

Effects and mechanisms of ziqi ruangan decoction on hepatic fibrosis

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Abstract: To investigate effect and mechanism of Ziqi Ruangan Decoction (ZQRGD) on hepatic fibrosis in rats. Rats were randomly assigned to blank group, model group, colchicine group, ZQRGD high-dose group, ZQRGD middle-dose group, and ZQRGD low-dose group. All groups except group A were intraperitoneally injected with 40% CCl₄/olive oil for 8 weeks; group C was then given intragastric colchicine administration. Groups D, E, and F were intragastrically dosed with ZQRGD. Compared with the colchicine group, the superoxide dismutase (SOD) activity of each dose group of ZQRGD significantly increased. TNF- α and IL-6 concentration significantly decreased in each drug intervention group, while these significantly decreased in the high-dose and medium-dose ZQRGD groups. The expression of α -SMA and collagen I significantly decreased in the drug treatment group compared with the model group, as did the expression of PI3K, AKT, and mTOR. Ziqi Ruangan Decoction had a favorable anti-liver fibrosis effect and the mechanism is related to anti-oxidative stress, anti-inflammation, the inhibition of the PI3K/Akt/mTOR signaling pathway, and the inhibition of hepatic stellate cell activation.

Keywords: Ziqi ruangan decoction, hepatic fibrosis, anti-oxidative stress, anti- inflammation, autophagy.

INTRODUCTION

Liver fibrosis is a pathological process, during which the extracellular matrix (ECM) accumulates due to an increase in synthesis and a reduction in degradation (Xu *et al.*, 2016). The activation of Hepatic stellate cells (HSCs) is a core event of liver fibrosis, and inhibiting this is considered to be an important strategy for intervention in liver fibrosis (Elpek, 2014). The liver is subjected to a variety of damaging factors: Cells such as hepatocytes, Kuffer cells, sinusoidal endothelial cells secrete various cytokines including transforming growth factor beta (TGF- β), insulin growth factor-1 (IGF-1), and tumor necrosis factor alpha (TNF- α). Subsequently, lipid peroxides and oxygen free radicals are produced that stimulate the activation and proliferation of HSC through the transcription factors c-myc and NF- κ B (Elpek, 2014). HSCs then transform from quiescent, lipid-containing cells into myofibroblast-like cells with fewer lipid droplets. The myofibroblast-like cells then migrate and accumulate in the damaged parts of liver tissue and secrete various proinflammatory factors and profibrotic factors that promote the synthesis of ECM. HSCs are continuously activated, and ECM in the liver can thus excessively accumulate to finally result in liver fibrosis. The contribution of HSC activity to liver fibrosis makes this a key target for treatment of this disease (Marra, 1999).

Ziqi Ruangan Decoction (ZQRGD) can treat liver fibrosis based on the “damp heat and stagnancy and toxin” theory, which has been confirmed by previous clinical and experimental research. This study aimed to investigate effect and mechanism of ZQRGD on hepatic fibrosis in rats.

MATERIALS AND METHODS

Animals

Sixty SD rats, aged 6–8 weeks, half male and female, clean grade, and body weight of 200 $\pm$ 20 g were obtained from Zhejiang Experimental Animal Center (animal certificate number: SCXK, Zhejiang, 2014-0001). All rats were housed in standard rat cages and fed freely with drinking water; the ambient temperature was maintained at 18–22°C, humidity at 40%–60%, with an alternating 12 h day and night. Institutional Review Board of the Ethics Committee of Affiliated Hospital of Nanjing University of Chinese Medicine approved our study. All animals were treated according to the National Research Council's Guide for the Care and Use of Laboratory Animals (1996).

Drugs

ZQRGD is composed of traditional Chinese medicines including astragalus, *Polygonum cuspidatum*, tangerine peel, red peony, comfrey, and notoginseng powder in a ratio of 10:15:6:10:10:4. After mixing, the herbs were immersed in water for 30 minutes and then boiled. The herbs were then simmered for 40 minutes, the liquid was

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removed and kept, and further water was then added to repeat the boiling and simmering steps. The slag was filtered, and the two boiled liquid fractions were combined and concentrated with a rotary evaporator to a crude liquid drug at 1 g/mL.

