Study on the effect of Mongolian Medicine Qiwei Qinggan Powder on Hepatic Fibrosis through JAK2/STAT3 Pathway

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\section*{ABSTRACT}

\textbf{Background}: To study the anti-hepatic fibrosis effect and explore the mechanism of Qiwei Qinggan Powder (QGS-7) in vivo and in vitro.

\textbf{Methods}: Carbon tetrachloride (CCl\textsubscript{4})-treated rats and hepatic stellate cells (HSCs) were used. Alanine Aminotransferase (ALT), Aspartate transaminase (AST) and Alkaline Phosphatase (ALP) were detected in serum of rats in each group, hydroxyproline (HYP) was detected in liver tissue. Formalin-fixed liver specimens were stained with hematoxylin and eosin (H&E) reagent, Masson trichrome, and then analyzed. The expression of Alpha smooth muscle actin (\textalpha-SMA) in liver was detected by immunohistochemistry. The expression level of Collagen I, \textalpha-SMA, Janus kinase 2 (JAK2), and signal transducer and activator of transcription 3(STAT3) mRNA were determined by real Time polymerase chain reaction (RT-qPCR).

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Meanwhile, the protein expression levels of $\alpha$-SMA, Collagen I, JAK2, phosphorylation-JAK2 (p-JAK2), STAT3 and phosphorylation-STAT3 (p-STAT3) were determined by Western Blot. The proliferation of HSC was detected by MTT and the apoptosis was detected by flow cytometry.

**Results:** QGS-7 treatment significantly improved the liver function of rats as indicated by decreased serum enzymatic activities of ALT, AST and ALP. Meanwhile, the HYP of liver was significantly decreased. Histopathological results indicated that QGS-7 alleviated liver damage and reduced the formation of fibrosis septa. Moreover, QGS-7 significantly attenuated expressions of $\alpha$-SMA, Collagen I, JAK2, p-JAK2, STAT3, p-STAT3 relative mRNA and protein level in the rat hepatic fibrosis model and HSCs. And QGS-7 can inhibit HSCs proliferation and promote it apoptosis.

**Conclusion:** Mongolian medicine QGS-7 has the effect of treating hepatic fibrosis and can inhibit the activation, proliferation and promote apoptosis of HSCs.

Meanwhile, in the process of anti-hepatic fibrosis, QGS-7 can reduce the expression of JAK2, p-JAK2, STAT3 and p-STAT3 in JAK2/STAT3 signaling pathway. Therefore, we speculate that QGS-7 may affect HSCs through JAK2/STAT3 signaling pathway, so as to play an anti-hepatic fibrosis role.

**Key words:** hepatic fibrosis, QGS-7, HSCs, serum containing drugs, JAK2/STAT3 signaling pathway

**Background**

Hepatic fibrosis is an important health problem in the world. About 1.5 million people die of cirrhosis and primary liver cancer every year [1]. Hepatic fibrosis is mainly
related to chronic hepatitis B or C infection, alcoholic steatohepatitis, nonalcoholic steatohepatitis and biliary diseases [2]. With the progress of fibrosis, cirrhosis will occur, and even develop into HCC [3-4]. At present, most of the drugs for the treatment of hepatic fibrosis are expensive, with many side effects, and there is no clearly recognized effective drug for the treatment of various types of hepatic fibrosis [5]. Therefore, it is urgent to study the therapeutic drugs and mechanism of hepatic fibrosis. Traditional Chinese medicine has the characteristics of low toxicity, less adverse reactions and good patient tolerance. Mongolian medicine is an important part of traditional Chinese medicine. It is the cream of traditional culture. It has unique theoretical system, special curative effect for many diseases, and great potential for development.

Mongolian medicine QGS-7 is a classic prescription of Mongolian Medicine [6], which can be used to treat various liver diseases [7]. It has been shown that QGS-7 has a good therapeutic effect on acute liver injury [8-9]. However, its therapeutic effect and mechanism on hepatic fibrosis have not been reported.

