Effect of Qizhu Granule on Liver Sinusoidal Endothelial Cells Capillarization in Carbon Tetrachloride-Induced Rats Fibrosis.

Rong Zhang (1695724998@qq.com)
China Academy of Chinese Medical Sciences Guanganmen Hospital https://orcid.org/0000-0001-9601-4535

Shao Neng Liu
China Academy of Traditional Chinese Medicine Guanganmen Hospital: China Academy of Chinese Medical Sciences Guanganmen Hospital

Zheng Ji Ma
China Academy of Traditional Chinese Medicine Guanganmen Hospital: China Academy of Chinese Medical Sciences Guanganmen Hospital

Yuan Jia Ding
China Academy of Traditional Chinese Medicine Guanganmen Hospital: China Academy of Chinese Medical Sciences Guanganmen Hospital

Min Hui Liu
China Academy of Traditional Chinese Medicine Guanganmen Hospital: China Academy of Chinese Medical Sciences Guanganmen Hospital

Ling Zhang
China Academy of Traditional Chinese Medicine Guanganmen Hospital: China Academy of Chinese Medical Sciences Guanganmen Hospital

Dong Ying Lu
China Academy of Traditional Chinese Medicine Guanganmen Hospital: China Academy of Chinese Medical Sciences Guanganmen Hospital

Research

Keywords: Qizhu granule, liver fibrosis, liver sinus endothelial cells, Angiogenesis, fenestration.

Posted Date: September 17th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-76714/v1

Effect of Qizhu Granule on Liver Sinusoidal Endothelial Cells Capillarization in Carbon Tetrachloride-induced Rats Fibrosis

Rong Zhang[1], Shaoneng Liu[1], Jizheng Ma, Jiayuan Ding, Huimin Liu, Ling Zhang, Yingdong Lu.

Guanganmen Hospital of Chinese Academy of Traditional Chinese Medicine

Gastroenterology Department, Guanganmen Hospital of Chinese Academy of Traditional Chinese Medicine,Beijing,China.

Corresponding author at: Guanganmen Hospital of Chinese Academy of Traditional Chinese Medicine, No. 5, North Line Pavilion, Xicheng District, 100053 Beijing, China.

E-mail address: liushaoneng@sohu.com (Shaoneng Liu).

[1] Both authors contributed equally to this work.

Abstract

Background: Qizhu granule, a traditional Chinese medicine, has been widely used in clinic as a complementary and alternative medicine to treat liver fibrosis.However, the mechanism underlying its anti-hepatic fibrosis is still not clear. Liver fibrosis accompanied by liver sinusoidal pathological angiogenesis has been highlighted as novel therapeutic targets for the treatment of chronic liver disease. In this study, we investigated the mechanism of anti-capillarization of this herbal drug against liver fibrosis.

Materials and methods: The liver fibrosis rats model induced by 4-week of intervention with 40% CCl_4 was employed in this study. Meanwhile, low, medium and high dose serum containing Qizhu granules were prepared. Liver tissues were acquired, and liver samples were subjected to histological studies.LSECs were isolated from liver fibrosis rats and were routinely cultured for 48h in low, medium and high dose of Qizhu granules-containing serum. The fenestration of LSECs in liver fibrosis rats were observed under scanning electron microscopy. The expression of the endothelial cell surface markers CD31, SE-1 and LSECs integrin αVβ3, FAK, p-FAK, Ras, MAPK, p-MAPK were measured by western blot.

Results: Compared with the control group, the loss of fenestration of LSECs in the model group increased. After intervention of Qizhu granule-containing serum, the fenestration of the structure of LSECs in liver fibrosis induced by CCl_4 increased, especially in the high-dose Qizhu granules group. Compared with the control group, the expression levels of SE-1 and CD31 in LSECs in the model group were significantly increased ($P < 0.05$). Compared with the model group, the expression levels of SE-1 and CD31 in LSECs of rats with hepatic fibrosis induced by CCl_4 decreased after the treatment of low, medium and high dose serum containing Qizhu granules ($P < 0.05$). Among them, the expression levels of SE-1 and CD31 in LSECs of Qizhu granules group decreased with the increase of dosage, showing a dose-dependent relationship to a certain extent. Compared with the normal control group, the expression of integrin αVβ3, Ras, p-FAK and p-MAPK protein increased in the LSECs of model group($P <0.05$). After treatment with Qizhu granule-containing serum, the expression of integrin αVβ3, p-FAK and p-MAPK protein in LSECs of liver fibrosis rats induced by CCl_4 were reduced ($P <0.05$), and the expression of FAK, Ras and MAPK protein decreased ($P> 0.05$).

Conclusions: Qizhu granule could reduce the loss of fenestration of LSECs, transforming the cell phenotype of LSECs, and ameliorating the pathological remodeling of hepatic sinus capillarization in hepatic fibrosis induced by CCl_4 in rats. It was found that Qizhu granules played an anti-fibrosis role by suppressing the expression of integrin αVβ3-FAK-Ras/MAPK signaling pathway of LSECs in CCl_4-induced fibrosis rats.

Keywords: Qizhu granule; liver fibrosis; liver sinus endothelial cells; Angiogenesis; fenestration.