Colchicine is known to inhibit hepatic fibrosis and was used for the positive control group (Liver Fibrosis Group of Chinese Society of Liver Diseases, 2007). Each piece of colchicine tablet (0.5 mg, Yunnan Haobang Pharmaceutical Co. Ltd., Kunming, Yunan, China; batch number: H53021798) was thoroughly mixed with 5 ml of distilled water prior to use.

Grouping, modeling, and administration methods

Rats were randomly assigned to six groups, 10 in each group: a blank group (group A); a CCl₄-induced mouse liver fibrosis model group (group B); a colchicine group (group C); a high-dose ZQRGD group (group D); a middle-dose ZQRGD group (group E); and a low-dose ZQRGD group (group F). Except for the blank group, the other groups were intraperitoneally injected with 40% CCl₄/olive oil at 1 mL/kg, twice a week for 8 weeks. The blank group was intraperitoneally injected with the same amount of olive oil for 8 weeks. Post-modeling administration: the blank group and the model group were intragastrically administered with distilled water 20 mL/kg·d, and the colchicine group was intragastrically administered with colchicine at a dose of 1 mg/kg·d, the dosage volume was 0.2 mL/kg. The high, medium, and low-dose groups of ZQRGD were given a one-time intragastric administration at a dose of 26, 13 and 6.25 g/kg·d respectively. Continuous administration lasted for 4 weeks.

Specimen collection

Blood was drawn from femoral artery within 24 h after the end of the last administration. The serum was taken for testing of the hepatic function. The rats were euthanized by cervical dislocation, and the liver was dissected and morphological changes of the gross specimens were observed. The tissues were fixed with 4% neutral formaldehyde solution, and histopathological and immunological analyses were performed. The other liver tissues were quickly frozen and stored in a refrigerator at -80°C for protein and mRNA detection.

Observation indicators

General information

The indexes included vitality, movement, diet, hair, weight, and mortality of the rats.

Biochemical indexes

The indexes included 1) liver function serum indicators: alanine aminotransferase (ALT); aspartate aminotransferase (AST); alkaline phosphatase (ALP); and γ -glutamyltransferase (GGT). 2) liver fibrosis index:

hyaluronic acid enzyme (HA); type III procollagen (PIII); and type IV collagen (IV-C) using an automatic biochemical analyzer.

Liver histopathology

and fibrosis. Referring to the standards established by the Chinese Society of Liver Diseases (Liver Fibrosis Group of Chinese Society of Liver Diseases, 2007): S0 stage, the liver is normal without any fibrosis; S1 stage, there is localized fibrosis in the portal area or its surrounding area or hepatic lobules, fiber interval is not formed; S2 stage, the fiber interval has formed, but the hepatic lobule structure is basically intact; S3 stage, there are more fibrosis intervals and the hepatic lobular structure is disordered; S4 stage, cirrhosis has formed, and the separated hepatocytes form different degrees of nodules. Transmission electron microscope was used to observe the existence of autophagosomes.

Determination of superoxide dismutase (SOD), malondialdehyde (MDA), TNF- α , interleukin-6 (IL-6), and interleukin-10 (IL-10) in the liver tissue. A portion of the liver tissue was rinsed with isotonic saline several times, blood was removed, the water blotted with a filter paper, and then 200 mg of tissue was placed in a small beaker which was already pre-cooled. The pre-cooled isotonic saline was pipetted into the beaker, with the volume of isotonic saline nine times the weight of the tissue block. The tissue block was cut with small scissors as soon as possible and ground to a 10% tissue homogenate by using a tissue homogenizer at a speed of 1,000–1,500 rpm. Concentration of SOD, MDA, TNF- α , IL-6, and IL-10 in the supernatant was detected by ELISA.

MRNA expression

Real-time qPCR (Thermal Cycler Dice Real Time System, Takara Code: TP800) was used to detect the expression of relative mRNAs of smooth muscle actin (α -SMA), type I collagen (Colla I), PI3K, AKT, and mTOR.