We calculated the liver index of rats, measured the contents of ALT, AST and ALP in serum, HYP in liver, observed the liver injury by HE staining, and counted the collagen content by Masson staining. It was confirmed that QGS-7 has a good effect on repairing liver injury and alleviating hepatic fibrosis. In addition, we also used immunohistochemistry to detect α-SMA in liver tissue, RT-qPCR and Western blot to detect the expression of α-SMA and Collagen I from mRNA and protein levels, and confirmed the change of expression of hepatic fibrosis marker protein.
In order to study the mechanism of QGS-7 in the treatment of hepatic fibrosis, we extracted RNA from the liver tissue of each group and analyzed the transcriptome sequence. Using bioinformatics, we screened out the signaling pathways with significant changes between the model group and QGS-7 group. After a large number of literature review, we selected JAK2/STAT3 signaling pathway for research. As the important members of this pathway, JAK2 and STAT3 play an important role in signal transduction. At present, JAK2/STAT3 signaling pathway has made great progress in renal and bone marrow fibrosis [10-14]. At the same time, there are some studies on liver injury and fibrosis [15-16], but it is the first time to use Mongolian medicine prescription to intervene hepatic fibrosis through JAK2/STAT3 signaling pathway. We hope to provide experimental data for the effect, target and mechanism of QGS-7 in the treatment of hepatic fibrosis.

**METHODS**

The Minimum Standards of Reporting Checklist contains details of the experimental design, and resources used in this study (Additional file 1).

*Composition and preparation of Qiwei Qinggan Powder*

QGS-7 is comprised of the following seven herbs: *Carthami Flos* (Honghua), 180g; *Scabiosa comosa* inflorescence (Lanpenhua), 60g; *Dracocephalum moldavica L.* (Xiangqinglan), 60g; *Wulingzhi* (Wulingzhi), 60g; artificial *Bovis Calculus* (artificial Niuhuang), 80g; *Dianthus superbus L.* (Qumai), 60g; *Gypsum Fibrosum* (Shigao), 180g. In addition to artificial Calculus Bovis, all herbal medicine are crushed, then with artificial Calculus Bovis are mixed, sifted and evenly mixed. The total used was
1.5~3g which is the common dose for adult humans. All the herbs were purchased from Inner Mongolia Tiansheng Mongolian Traditional Chinese Medicine Co., Ltd (Inner Mongolia, China).

**Experimental animals**

Male Wistar rats of Specific pathogen-free (SPF) grade, weighing 190-220 g were obtained from the Experimental Animal Center of Inner Mongolia Medical University, China [Certificate of quality No. SCXK (Jing) 2016-0006] and kept in a 18~22 °C and 70% humidity controlled room with 12 h light-dark cycle. The animals were fed on regular sterile chow diet and water ad libitum.

**Hepatic fibrosis model replication and QGS-7 treatment**

Fifty rats were randomly divided into the five groups (10 rats per group): Blank group, model group and QGS-7 [135, 270, 405 mg/(kg · d)] groups. Hepatic fibrosis was generated by 10 weeks of treatment with CCl₄ [CCl₄/peanut oil 1:1 (vol/vol), a mixture of pure CCl₄ and peanut oil at 2ml/kg body weight by gavage twice weekly [17-18]. At the same time, QGS-7 was given once a day. The model group and the blank group received equal volume of 0.5% sodium carboxymethylcellulose solution. At the end of the experimental period, all rats were sacrificed under chloral hydrate anesthesia. Blood was obtained from the abdominal aorta, and the liver was excised. The liver was immediately frozen for biochemical measurements or fixed in formalin for histochemical examination.

**Preparation of QGS-7 containing serum**

Wistar rats were randomly divided into two groups (8 rats per group): Control group
and QGS-7 groups. QGS-7 was given once a day according to 10 times of the lowest adult dose [1350 mg/(kg · d)]. On the 7th day, the rats fasted 12 h before gavage and carried out the experiment within 1-2 h after gavage. The blood was collected from abdominal aorta, and then placed for 20 minutes, centrifuged in a centrifuge (4 °C, 3000 r/min, 15 min). After centrifugation, the serum was filtered with 0.22 μM filter membrane, which was called drug serum. In the control group, the drug was replaced by normal saline, and the preparation method was the same as before. After being inactivated at 56 °C for 30 minutes, the drug serum was stored in a refrigerator at 80 °C.

**Cell culture**

The hepatic stellate cell line (HSC-T6 cells) was purchased from Beijing Beina Science&Technology Co., Ltd (Beijing, China). Cells were cultured in DMEM supplemented with 10 % FBS (Thermo Fisher Scientific, Shanghai, China) at 37 °C with 5 % CO₂.

**Calculations of liver index**

Liver index was calculated according to the formula: (liver weight / body weight) ×100%.