1. Background

Hepatic fibrosis, which is a common pathological process involved in the development of various etiologies including infection, alcohol, cholestasis, drugs, metabolic disorders, or immune attack[1-4], is far from an ideal effect in their effective prevention and treatment. Its end stage, cirrhosis, is a major clinical problem worldwide due to its life-threatening complications of portal hypertension, liver failure, and hepatocellular carcinoma[5]. To

date, no approved antifibrosis therapy is used in clinical practice, largely owing to the various side effects and uncertain positive clinical outcomes[2, 6]. For liver cirrhosis, the end-stage of liver fibrosis, liver transplantation is the only curative therapy. Therefore, effective anti-fibrosis therapeutics are required urgently.

Liver fibrosis is characterized by the deposition of extracellular matrix proteins and increased intrahepatic angiogenesis induced by the activation of hepaticstellate cells(HSCs) and liver sinusoidal endothelial cells (LSECs)[7]. Hepatic fibrosis is accompanied by angiogenesis. Recent data indicate that hepatic angiogenesis, regardless of the etiology, takes place in chronic liver diseases (CLDs) that are characterized by inflammation and progressive fibrosis. Because antiangiogenic therapy has been found to be efficient in the prevention of fibrosis in experimental models of CLDs, it is suggested that blocking angiogenesis could be a promising therapeutic option in patients with advanced fibrosis[7-10].

Traditional Chinese herbal medicines (TCM) are multiingredient extracts with low adverse effects in the treatment of chronic liver diseases and are effective in preventing fibrogenesis and other chronic liver injuries[4, 11-13]. Qizhu granule is a complex prescription of Chinese herbal medicine consisting of 10 medical herbs (Astragalus Membranaceus, Curcuma Zedoary, Atractylodes Macrocephala, Bupleurum Chinensis, Salvia Miltiorrhiza, Peach Kernel, Oriental Wormwood, Radix Curcumae, Rhizoma Menispermi and Radix Liquiritiae). It is a TCM formula for the treatment of chronic liver disease. The clinical prevention and treatment of hepatic fibrosis and early hepatic cirrhosis also confirm its obvious anti-fibrosis effects. However, the function and mechanism of Qizhu granule in treating hepatic fibrosis are still unclear. The aim of the current study was to determine the anti-fibrogenic effects of Qizhu granule in terms of pathological alterations of the fenestration and phenotypes of the LSECs in CCl_4-induced fibrotic rat.

2.Materials and methods

2.1 Materials

Pathology Microtome (Leica Instruments Co.,Ltd.,RM2016, Shanghai, China), Embedding Machine (Junjie Electronics Co.,Ltd., JB-P5,Wuhan, China), Dehydration Machine (Junjie Electronics Co., Ltd., JJ-12J, Wuhan, China), Freezing Table (Junjie Electronics Co., Ltd., JB-L5, Wuhan, China), Organizing Spreader (Kodi Instrument Equipment Co., Ltd.,KD-P,Zhejiang,China), Oven (Huitai Instrument Manufacturing Co., Ltd., DHG-9140A, Shanghai, China), Slides and Coverslips (Shitai Experimental Equipment Co., Ltd., 10212432C, Jiangsu, China), Upright Optical Microscope (Olympus, IX71), CCl_4 (Aladdin Bio, C112041-500mL), Absolute Ethanol, Xylene, Hydrochloric Acid, Ammonia, Neutral Gum (Sinopharm Group Chemical Reagent Co., Ltd., China), Masson Staining Kit (Solaibao, G1340-7, Beijing, China), Toluene Ammonia Blue Staining Solution (Google Biotechnology, G1032, Wuhan, China), Ultra-clean Workbench (SW-CJ-1FD, Suzhou, China), CO_2 incubator (SANYO (XD-101), Japan), Biological Inverted Microscope (OLYMPUS (IX71), Germany), Desktop Low-speed Centrifuge (Medical Equipment Co., Ltd. Medical Equipment Factory (80-2), Shanghai, China), Oscillator (Shanghai West Analytical Instrument Factory (WH-2), China), Vertical Pressure Cooker (BoXun (YXQ-LS-50), Shanghai, China), Cell Culture Flask (FALCON China (353014)), Electrophoresis Instrument (Tanon, EPS300), Enzyme Standard Instrument (Thermo, muLISKANMK3), 4 ℃ Centrifuge (Eppendorf, Centrifuge 5415R), Scanning Electron Microscope (Hitachi, SU8010).

2.2 Preparation of Qizhu granule

Qizhu granule (Document No.: Beijing medicine Z20063189) were prepared and provided by the pharmaceutical preparation room of Guanganmen Hospital of Chinese Academy of traditional Chinese medicine (Beijing, China). The clinical usage was to take 6g each time, twice a day, with a total daily dosage of 12g. The dose conversion coefficient for each Kg of body weight of animals and humans was 6.25 (refer to modern medical experimental zoology edited by shi xinyou), and the dosage for rats was 1.25g/Kg/d.

2.3 Animal models

Seventy male Wistar rats (180–200g) were provided by the laboratory animal center of the academy of military medical sciences(Beijing, China). The rats were kept in cages in an environmentally controlled room (20±2℃, relative humidity 36%) on a 12-h light/dark cycle and allowed free access to food and water, fed with standard synthetic feed, and drink sterile water specially. All protocols of this study were approved by the Animal Ethics Committee of Guanganmen Hospital of Chinese Academy of Traditional Chinese Medicine.