Western-blot

Briefly, protein was extracted from rat liver tissue. The membrane was then incubated with primary antibodies to PI3K, p-PI3K, AKT, p-AKT, mTOR, p-mTOR ULK-1, Beclin-1, LC3I and LC3II. Protein bands were visualized by the Micro Chemi 4.2 system and quantified by Quantity One.

STATISTICAL ANALYSIS

Numerical data were expressed as mean $\pm$ standard deviation (SD) and analyzed using Student's T-test. The count data were tested by chi-square. A difference was considered statistically significant with a P-value of less than 0.05. The statistical data were analyzed by using SPSS 17.0 statistical software.

RESULTS

General information

No rats died during the experiment. The rats in the blank group had good vitality, quick activity, shiny hair, and good appetite. After modeling with CCl₄, the rats exhibited different degrees of irritability, irritation, slow movement, yellow hair without luster, loss of appetite, weight loss, and loose stools. The mental activity, hair, weight and other conditions of each drug intervention group were improved to varying degrees. Specifically activity, hair, weight, and appetite of groups C, D, E, and F were improved, with group D showing the best progress.

Comparison of serum liver function and liver fibrosis indexes

The liver function indexes of the model group B were higher than those of the blank group A. Compared with group B, the AST and ALP levels in the ZQRGD dosing groups D, E, and F decreased. Compared with the colchicine treatment group C, only the level of AST of groups D and E had decreased (fig. 1A).

The levels of the liver fibrosis indexes HA, PIII, and IV-C for group B were higher than those for group A. The levels of HA, PIII, and IV-C decreased in each drug intervention group compared with both group B and group C; however, only the level of IV-C in group F decreased in comparison with group C (fig. 1B).

Liver histology

HE staining in the model group A (fig. 1C) had normal hepatocyte morphology with a clear boundary of hepatocyte cord, radial distribution around the central vein, intact hepatic lobule, no inflammation in the portal area, and hyperplasia of fibrous connective tissue; Masson staining showed a small amount of fibrous connective tissue around the central vein.

In the model group B, HE staining showed a small amount of chronic inflammatory cell infiltration in the liver portal area, interfacial inflammation formation, and fibrous tissue hyperplasia connected to two portal areas. Masson staining showed increased fibrous tissue.

HE staining of the colchicine treatment in group C revealed mild hydropic degeneration and a small amount of inflammatory cell infiltration; Masson staining showed that the fiber area was reduced compared with the model group.

HE staining of the high-dose ZQRGD group D showed little inflammatory cell infiltration and a small amount of cholestasis, while Masson staining showed a small amount of fibrous connective tissue around the central vein.

Table 1 shows the different liver fibrosis stage distribution among these groups; there was a statistical difference between groups D and B by chi-square test.

Table 1: Stage of liver fibrosis in each group

Group	S0	S1	S2	S3	S4
A	10	0	0	0	0
B	0	3	4	3	0
C	0	8	1	1	0
D*	0	9	1	0	0
E	0	7	3	0	0
F	0	7	3	0	0

Note: Compared with group B, *P<0.05.

Comparison of oxidative stress response in different groups

The SOD concentration of the blank group B was lower than that of the model group A, while the MDA concentration was higher than that of group A (fig. 1E, F). The SOD concentration of each drug intervention group was higher than that of group B, though only groups D and E had a lower MDA concentration than that of group B. Compared with group C, the SOD concentration of groups D, E, and F all increased, while the MDA concentration of group D had decreased.

Comparison of inflammation status in different groups

The concentration of TNF- α and IL-6 was elevated in the model group B compared with the blank group A, while the IL-10 concentration was reduced (fig. 1D). Compared with group B, TNF- α and IL-6 concentration was reduced in each drug intervention group, while IL-10 increased. Compared with the colchicine group C, the concentration of TNF- α and IL-6 were reduced, while that of IL-10 was elevated in groups D and E; TNF- α concentration was only elevated in group F, the lowest dose of ZQRGD.

Comparison of α -SMA and Colla I

The mRNA expression of α -SMA and Colla I in group B were higher than those in group A, but lacked a significant difference (fig. 2). The mRNA expression of α -SMA in dose groups D and E was significantly lower than that in group B ($P < 0.05$). The mRNA expression of Colla I in each drug-intervention group was significantly lower than that in group B ($P < 0.05$).