**Measurements of serum AST, ALT, ALP and tissue HYP**

The activities of ALT, AST, ALP and HYP content were measured by Visible light colorimetry. An Ultraviolet spectrophotometer and commercial kits (Nanjing Jiancheng Corporation, Nanjing, China) were used for all analyses. ALT, AST and ALP activities were expressed as U/L and HYP level was expressed as μg/g.
Histopathological changes

Liver sections fixed in formalin were embedded in paraffin and cut to a thickness of 4-5 μm. Hematoxylin-eosin and Masson’s trichrome was performed according to standard procedure. Sections were visualized by a microscope and the ratio of collagen deposition (blue color area) over the whole field area was quantified by ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Immunohistochemical examination

For Immunohistochemistry, sections were incubated with α-SMA primary antibody (Proteintech Group, Wuhan, Hubei, China) overnight at 4°C, followed by incubation with secondary antibody (Maixin Biotechnology Development Co., Ltd, Fuzhou, Fujian, China) for 1 h. Finally, the expression of α-SMA was observed under microscope.

Screening signal pathway by transcriptome sequencing

The first step is to extract RNA from liver tissue and evaluate its quality, then purify, fragment and synthesize the first and second strands of cDNA, then repair the end of cDNA and place it in DNA add 'A' at the 3 'end, then connect the DNA segment with the connector and purify the cDNA template, then enrich and purify the cDNA template by PCR, and then check the DNA library and sequence after completion; determine the signal path for further research by analyzing the RNA sequencing results (processing the original data, calculating the gene expression amount, and bioinformatics analysis).

RT-qPCR Assay
Total RNA was extracted from rat liver and HSC respectively. After the quality test was qualified, the reverse transcription kit (TIANGEN BIOTECH Co., Ltd, Beijing, China) was used for reverse transcription. Then the relative mRNA expression of α-SMA, collagen I, JAK2 and STAT3 in rat liver and HSC was detected by RT-qPCR. The data were processed by $2^{-\Delta\Delta C_t}$ method. The primers (Shanghai Sengon Biological and Technological Company, Shanghai, China) for each gene are shown in the Table 1.

**Western blot analysis**

The protein in rat liver and HSC was extracted respectively. After quantitative analysis by BCA method, the sample buffer was added. After protein boiling, the sample was loaded and electrophoresis was carried out. The protein expression levels of α-SMA, collagen I, JAK2, STAT3 (Proteintech Group, Wuhan, Hubei, China), p-JAK2 and p-STAT3 (BOSTER Biological Technology Co., Ltd, Wuhan, Hubei, China) were detected, and the gray value of the item was analyzed by image studio software. The ratio of the gray value of the target protein band to the gray value of the internal reference band was used as the expression amount of the target protein, each group repeated three times, and the follow-up statistical analysis was carried out.

**Detection of HSC proliferation by MTT**

The logarithmic phase cells were collected and seeded in 96 well plates. Each group was set with 5 duplications and cultured for 12 hours. Discard the original culture medium, and add the corresponding concentration of serum culture medium into each well for 24 hours. Remove the supernatant, add 90 μl fresh culture medium, add 10 μl
MTT solution, and continue to culture for 4 hours. Suck off the supernatant, add 110 μl formazan, shake it on the shaking table at low speed for 10 min, and measure the absorbance value of each well at 490 nm of the enzyme labeling instrument.

**Apoptosis of HSC-T6 cells detected by Annexin V-FITC and PI double staining**

The logarithmic phase cells were collected and seeded in 6 well plates. Each group was set with 3 duplications and cultured for 12 hours. Discard the original culture medium, add the corresponding concentration of serum culture medium for 24 hours. Trypsin digests cells, centrifuges, discards supernatant. Add 390 μl Annexin V-FITC binding solution and gently resuspend the cells. Add 5 μl Annexin V-FITC and mix gently. Add 10 μl PI and mix gently. Incubate at room temperature in dark for 10-20 min, then place in ice bath. Detection on flow cytometry.

**Statistical analysis**

All results were presented as mean ± standard deviation (SD). Statistical analysis was performed with SPSS software (version 24). The statistical significance between groups was analyzed using one way ANOVA. The difference was considered significant at $P < 0.05$.

**RESULTS**

**QGS-7 attenuated CCl₄-induced liver fibrosis in rats**

In order to study the anti-fibrosis effect of QGS-7, we first studied the therapeutic effect of QGS-7 on CCl₄ induced hepatic fibrosis in rats. At the end of the experiment, no rats in each experimental group died. However, all the rats in the model group had reduced diet, sluggish action, depressed spirit, weight loss, disordered fur and
sometimes irregular stool. At the same time, as shown in Table 2, the rats in model group has higher liver index compared with other groups. Meanwhile, the rats in model group had significantly higher levels of serum ALT, AST and ALP, which represented a decrease in liver function. Liver tissue levels of HYP are surrogate markers of hepatic fibrosis. Model rats also exhibited higher levels of HYP. Each dose group of QGS-7 significantly decreased the elevated ALT, AST and HYP levels, while Low and middle dose group (135, 270 mg/kg) had no obvious effect in ALP, and middle and high dose (135, 405 mg/kg) had no obvious effect in liver index.