2.3.1 Preparation of drug serum

Fifty Wistar rats were randomly divided into 5 groups:the first group consisted of 10 rats. These rats were treated with high dose of Qizhu granule (gavage twice a day+ 5 times+ treatment with Qizhu granule 2.5g/Kg/d). The second group consisted of 10 rats, and these rats were treated with medium dose of Qizhu granule (gavage twice a day+ 5 times+ treatment with medium dose of Qizhu granule 1.25g/Kg/d). The third group consisted of 10 rats, and these rats were treated with low dose of Qizhu granule (gavage twice a day+ 5 times+ treatment with low dose of Qizhu granule 0.625g/Kg/d). The fourth group consisted of 10 rats,and these rats were treated with three steamed water(gavage twice a day+ 5 times+ treatment with three steamed water). The fifth group consisted of 10 rats,these rats were treated with three steamed water (gavage twice a day+ 5 times+ treatment with three steamed water). All rats were given 1.0ml/100g gavage.Blood was collected from the heart in super clean taichung within 1 hour after the last gavage. Blood samples were stored in refrigerator vertically standing for 3 hours at 4 ℃. Those samples were centrifuged at 3000 RPM * 15 min 4 ℃. The centrifugal aseptic conditions after the serum was isolated and in 56 ℃ water bath inactivated serum 30 min, inactivated after using 0.22 um microporous membrane filter in addition to bacteria.

2.3.2 Establishment of liver fibrosis model

The remaining 20 rats were randomly divided into two groups, one group was injected intraperitoneally with 40% CCl_4 (diluted in olive oil) at 3ml/kg body weight twice weekly for 4 weeks to establish the liver fibrosis model. and the other group was injected with olive oil solution (3ml/kg weight) twice a week for 4 weeks. On the fourth weekend, 2 rats were randomly killed in each group to observe whether CCl_4 induced liver fibrosis was successful. After observing the successful modeling, the remaining two groups of rats were sacrificed. Pentobarbital anesthesia before surgery (0.03 ml/10g, intraperitoneal injection). The livers were rapidly removed, and liver tissue was taken from the right lobe of the livers, fixed in 10% phosphate-buffered formaldehyde, and processed for embedding into paraffin. The liver tissue sections were stained with hematoxylin and eosin (HE) for morphological evaluation. Masson trichrome and Sirius Red staining were used to assess the degree of liver fibrosis.

2.3.3 LSECs Isolation and Culture

After the liver fibrosis rats were successfully made, the rats were anesthetized to death, a sterile 30G needle was inserted into the portal vein and the inferior vena cava was cut. HBSS containing 0.5mm EDTA and containing no Ca^{2+} or Mg^{2+} was injected for 10min at a speed of 5ml/min. Then annotated with collagenase and Ca^{2+} and Mg^{2+} HBSS containing 0.02% type IV for 10 min at a rate of 5 ml/min. At the end of perfusion, the liver was carefully removed and transferred to the DMEM containing 5% serum, and the liver capsule was torn open with sterile forceps to allow the outflow of liver parenchymal and non-parenchymal cells. The collected cell suspension was centrifuged at a low speed of 50×g for 10min, supernatant was collected, and the precipitation was centrifuged at 400×g for 10min, and the precipitation was resuspended with a complete DMEM medium and carefully added to centrifuge tubes containing 50% percoll and 25% percoll. After centrifugation at a high speed of 900×g for 20 minutes (without brakes), obvious bands of non-essential cells were observed between 25% percoll and 50% percoll. To suck out it carefully after DMEM medium heavy completely suspended and centrifugal wash away the remnants of percoll liquid. Non-parenchymal cells suspension was added to 24-orifice plate and placed in the cell cultivation for 20 min, did not stick with containing 1% after parietal cells suspension centrifugal blood

endothelial growth factor (ECGS) and 5% of endothelial cell culture medium (ECM) according to the 2×10^6/ml after suspension inoculation in rat tail collagen package is 6 orifice or in 96 - well plates. The cells were cultured at 37℃ with 5% CO_2.

2.4 LSECs identification

2.4.1 Trypan blue staining

Trypan blue staining was performed on the LSECs of each group of rats. Slides were photographed and observed under an inverted microscope to calculate the percentage of the total number of living cells (number of unstained cells). Cell survival rate = (total number of cells-blue cells) / total number of cells×100%.

2.4.2 Flow cytometry

When the cells grow to cover 80% or more of the culture flask, discard the culture solution and wash the cells once with PBS. Digest with 0.25% trypsin, then add anti-rabbit CD31 and anti-mouse SE-1 to the cells, and incubate overnight at 4℃. Centrifuge, rinse with PBS, add secondary antibody, and measure the expression rate of PE-CD31 and FITC-SE-1 by flow cytometry.

2.5 LSECs experimental protocol

Control group: Normal rats liver sinusoidal endothelial cells, plus normal drug-free serum. Model group: liver sinusoidal endothelial cells with fibrosis, plus normal drug-free serum. Low dosage group: liver sinusoidal endothelial cells with fibrosis, plus serum containing low dose of Qizhu granules. Medium dosage group: liver sinusoidal endothelial cells with fibrosis, plus serum containing middle dose of Qizhu granules. High dosage group: liver sinusoidal endothelial cells with fibrosis, plus serum containing high dose of Qizhu granules. Serum addition amount is 10%, cultured in 37℃, 5% CO_2 incubator, changing fluid after adherence, and testing for relevant indicators after 48 hours of culture.