Western blotting showed that that protein expression of α -SMA and Colla I was higher in group B, while the expression of α -SMA and Colla I in each drug intervention group was lower than that in group B; expression of α -SMA and Colla I was lowest in group C.

Expression of PI3K/AKT/mTOR

mRNA expression of PI3K and AKT was significantly higher in group B than those in group A (fig. 3). Compared with group B, mRNA expression of PI3K and AKT decreased in each drug intervention group, but

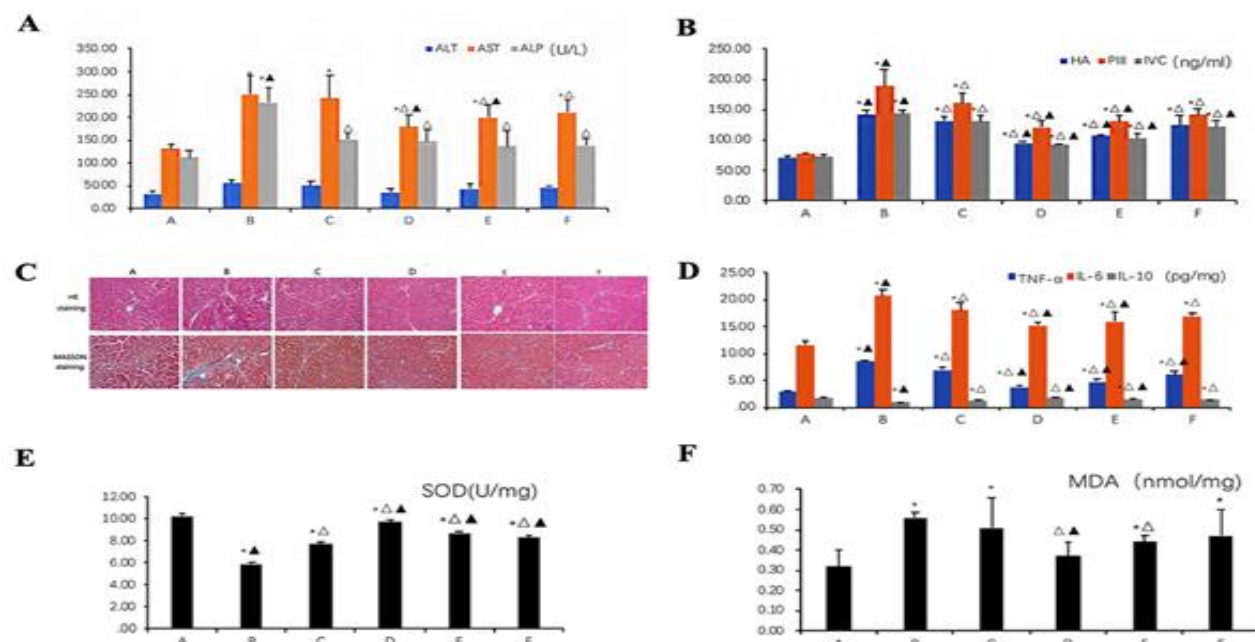


Fig. 1: **A:** Comparison of liver function indicators in each group. A is blank control group, B is model group, C is colchicine group, D, E, and F are ZQRGD high-, medium-, and low-dose group, respectively, and the following figures are the same. Compared with group A, * $P < 0.05$; compared with group B, $\Delta P < 0.05$; compared with group C, $\blacktriangle P < 0.05$. **B:** Comparison of liver fibrosis indicators in each group. Compared with group A, * $P < 0.05$; compared with group B, $\Delta P < 0.05$; compared with group C, $\blacktriangle P < 0.05$. **C:** Comparison of liver tissue histology changes in each group of rats (HE, $\times 100$; Masson, $\times 100$). HE staining of groups E and F showed low inflammatory cell infiltration and a minimal amount of cholestasis; Masson staining of both groups showed a small amount of fibrous connective tissue, which is less than that in the model group. **D:** Comparison of immunological indicators in each group. Compared with group A, * $P < 0.05$; compared with group B, $\Delta P < 0.05$; compared with group C, $\blacktriangle P < 0.05$. **E & F:** Comparison of SOD and MDA in each group. Compared with group A, * $P < 0.05$; compared with group B, $\Delta P < 0.05$; compared with group C, $\blacktriangle P < 0.05$.