Liver morphology can directly reflect the color, smoothness and hardness of liver surface, so as to preliminarily judge the damage of liver. The liver of the blank group showed bright red, smooth surface, no rough and granular feeling, and soft texture. In the model group, the liver was dark red or even yellow, with rough surface, obvious granular feeling and hard texture. The liver of rats in each dose group of QGS-7 was dark red, with rough surface, but no obvious granule sense. The liver state was between the blank group and the hepatic fibrosis model group. The results suggests that QGS-7 can improve the hepatic fibrosis of rats (Figure 1A).

To assess histological changes, hematoxylin and eosin (H&E) and Masson staining of liver tissue sections from each group were examined. H&E staining showed that in the blank group, the structure of liver lobule was complete, the hepatocytes were arranged orderly and the plasma was even. In the model group, the structure of liver lobule was destroyed, the arrangement of liver plate was disordered, a large number of inflammatory cells infiltrated and the balloon like changes of liver
cells could be seen in liver tissue, and some samples even appeared and pseudolobule. The infiltration of inflammatory cells and the decrease of cell degeneration and necrosis in liver tissue of rats in each dose group of QGS-7 (Figure 1B). Masson staining showed that the hepatocytes of the blank group were intact without abnormal fibrous tissue proliferation. In the model group, a large number of fibroblasts appeared in the liver tissue, the arrangement of liver cords was disordered, the connective tissue and fibrous tissue proliferated obviously, and the pseudolobule was formed. The rats in each dose group of QGS-7 were improved in varying degrees, the proliferation of fibrous tissue was reduced, and the structure of liver tissue tended to be normal (Figure 1C). After statistical analysis of pathological sections stained by Masson, it can be observed that compared with the blank group, the collagen content in the liver of the model group increased significantly; compared with the model group, the collagen content in the liver tissue of each group of QGS-7 decreased significantly (Figure 1D). Furthermore, intervention with QGS-7 also inhibited the up-regulation of α-SMA, collagen I (Figure 1E–G), indicating that QGS-7 treatment inhibited established hepatic fibrosis.

Transcriptome sequencing

The gene expression level of blank group, model group and QGS-7 group was analyzed by transcription sequencing, and the overall distribution map of different transcripts or genes was obtained (q value <0.05) (Figure 2). Compared with the model group, there were 63 up-regulated mRNA and 126 down regulated mRNA in QGS-7 group. Through further enrichment analysis of GO and KEGG, and combined
with comparison with the blank group, it was found that there were many signal pathways with significant changes. Combined with literature search, the JAK2/STAT3 signaling pathway possibly related to QGS-7 anti-fibrosis effect was obtained, which was verified by subsequent experiments.

*Effect of QGS-7 on iJAK2, STAT3 mRNA expression and JAK2, p-JAK2, STAT3, p-STAT3 protein expression in vivo*

The RT-qPCR results showed that compared with the blank group, the expression of JAK2 and STAT3 mRNA in the liver tissue of the model group increased significantly; compared with the model group, the expression of JAK2 mRNA in the low dose group of QGS-7 decreased significantly, and the expression of STAT3 mRNA in the high dose group of QGS-7 decreased significantly, as shown in Figure 3A. The results showed that QGS-7 could significantly inhibit the expression of JAK2 and STAT3 mRNA in liver tissue.

Western blot results showed that compared with the blank group, the expression level of JAK2, p-JAK2, STAT3 and p-STAT3 protein in the liver tissue of the model group was significantly increased; compared with the model group, the expression of JAK2, p-JAK2 and STAT3 protein in each group of QGS-7 was significantly decreased, and the expression of p-STAT3 protein in the high and middle dose groups of QGS-7 was significantly decreased (Figure 3B). It is suggested that QGS-7 can not only reduce the protein content of JAK2 and STAT3 in liver tissue, but also reduce the expression level of p-JAK2 and p-STAT3.

*In vitro experiments verify that QGS-7 has anti fibrosis effect through JAK2/STAT3*
The results of RT-qPCR showed that compared with the control group, the relative mRNA expression of α-SMA and collagen I in the low and high dose serum group decreased significantly, and the relative mRNA expression of α-SMA and collagen I in the middle dose serum group decreased significantly (Figure 4A).

Western blot results showed that compared with the control group, the expression level of α-SMA and Collagen I protein in the low, middle and high dose serum groups decreased significantly (Figure 4B).