2.6 Observe Content and Methods

2.6.1 Western blot detection of CD31, SE-1, αvβ3, FAK, p-FAK, Ras, MAPK and p-MAPK protein expression

The collected cells were lysed with lysate containing protease inhibitors (Complete, Roche) (50 mM Tris-Cl, pH 7.4, 1 mM EDTA pH 8.0, 250 mM NaCl, and 1% Triton-X). The protein lysate concentration was measured using the BCA method (Beyotime P0011). After mixing 10 μg cell lysate with 5x sample buffer (15 g SDS, 15.6 ml 2 M Tris pH 6.8, 57.5 g glycerol,16.6 ml b-mercaptoethanol), load the sample into a 10% polyacrylamide gel , SDS-PAGE separation and transfer to PVDF membrane (Bio-Rad no.162-0177). After blocking with 4% milk containing 0.1% Tween, add the following antibodies and incubate overnight at 4℃: SE-1 antibody (1:1000; Novus no.NB110-68095SS), CD31 antibody (1: 500; Affinity no.AF6191), αvß3 antibody (1: 1000; Affinity no. DF6815), FAK antibody (1: 1000; Affinity no. AF6397), p-FAK antibody (1: 1000; Affinity no. AF3398), MAPK antibody (1: 1000; Affinity no. AF6456), p-MARK antibody (1:1000; Affinity no. AF4001), GAPDH antibody (1: 1000; Affnity no. AF7021). After washing the membrane 3 times with PBS solution containing 0.1% Tween, add 4% milk containing 0.1% Tween and HRP secondary antibody (Abcam, ab6721) and incubate at room temperature for 2 hours. Remove the membrane, add ECL developing solution (Bio-Rad no.170-5060) dropwise to the

membrane and put it into the GelDoc imaging system (Bio-Rad) to take pictures. The protein expression level was normalized with the internal reference protein GAPDH.

2.6.2 Scanning electron microscope analysis

LSECs in each group were washed with PBS for 3 times, then 2ml of electron microscope fixing solution was added and stored in the refrigerator at 4℃, and the fenestrated structure of the hepatic sinus endothelium were observed by scanning electron microscope.

2.7 Statistical analysis

Mean ± standard deviation was used for statistical description of measurement data, and t test was used for measurement data. $P < 0.05$ indicated significant difference in the test. SPSS 17.0 software was used for statistical analysis

3. Results

3.1 Establishment of rats liver fibrosis model

The rats were killed after 4 weeks, and the liver tissues of each group were stained with HE and Masson (Figure 1) to observe the damage of the liver tissues and the liver fibrosis. The Control group showed normal lobular architecture, liver cells with well-preserved cytoplasm and well-defined nucleus.However, typical steatosis and portal and lobular inflammation were observed in the CCL_4 group after 4 weeks of CCl_4-induced,which were full fat vacuoles in lobule cells, infiltration of inflammatory cells, cell swelling and lipid degeneration in the central region of the lobules. Masson staining showed that normal rats had no fibrous hyperplasia, while the model group had proliferated fibrous tissue and the formation of fibrous septa, indicating that the animal model of hepatic fibrosis was successfully established.

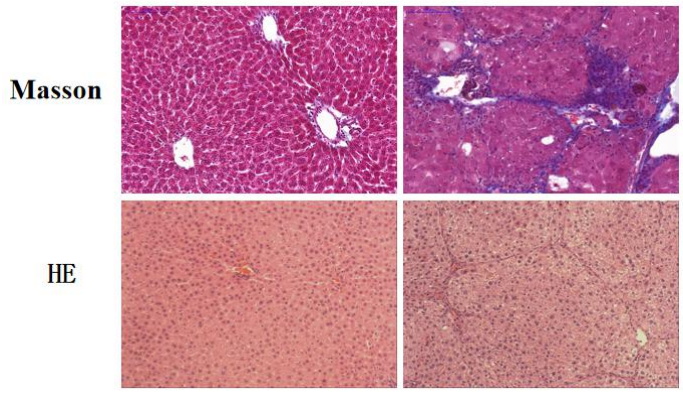

Figure 1 The rat liver tissue, Stain, Masson; scale bar, 2 μm. HE stain,×200

3.2 LSECs observation

In this study, trypan blue staining and flow cytometry were used to identify the obtained LSECs. The results of trypan blue staining showed that there was no significant difference in the cell survival rate of LSECs between the liver fibrosis model group and the normal control group.Cell viability was about 80% in both groups (see figure 2). Flow cytometry was used to detect the proportion of CD31+ cells and SE-1+ cells in rat hepatic sinus endothelial cells in each group. As can be seen from the results, the proportion of CD31+ cells and SE-1+ cells in LSECs in both the control group and the hepatic fibrosis group was over 90%. Compared with the control group, both the proportion of CD31+ cells and the proportion of SE-1+ cells in the LSECs group of liver fibrosis were significantly increased (see figure 3).

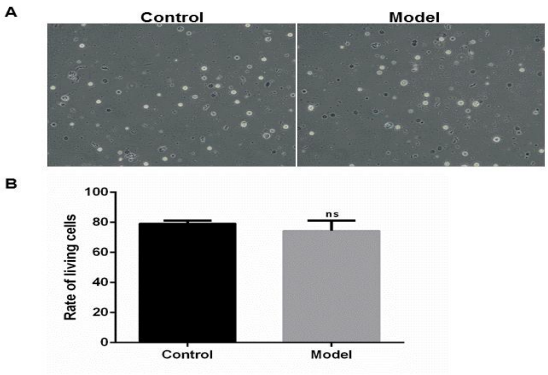

Figure 2 A. Trypan blue staining of hepatic sinus endothelial cells.