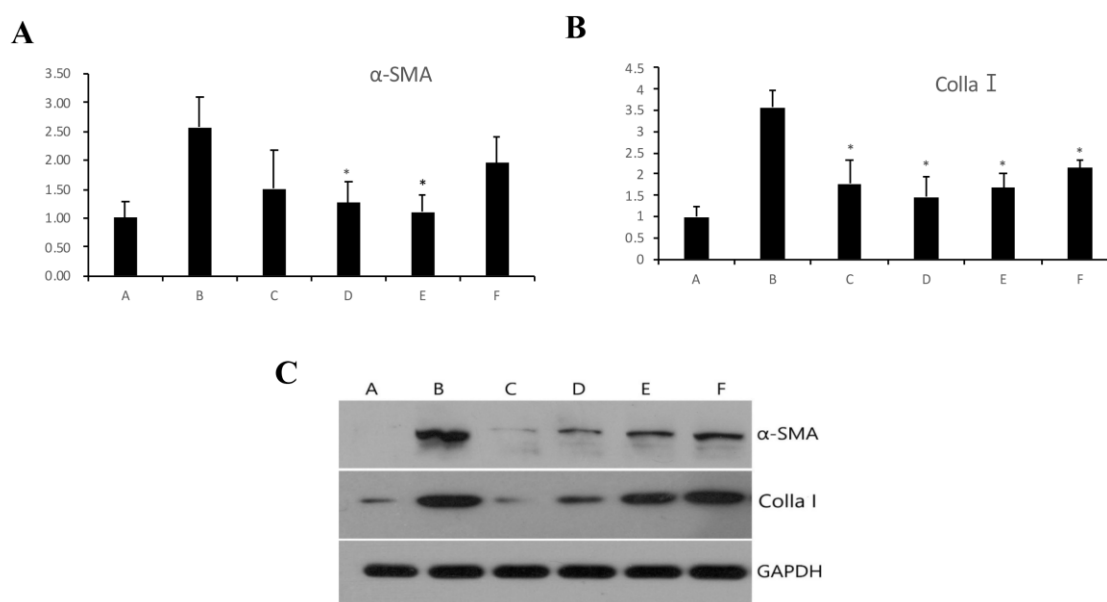


Fig. 2: Expression of α -SMA and Colla I in each group. **A:** mRNA of α -SMA. **B:** mRNA of Colla I. **C:** Protein expression of α -SMA and Colla I. Compared with group B, * $P < 0.05$.

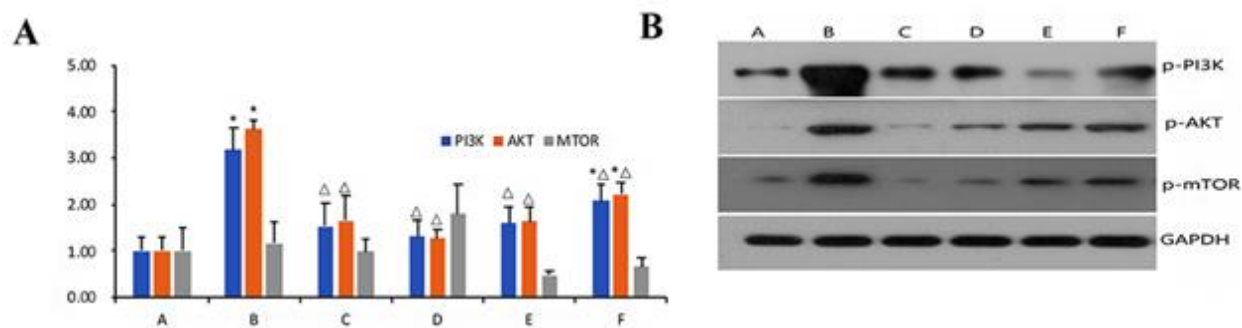


Fig. 3: Expression of p-PI3K, p-AKT, and p-mTOR in each group. **A:** mRNA expression. **B:** Protein expression. Compared with group A, * $P < 0.05$; compared with group B, $\Delta P < 0.05$.