The results of RT-qPCR showed that compared with the control group, the relative mRNA expression of JAK2 and STAT3 in each dose serum group decreased significantly (Figure 4C). The results showed that QGS-7 could reduce the expression of JAK2 and STAT3 mRNA in HSC.

Western blot results showed that compared with the control group, the protein expression of JAK2, p-JAK2, STAT3 and p-STAT3 in the low and middle dose serum groups decreased significantly; the protein expression of p-JAK2 and p-STAT3 in the high dose serum group decreased significantly (Figure 4D). This suggests that QGS-7 can significantly reduce the expression level of JAK2 and STAT3 protein, and p-JAK2 and p-STAT3 in HSC.

The results of MTT showed that after 24 h treatment the OD values of the low, middle and high dose serum groups decreased significantly, and the inhibition rates were 42.95%, 50.89% and 44.93% respectively; after 48 h treatment, the OD values of the middle and high dose serum groups decreased significantly and the inhibition
rates were 50.89% and 33.55%; the OD values of the low, middle and high dose serum groups decreased significantly after 72 hours treatment, the inhibition rates were 21.36%, 33.26% and 33.00% (Table 3).

FITC labeled Annexin and PI double staining can be used to distinguish three types of cells: living cells, early apoptotic cells and late apoptotic and necrotic cells. The results showed that compared with the control group, the apoptosis rate of the low dose serum group was significantly higher; the apoptosis rate of the high dose group was significantly higher (Figure 4E).

**Discussion**

Fibrosis is a process closely related to organ damage, which plays a role in preventing organ tissue from disintegration in the process of chronic inflammation. With the repair of tissue damage, fibrosis can be reversed in a few weeks [19]. However, this ability to reverse fibrosis is limited. When ECM is widely accumulated and cross-linked, fibrinolysis is blocked and cell components that can eliminate scar tissue are lost, it will make hepatic fibrosis difficult to be reversed [20]. Although people have a deeper understanding of the pathogenesis of hepatic fibrosis, there is still a lack of effective anti-hepatic fibrosis drugs [21].

QGS-7 is a classic prescription of Mongolian Medicine, also known as Eligen-7 (ELG-7) [6], which can be used to treat various liver diseases in clinical practice [7]. Although QGS-7 has been used in the clinical treatment of hepatic fibrosis, there is no preliminary in vitro and in vivo experimental data, and the mechanism of its treatment of hepatic fibrosis is not clear, so it is particularly important to supplement this data.
Hepatic fibrosis is an important node in the development of liver diseases. Correct understanding and timely treatment can effectively control the development of liver diseases. It has been reported that CCl₄ induction method is the most classical one among the chemical drug induction methods [22]. The method of gavage is simple in operation and well tolerated by animals, so we choose it to establish hepatic fibrosis model [17-18]. In the development process of hepatic fibrosis, the liver will increase and become heavier, so the liver index can reflect the status of the liver. Compared with the blank group, the liver index of the model group increased significantly; compared with the model group, the liver index of the low dose group decreased significantly. The process of liver injury is accompanied by the degeneration, necrosis and rupture of hepatocytes, and then the enzymes existing in the cells enter into the serum. Therefore, the content of enzymes in the serum can reflect the damage and damage degree of hepatocytes [23]. The changes of serum enzymes (ALT, AST, ALP) are often used to reflect the liver function to judge the degree of liver damage. Therefore, the above indicators are also selected in the study of the therapeutic effect of QGS-7 on hepatic fibrosis. In addition, HYP is a characteristic amino acid component of collagen. When hepatic fibrosis occurs, a large number of ECM accumulates, and the collagen content increases, which leads to a significant increase of HYP, which can be used as one of the indicators to investigate the occurrence of hepatic fibrosis [24]. The results of this study showed that compared with the blank group, the liver tissue was hard and felt granule, and the contents of ALT, AST, ALP and HYP in serum were significantly increased. Compared with the model group, the
liver condition of rats in each dose group of QGS-7 was improved, the ALT, AST, HYP content in each dose group and the ALP content in high dose group were significantly reduced. It shows that QGS-7 has a certain protective effect on liver injury. In addition to the above indicators, pathological section observation can more accurately determine the degree of hepatic fibrosis. Clinically, it is also an important indicator to evaluate liver injury and differential diagnosis of hepatic fibrosis. H&E staining can observe the infiltration of inflammatory cells, balloon like changes, pseudolobules and other pathological states, while Masson staining can intuitively reflect the proliferation of collagen fibers, connective tissue hyperplasia, hepatic cord disorder and pseudolobules and other pathological states in the liver tissue, so as to judge the situation of hepatic fibrosis. Compared with the blank group, inflammatory cell infiltration, pseudolobule and bridge connection can be seen in the liver pathological section of the model group, Masson staining can see a large number of fibrogenesis, wrapping the damaged hepatocyte to form pseudolobule, indicating the success of the hepatic fibrosis model. Compared with the model group, the pathological manifestations of QGS-7 group were alleviated, and the blue collagen fibers were also decreased by Masson staining. It is suggested that QGS-7 has a certain therapeutic effect on rats with hepatic fibrosis.