A. Trypan blue staining of hepatic sinus endothelial cells in each group,image magnification: 400 times. The Control group represented the normal Control group, and the Model group represented the liver fibrosis Model group. B. Comparison of cell survival rate between the two groups, and T-test was used for data analysis, with no statistical significance.

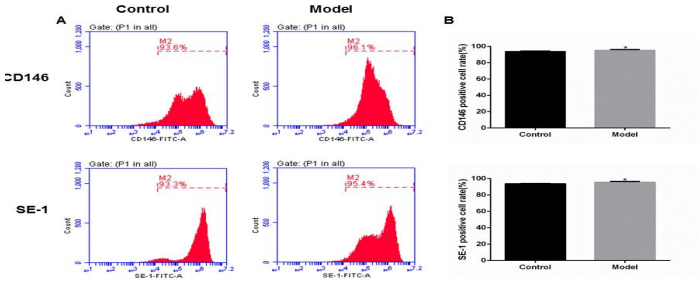

Figure 3. Identification of LSECs by flow cytometry.

A. LSECs were identified by flow cytometry. B. The LSECs flow test results of each group were analyzed, and the data were analyzed by t-test, * denoting $p<0.05$ (vs Control).

3.3 Effect of Qishu granules on angiogenesis of hepatic sinus endothelial cells.

3.3.1 Effect of Qishu granules on the fenestrae of LSECs.

Numerous fenestrae on the surface of normal LSECs were apparent under a scanning electron microscope(Figure4 A). The LSECs from Model group were in a fibrotic state and exhibited disappearance of LSEC fenestrae (Figure4 B).The LSECs from Model group treated with low dosage of Qizhu granule (48h, 0.625g/Kg/d)containing drug serum presented with fenestrae of LSECs similar to those of normal control rats(Figure4 C). The LSECs from Model group treated with middle dosage of Qizhu granule (48h, 1.25g/Kg/d)containing drug serum presented with fenestrae of LSECs similar to those of normal control rats(Figure4 D). The LSECs from Model group treated with high dosage of Qizhu granule (48h, 2.5g/Kg/d)containing drug serum demonstrated fenestrae of LSECs (Figure4 E)similar to control group and cell junctions between hepatocytes similar to control rats.

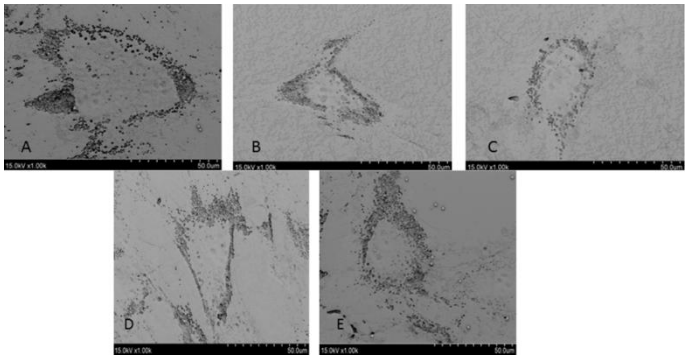

Figure 4. The fenestration on LSECs in each group was observed by scanning electron microscopy(scale bar = 50 μm).

A. LSECs in normal group,plused normal serum without drugs. B. Model group,model LSECs,plused normal drug-free serum. C. Qizhu granules low-dose group: model LSECs and added low-dose Qizhu granule-containing serum for treatment. D. Qizhu granules middle-dose group: model LSECs, plused medium-dose Qizhu granule-containing serum treatment. E. Qizhu granules high-dose group: model LSECs and added high-dose Qizhu granule-containing serum for treatment.

3.3.2 Effect of Qishu granules on the phenotype of LSECs.

CD31 and SE-1 were commonly used as vascular endothelial markers and these were rarely expressed in normal liver. However, these indicators were increased in expression of hepatic fibrosis after sinusoidal capillary vascularization. Immunohistochemical staining was used to detect the expression of CD31 and SE-1 in sinusoidal endothelial cells of each group. The results showed that the expression of CD31 and SE-1 in sinusoidal endothelial cells of CCl_4 group was significantly higher than that in normal group ($P<0.05$). After administration of Qizhu granules,the expression of CD31 and SE-1 was significantly decreased in hepatic sinusoidal endothelial cells($P<0.05$). The results showed that Qishu Granules could inhibit the expression of CD31 and SE-1 in hepatic sinusoidal endothelial cells of rats with hepatic fibrosis (Figure 5).

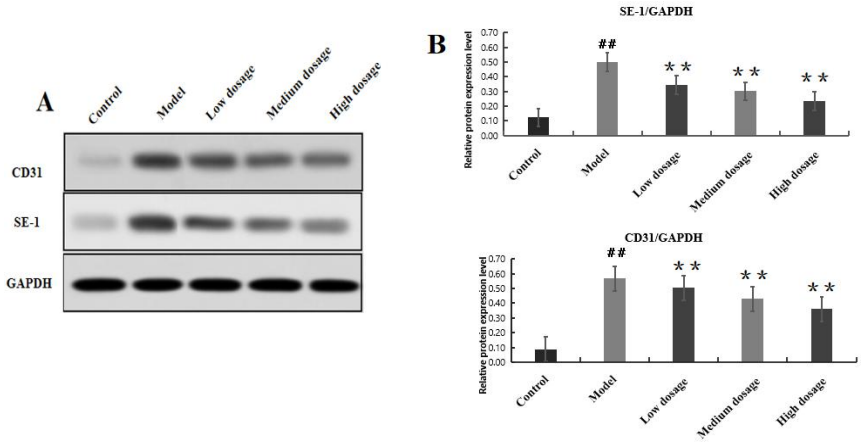

Figure 5 Expression of SE-1 and CD31 in LSECs of each group.