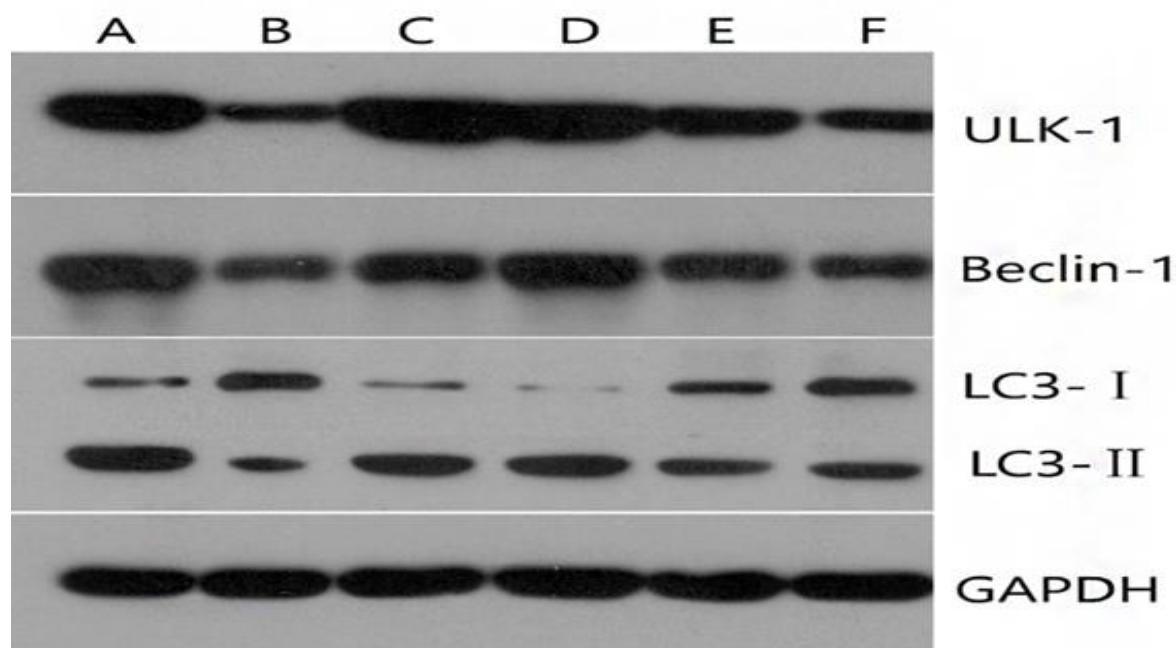


Fig. 4: Protein expression of ULK-1, Beclin-1, LC3I and LC3II in each group.