In the process of hepatic fibrosis, HSC is activated, α-SMA is expressed in large quantities, ECM is synthesized and secreted [25] and collagen IV is replaced by collagen I, which can form scar tissue. When HSC is activated and proliferated, the expression level of collagen I mRNA is 60-70 times that of resting [26], which is the
most important part of ECM. Therefore, the increase of collagen I can reflect the degree of hepatic fibrosis [27]. The results of immunohistochemistry can not only reflect the position of protein expression in cells, but also directly reflect the expression of α-SMA in liver tissue of rats in each group. α-SMA is mainly expressed in HSC cytoplasm activated in the portal area. Compared with the blank group, the positive expression area of the model group increased greatly. And the α-SMA in the liver tissue of each dose group of QGS-7 decreased compared with the model group. The results of RT-qPCR and Western blot suggested that QGS-7 might play an anti-fibrosis role by inhibiting the activity of HSC and reducing the secretion of collagen.

Mongolian medicine is similar to other traditional medicine prescriptions which has the characteristics of multi target and multi mechanism. In order to better define the target and mechanism of QGS-7 against hepatic fibrosis, we extracted RNA from rat liver tissue and sequenced the transcriptome gene. The JAK2/STAT3 signaling pathway is one of the possible mechanisms of QGS-7 and anti-fibrosis by bioinformatics analysis and a large number of literature search. We have carried out subsequent tissue and cell verification.

JAK2/STAT3 signaling pathway is mediated by cytokines, mainly involved in cell proliferation, differentiation, apoptosis and immune regulation [28]. JAK2/STAT3 has been widely confirmed to play an important role in the development of organ fibrosis in recent years [14, 29-31]. Through a large number of literature searches, combined with transcriptome results, we found the JAK2/STAT3 signaling pathway changed
significantly after hepatic fibrosis. The results of transcriptome reflect the common effects of all cells in the liver tissue, but the key point for the treatment of hepatic fibrosis is the role of HSC, so it is necessary to detect the activation, proliferation and apoptosis of HSC in vitro. At the same time, HSC accounts for about 8% - 15% of the total number of liver cells in the normal liver, but with the occurrence of chronic fibrosis injury, HSC rapidly proliferates to several times of the normal state [32]. Therefore, we preliminarily determined that the change of JAK2/STAT3 pathway was related to the change of HSC in the transcriptome sequencing results, and speculated that JAK2/STAT3 signaling pathway was related to the anti-fibrosis effect of QGS-7. In JAK2/STAT3 signaling pathway, p-JAK2 and p-STAT3 are the activation forms of JAK2 and STAT3, respectively. In this study, the changes of p-JAK2 and p-STAT3 protein can reflect the activation degree of JAK2/STAT3 signaling pathway. The expression of JAK2, STAT3, p-JAK2 and p-STAT3 could be down regulated by QGS-7 in vivo. What's more, JAK2-mediated fibrosis signal is caused not only by the increase of JAK2 expression, but also by the p-JAK2 expression. HSC is considered to be the main fibroblast type of liver and the main source of ECM [33]. At the same time, the activation of HSC is also a key step of hepatic fibrosis. Therefore, in order to confirm that QGS-7 plays an anti-fibrosis role by affecting HSC, we carried out a series of experiments in vitro with HSC-T6 cell line. HSC-T6 is an immortalized rat HSC line transfected by simian virus 40 (SV40). It is known that HSC-T6 has almost all functions of activating HSC, such as expression of α-SMA, collagen I, matrix metalloproteinases (MMP), tissue inhibitor of matrix
Metalloproteases (TIMP-1), and produce endogenous TGF-β1. The morphology of fibroblasts was observed under the microscope, which can proliferate rapidly in the process of culture [34-35]. These characteristics of HSC-T6 are typical of activated astrocytes. We also observed under the inverted microscope that HSC morphology showed stretching state, pseudopodia increased with star like change, and the connection between cells became loose obviously, showing a significant activation state. The expression of α-SMA mRNA and protein was detected by RT-qPCR and Western blot. So we did a follow-up experiment without induction.