A: SE-1 and CD31 expression in each group. Control group: normal control group LSECs, plused normal drug-free serum. Model group: Model group LSECs, plused normal drug-free serum. Low dosage: model LSECs, plused low-dose drug serum of Qishu granules. Medium dosage: LSECs for modeling, plused medicinal serum for Qishu granules dose. High dosage: model LSECs, plused high-dose drug serum of Qishu granules. **B**: Analysis of the expression levels of SE-1, CD31 in each group.Compared with the normal control group $^{\#\#}P<0.05$, compared with the model group $^{**}P<0.05$.

3.4 Effect of Qizhu Granules on integrin αVβ3-FAK-Ras / MAPK signaling pathway of liver sinusoidal endothelial cells.

Compared with the normal control group, the integrin αVβ3 protein level in LSECs in the model group increased significantly (P <0.05), and the expression levels of Phosphor-FAK(p-FAK), Phosphor-MAPK(p-MAPK) and Ras protein were also all increased (P <0.05). These results indicated that the expression of integrin αVβ3, FAK, Ras and MAPK proteins in LSECs were activated during hepatic fibrosis.Compared with model group, the low, middle and high dose of Qizhu Granules attenuated the up-regulation of integrin αVβ3, FAK, Ras, MAPK in LSECs of liver fibrosis rats induced by CCl_4. The low, middle and high dose of Qizhu Granules suppressed integrin αVβ3, p-FAK and p-MAPK in LSECs in fibrosis rats induced by CCl_4 simultaneously (P<0.05).(Figure 6)

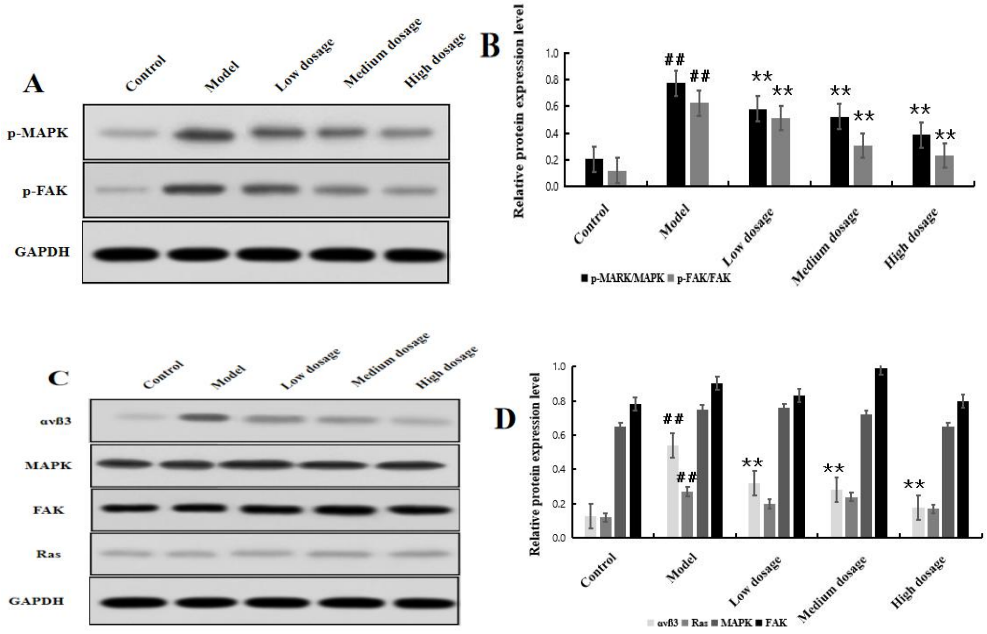

Figure 6. Qizhu Granules suppressed angiogenesis via inhibiting αVβ3-FAK-Ras / MAPK signaling pathway. Western blot for FAK and MAPK phosphorylated (A)and total integrin αVβ3, FAK, Ras and MAPK expression (C) were shown.GAPDH was used as loading control. The experiments were repeated at least three times. Data are presented as means±SEM (B, D). Compared with the control group $^{\#\#}$P<0.05, compared with the model group** P<0.05.

4. Discussion

Liver fibrosis can result from chronic liver injuries of any etiology. Perpetuation of the fibrotic reaction can lead to severe hepatic dysfunctions and even life-threatening scenarios such as end-stage liver disease, cirrhosis and hepatocarcinoma, whose incidence is increasing worldwide[2]. Globally, 1.4 million deaths occur annually as a result of chronic liver disease[13]. Effective medical treatment for liver fibrosis is much needed. Currently, orthotopic liver transplantation is the only definitive therapeutic option for terminal liver diseases[4]. However, its clinical application is limited due to poor long-term graft survival, shortage of donor organs, and high costs associated with the procedure[14]. Despite this, no antifibrotics are currently licensed for use in humans[5]. Western medicines lack targeted drugs that can reverse liver fibrosis and repair injured livers in any meaningful way[6].