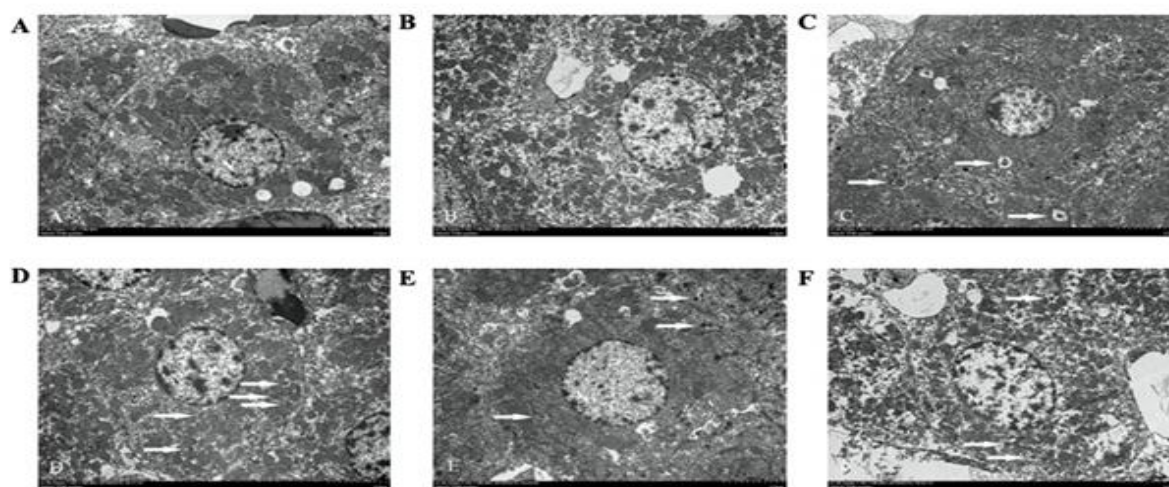


Fig. 5: Transmission electron microscope images of autophagy. N: cell nucleus. →: autophagosome.

there was no significant difference in mRNA expression of mTOR between these groups.

Western blotting showed that the protein expression of p-PI3K, p-AKT, and p-mTOR in group B was higher than that in group A. Compared with group B, p-PI3K, p-AKT and p-mTOR were reduced by different degrees in each drug intervention group.

Expression of autophagy-related proteins and the presence of autophagosomes

Western blotting showed that expression of ULK-1, Beclin-1, and LC3II/LC3I in group B was reduced to different extents than those in group A. Expression of ULK-1, Beclin-1, and LC3II/LC3I in each drug intervention group was increased to different degrees compared with group B, among which Beclin-1 and LC3II/LC3I were the highest in group D, while ULK-1 was the highest in group C (fig. 4). In addition, transmission electron microscope images also show the presence of autophagosomes (fig. 5).

DISCUSSION

In this study, the liver function indicator ALT was unchanged among all the groups, which may be because the highest ALT level was reached at 72 h after CCl₄ injection and then decreased back to the normal level. We found that ZQRGD can increase the concentration of SOD and reduce the concentration of MDA in the liver of rats with hepatic fibrosis, indicating that ZQRGD can eliminate excess oxygen free radicals and inhibit lipid peroxidation, thereby protecting the liver. Our study also shows that ZQRGD can reduce the concentration of IL-6 and TNF- α while increasing IL-10 levels in the liver.

Autophagy is a highly conserved intracellular metabolic process, and during liver fibrosis, is associated with the activation of HSC transforming into collagen-producing myofibroblasts (Thoen *et al.*, 2011). The expression of the autophagy-related gene LC3II in liver tissue was significantly increased in the liver fibrosis, indicating that autophagy activity was significantly enhanced in the liver fibrosis process (Chen *et al.*, 2010). When HSCs were treated with the autophagy inhibitor 3MA (Wang *et al.*, 2017), or when the autophagy-related gene Atg5 was knocked out in HSC, the number of lipid droplets in the cells and cell volume increased significantly, suggesting that inhibition of autophagy can inhibit the activation of HSC. Therefore, selectively blocking autophagy of HSC and liver fibroblast-like cells is considered to be one of the strategies for preventing and treating liver fibrosis.

PI3K/AKT signaling pathway regulates the proliferation and apoptosis of HSC and can adjust the synthesis and degradation of ECM to affect the progression of liver fibrosis (Wang *et al.*, 2015). Phosphorylated AKT

activates mTORC1, which inhibits the formation of the ULK1 complex and ultimately inhibits autophagy (Wang *et al.*, 2018). Our research shows that ZQRGD can s the protein expression levels of PI3K and Akt and decrease the phosphorylation levels of PI3K, Akt, and mTOR, while increasing the protein expression of ULK-1, Beclin-1 and LC3II/LC3I, indicating that ZQRGD can inhibit the PI3K/Akt/mTOR signal and further activate the autophagy pathway, thereby inhibiting HSC activation and exerting anti-fibrosis effects.

CONCLUSION

Ziqi ruangan decoction has favorable anti-liver fibrosis effects that is related to anti-oxidative stress reaction, the inhibition of an inflammatory response and the PI3K/Akt/mTOR signaling pathway, the activation of the autophagy pathway, and finally the inhibition of HSC activation. The causal relationship between autophagy and hepatic fibrosis remains unclear and is worthy of further study.

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