When Mongolian medicine acts on cells, we adopt the drug containing serum administration method, which can simulate the absorption, distribution, metabolism and excretion of oral drugs through a series of processes, so that there are not only prototype components of compound formula in serum, but also products after metabolism, which can fully reflect the changes of drug compatibility [36-37].

The activation of HSC is the central link of hepatic fibrosis and α-SMA is the marker of HSC activation. After HSC activation, α-SMA protein is highly expressed, which will further increase the synthesis and accumulation of ECM dominated by collagen I, and finally lead to hepatic fibrosis [38]. This study found that QGS-7 can inhibit the activity of HSC and reduce the production of ECM by reducing α-SMA and collagen I in HSC.

Subsequently, in order to determine whether the changes of JAK2/STAT3 pathway in transcriptome sequencing results are related to the changes of HSC, we used RT-qPCR and Western blot assay to detect the changes of pathway related factor’s
mRNA and protein level in HSC. The results of the experiments showed that the effect of QGS-7 on hepatic fibrosis might be influenced by the JAK2/STAT3 signaling pathway in HSC.

According to the literature, JAK2/STAT3 signaling pathway participates in the process of cell differentiation, thus promoting the fibrotic response and leading to HSC activation [39]. In this study, it was also confirmed that the expression of α-SMA in HSC increased significantly after hepatic fibrosis. Meanwhile, the downstream signal of JAK2/STAT3 affects the expression of many genes, including some genes related to cell proliferation, migration and apoptosis [40-44]. Therefore, we used MTT method to detect the proliferation of HSC-T6 cells. The results showed that the inhibition rate of HSC-T6 cells in the serum containing drugs increased. In addition, Annexin V-FITC and PI double staining technique showed that the apoptosis rate of the low dose and high dose group was significantly higher than control group. This suggests that JAK2/STAT3 signaling pathway can inhibit HSC proliferation and promote HSC apoptosis to produce anti fibrosis effect.

There were some limitations to the study as we did not use the HPLC to analysis effective components of QGS-7. A follow-up study using HPLC to analysis effective components of QGS-7 has already been planned.

**Conclusions**

So far, eliminating the root cause of liver disease is still the most effective way to prevent hepatic fibrosis. However, in the process of hepatic fibrosis, drugs and means for the treatment of HSC and disease molecular mechanism are particularly important.
Through the above research, it is confirmed that the Mongolian medicine QGS-7 has the effect of treating hepatic fibrosis; the Mongolian medicine QGS-7 can inhibit the activation, proliferation and promote apoptosis of HSC; the Mongolian QGS-7 can reduce the expression of JAK2, p-JAK2, STAT3 and p-STAT3 in JAK2/STAT3 signaling pathway in the process of anti-hepatic fibrosis; we speculate that the Mongolian medicine QGS-7 may be through JAK2/STAT3 signaling pathway affects HSC and plays an anti-fibrosis role.

**Abbreviations**

QGS-7: Qiwei Qinggan Powder; HSC: hepatic stellate cell; ALT: Alanine Aminotransferase; AST: Aspartate transaminase; ALP: Alkaline Phosphatase; HYP: hydroxyproline; α-SMA: alpha smooth muscle actin; JAK2: janus kinase 2; STAT3: signal transducer and activator of transcription 3.

**Authors’ contributions**

LJ carried out experiments, analyzed data, wrote and revised the manuscript. Menggensilimu carried out experiments and revised the manuscript. YHW carried out pathological analysis. The contents of ALT, AST and ALP were determined and analyzed by WF. YXY and ZCY participated in Western Blot and RT-qPCR experiment. BXM and Hurilebagen were used to identify herbs. XR directed the experiment of molecular biology. WHS participated in the statistical analysis. JR guided in vivo experiments. MLJ participated in the design of the study. ZJY participates in RNA extraction. SXL participated in the statistical analysis and experiment in vivo. MYH designed the study, revised the manuscript and guided the
experiment.

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**Acknowledgements**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and materials**

I agree to share my data and materials

**Consent for publication**

Not applicable.
Ethics approval and consent to participate

All the animal protocols were approved by department of Basic Medical, the Inner Mongolia Medical University, China.

Funding

The present study was supported by the National Natural Science Foundation of China (Grant Nos. 81960759, 81560706), the Inner Mongolia Natural Science Foundation (Grant Nos. 2019MS08010, 2014MS0841), the Inner Mongolia Young Innovative Talents Training Program, the Inner Mongolia Medical University Talent Team Program.