A variety of small molecules and biological agents are being developed to treat hepatic fibrosis. However, as mediated by several pathogenic mechanisms mediate hepatic fibrosis, these agents have not yet been successfully employed in anti-hepatic fibrosis treatment regimens in a clinical setting. Many clinical and experimental studies have shown that TCMs are superior because they exert pharmacological effects by acting on multiple targets

during the treatment of complex disease[15, 16], which can delay or curb liver fibrosis and even cirrhosis. Thus, TCMs with anti-liver fibrosis properties are becoming the focus of studies on the prevention and treatment of this disease[17]. Qizhu granule, a Chinese medicinal formula, is used for the prevention and treatment of chronic liver disease. Moreover, Qishu granule-specific adverse drug reactions have not yet been observed in a clinical setting. However,at present, there is no study of the effect on hepatic sinusoidal endothelial cells of Qizhu granules. This experiment intends to study the anti-hepatic fibrosis mechanism of Qizhu granules from the perspective of capillarization and angiogenesis of hepatic sinusoidal endothelial cells.

The high metabolic activity of the liver demands an efficient vasculature to the organ. The intricate network of capillaries of the liver is lined by LSECs. LSECs are highly specialized fenestrated cells without a basement membrane. A specialized endothelial cell type is phenotypically different from vascular endothelial cells[18]. LSEC is a type of liver specific microvascular cell characterizes unique phenotype. They are unique among other vascular endothelia[19]. Fenestration is generally considered to be a reliable marker of LSECs, making them clearly distinguishable from all other types of liver cells including endothelial cells from larger vessels[19]. Fenestrations are their most significant morphological feature, but can only be visualized by scanning electron microscopy[20]. In normal liver, differentiated LSECs form capillaries of microvasculature and facilitate filtration by fenestrae as a selectively permeable barrier between liver parenchyma and sinusoid[21, 22].

Hepatic sinusoidal capillarization is a basic pathological change associated with hepatic fibrosis and cirrhosis[23-26]. Hepatic sinusoidal capillarization plays a key role in the pathogenesis and progression of this process. Defenestration of LSECs, a typical phenomenon that occurs during hepatic sinusoidal capillarization, plays a unique role in liver fibrosis and cirrhosis.This differentiation state is called capillarization and corresponding to the loss of LSEC-specific markers, and loss of LSECs fenestration.Restoration of LSECs phenotype could present a potential route for promoting the regression of fibrosis. Enhancing the LSECs could be a potential target for therapeutic purposes[27, 28] . In this study,the rats with liver fibrosis mediated by CCl_4, the expression of CD31 in hepatic sinus endothelial cells was significantly increased, and the expression of CD31 and SE-1 were decreased after the treatment with Qizhu granule containing serum.

Integrin αvβ3 is rarely expressed in normal liver tissues, but its expression is up-regulated during angiogenesis. Recent studies have shown that upregulation of integrin αvβ3 is a key receptor molecule in vascular reconstruction and a common biomarker for angiogenesis[29, 30] . In this study,compared with the normal group, the expression of LSECs.Integrin were significantly increased in the model group, which was consistent with the literature reports. Integrins can activate integrin-related cytoplasmic signaling proteins to initiate intracellular signal transduction[31]. FAK is an important integrin-related tyrosine kinase signaling protein. In the process of liver fibrosis, the level of integrin in LSECs increases, and FAK is an important cytokine in the integrin signaling pathway, which is involved in angiogenesis by regulating the downstream ras-mapk pathway[32] and plays a role in promoting fibrosis, among which drug inhibition of FAK can play an anti-fibrosis therapeutic effect[33]. The results of this study suggest that the antifibrosis effect of Qizhu granule may be related to its regulation of the integrin αVβ3-FAK-Ras / MAPK signaling pathway.

5. Conclusions

In the present study, our work further indicated that Qizhu granules attenuated LSECs capillarization in rats model of liver fibrosis induced by CCl_4. As there is no available approaches to inhibit sinusoidal capillarization and liver angiogenesis, Qizhu granules shed a light on treatment of liver fibrosis. Of course, the results based on in vitro and animal models cannot refect patient’s situation in a large context. Species, etiology, natural histology and distinct pathophysiological response between patients and animals make good experimental achievements cannot translate into clinical success. In the future, a clinical trial should be considered to assess the role of Qizhu granules on liver fibrosis.

Abbreviations

CCl4: carbon tetrachloride; CD31: Cluster of differentiation 31; Sinusoidal Endothelial-1: SE-1; LSECs: Liver Sinusoidal Endothelial Cells; αVβ3: Integrinαvβ3; FAK: Focal Adhesion Kinase; p-FAK: phospho-FAK; MAPK: mitogen-activated protein kinase; p-MAPK: phospho-MAPK; CLDs: chronic liver diseases ; TCM: Traditional Chinese herbal medicines.

Acknowledgements

Not applicable.

Authors' contributions

RZ and SNL conceived the original idea, JZM, JZD, HML, LZ, YDL conducted all the experiments, searched the databases, analyzed the data, created the illustrations, and drafted the manuscript, RZ and SNL had primary responsibility for final contengt. All authors read and approved the final manuscript.

Funding

This work was supported by grants from National Natural Science Foundation of China (Grant No. 81573917).

Availability of data and materials

All data generated or analyzed during this study are included in the text.

Ethics declarations

Ethics approval and consent to participate.