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Figure legends

Figure 1. QGS-7 attenuated CCl₄-induced liver fibrosis in rats. (A) Generation of hepatic fibrosis of rats in different groups. (B) Effects of QGS-7 on the histological changes of liver in CCl₄-induced hepatic fibrosis rats (100×, 400×). (C) Representative micrographs of Masson trichrome staining of liver tissues. (D) Quantification of liver fibrosis (ratio of blue color area). (E) QGS-7 ameliorated pathological changes in liver as shown by immunohistochemistry. (F) RT-qPCR for α-SMA and Collagen I. (G) Western blot analysis of α-SMA and Collagen I.

Notes: Compared with the blank group, *P <0.05, **P <0.01; Compared with the model group, *P <0.05, **P <0.01.

Figure 2. Volcanic map of differential gene comparison between QGS-7 group and model group.

Figure 3. Effect of QGS-7 on JAK2, STAT3 mRNA expression and JAK2, p-JAK2, STAT3, p-STAT3 protein expression in vivo. (A) JAK2 and STAT3 mRNA expression level. (B) JAK2, p-JAK2, STAT3 and STAT3 protein expression level.

Notes: Compared with the blank group, *P <0.05, **P <0.01; Compared with the model group, *P <0.05, **P <0.01.

Figure 4. In vitro experiments verify that QGS-7 has anti fibrosis effect through JAK2/STAT3 signaling pathway. (A) Relative mRNA expression of α-SMA and collagen I. (B) α-SMA and collagen I protein expression level. (C) JAK2 and STAT3 mRNA expression level. (D) JAK2, p-JAK2, STAT3 and STAT3 protein expression level. (E) HSC apoptosis rate.

Notes: Compared with the blank group, *P <0.05, **P <0.01; Compared with the model group, *P <0.05, **P <0.01.
Table 1 Objective gene primer design

| Gene                        | Forward primer                      | Reverse primer                      |
|-----------------------------|-------------------------------------|-------------------------------------|
| β-actin                     | ACCCGCGAGTACAACCTTCT                | TTCAGGGTCAGGATGCCTCT                |
| α-SMA                       | CATCCACGAAAACCACCTA                 | GGGCAGGAATGAATTTGGA                 |
| Collagen type I alpha 1 chain| TGTTGGTCCTGCTGGCAAGAATG             | GTCACCTTGTTCGCTGTCCTCAC             |
| JAK2                        | GTGCGTGCGAGCGAAGATCC                | ACTGCTGAATGAACCTGCAGAATC            |
| STAT3                       | CCAGTCTGTTGTATCTCAACAT              | CAGGTTCACAGGAGGCTTAGTG             |
Table 2 The expression level of liver index, ALT, AST, ALP and HYP ($\bar{x} \pm s$, $n=10$)

| Group         | n  | liver index (%) | ALT (U/L) | AST (U/L) | ALP (U/L) | HYP (μg/g) |
|---------------|----|-----------------|-----------|-----------|-----------|------------|
| Blank         | 10 | 3.06±0.21       | 11.73±6.16| 17.31±2.48| 14.63±4.36| 352.50±37.30|
| Model         | 10 | 3.91±0.62***    | 34.22±5.15**| 41.07±9.30***| 50.84±16.04**| 1151.00±173.90**|
| High dose     | 10 | 3.55±0.48       | 20.48±4.70**| 21.04±7.40**| 26.07±6.87**| 649.50±62.09**|
| Middle dose   | 10 | 3.47±0.30       | 19.31±5.93**| 23.74±9.34**| 40.56±10.42| 754.70±64.84**|
| Low dose      | 10 | 3.31±0.25*      | 20.16±4.74**| 21.2±6.49**| 42.02±9.43| 911.10±139.60**|

Notes: Compared with the blank group, *$P < 0.05$, **$P < 0.01$; Compared with the model group, *$P < 0.05$, **$P < 0.01$. 
Table 3: Effect of QGS-7 containing serum on proliferation of HSC-T6 cells ($\bar{x} \pm s$, n=3)

| Group              | OD values 24 h ($\bar{x} \pm s$) | OD values 48 h ($\bar{x} \pm s$) | OD values 72 h ($\bar{x} \pm s$) |
|--------------------|----------------------------------|----------------------------------|----------------------------------|
| Control            | 1.15±0.15                         | 1.49±0.06                        | 1.55±0.02                        |
| High dose serum    | 0.65±0.11**                       | 1.23±0.11                       | 1.22±0.08**                      |
| Middle dose serum  | 0.56±0.02**                       | 1.02±0.23**                     | 1.03±0.12**                      |
| Low dose serum     | 0.63±0.16**                       | 0.99±0.06**                     | 1.04±0.10**                      |

Notes: Compared with the blank group, *P < 0.05, **P < 0.01; Compared with the model group, *P < 0.05, **P < 0.01.