All animal protocols in the study were performed in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. All experiments involving animals were approved by the Institutional Animal Care and Use Committee of Guanganmen Hospital of Chinese Academy of Traditional Chinese Medicine. Ethics No. IACUC-GAMH-2020-003.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References:

1. Iredale, JP. Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. J Clin Invest. 2007; 117(3): 539-48. http://10.1172/JCI30542.
2. Hernandez-Gea V, Friedman SL. Pathogenesis of Liver Fibrosis. Annu Rev Pathol. 2011; 6: 425-56. http://10.1146/annurev-pathol-011110-130246.
3. Parveen N, Aleem AK, Habeeb MA, Habibullah CM. An update on hepatic stem cells: bench to bedside. Curr Pharm Biotechnol. 2011; 12(2): 226-30. http://10.2174/138920111794295765.
4. Dong S, Su SB. Advances in mesenchymal stem cells combined with traditional Chinese medicine therapy for liver fibrosis. J Integr Med. 2014; 12(3): 147-55. http://10.1016/S2095-4964(14)60022-4.

5. Fallowfield JA. Therapeutic targets in liver fibrosis. Am J Physiol Gastrointest Liver Physiol, 2011; 300(5): G709-15. http://10.1152/ajpgi.00451.2010.
6. Sobaniec-Lotowska ME, Lotowska JM, Lebensztejn DM. Ultrastructure of oval cells in children with chronic hepatitis B,with special emphasis on the stage of liver fibrosis: the first pediatric study. World J Gastroenterol. 2007;13(21): 2918-22. http://10.3748/wjg.v13.i21.2918.
7. Iwakiri Y, Shah V, Rockey DC. Vascular pathobiology in chronic liver disease and cirrhosis-current status and future directions. J Hepatol. 2014; 61(4): 912-24. http://10.1016/j.jhep.2014.05.047.
8. Fernández M, Semela D, Bruix J, Colle I, Pinzani M, Bosch J. Angiogenesis in liver disease. J Hepatol. 2009; 50(3): 604-20. http://10.1016/j.jhep.2008.12.011.
9. Ciupińska-Kajor M, Hartleb M, Kajor M, Kukla M, Wyleżoł M, Lange D, Liszka L. Hepatic angiogenesis and fibrosis are common features in morbidly obese patients. Hepatol Int. 2013;7(1): 233-40. http://10.1007/s12072-011-9320-9.
10. Shah VH, Bruix J. Antiangiogenic therapy: not just for cancer anymore? Hepatology. Hepatology. 2009; 49(4): 1066-8. http://10.1002/hep.22872.
11. Mu Y, Liu P, Du G, Du J, Wang G, Long A, Wang L, Li F. Action mechanism of Yi Guan Jian Decoction on CCl_4 induced cirrhosis in rats.J Ethnopharmacol. 2009; 121(1): 35-42. http://10.1016/j.jep.2008.09.032.
12. Lou JL, Jiang MN, Li C, Zhou Q, He X, Lei HY, Li J, Jia YJ. Herb medicine Gan-fu-kang attenuates liver injury in a rat fibrotic model. J Ethnopharmacol. 2009; 128(1): 131-8. http://10.1016/j.jep.2009.12.038.
13. Liu HL, Lv J, Zhao ZM, Liu CH, Tan Y, Glenn JS, Tao YY, Weng HL,Liu CH. Fuzhenghuayu Decoction ameliorates hepatic fibrosis by attenuating experimental sinusoidal capillarization and liver angiogenesis. Sci Rep. 2019; 9(1): 18719. http://10.1038/s41598-019-54663-4.
14. Zhao Q, Ren H, Zhu D, Han Z. Stem/progenitor cells in liver injury repair and regeneration. Biol Cell. 2019; 101(10): 557-71. http://10.1042/BC20080105.
15. Du CY, Roy CY, Zheng KY, Dong TT, Lau DT, Tsim KW. Yu ping Feng san,an ancient Chinese herbal decoction containing Astragali Radix,Atractylodis Macrocephalae Rhizoma and Saposhnikoviae Radix,regulates the release of cytokines in murine macrophages. PLoS One. 2013; 8(11): e78622. http://10.1371/journal.pone.0078622.
16. Guo SG, Zhang W, Jiang T, Dai M, Zhang LF, Meng YC, Zhao LY, Niu JZ. Influence of serum collected from rat perfused with compound Biejiaruangan drug on hepatic stellate cells. World J Gastroenterol, 2004; 10(10): 1487-94. http://10.3748/wjg.v10.i10.1487.
17. Yang MD, Chiang YM, Higashiyama R, Asahina K, Mann DA, Mann J, Wang CC, Tsukamoto H. Rosmarinic acid and baicalin epigenetically derepress peroxisomal proliferator-activated receptor γ in hepatic stellate cells for their antifibrotic effect. Hepatology. 2012; 55(4) : 1271-81. http://10.1002/hep.24792.
18. Elvevold K, Smedsrød B, Martinez I. The liver sinusoidal endothelial cell: a cell type of controversial and confusing identity. American Journal of Physiology Gastrointestinal Liver Physiology. 2008; 294(2): G391-400. http://10.1152/ajpgi.00167.2007.
19. Braet F, Wisse E. Structural and functional aspects of liver sinusoidal endothelial cell fenestrae: a review. Comp Hepatol. 2002; 1(1) :1. http://10.1186/1476-5926-1-1.
20. March S, Hui EE, Underhill GH, Khetani S, Bhatia SN. Microenvironmental regulation of the sinusoidal endothelial cell phenotype in vitro. Hepatology. 2009; 50(3): 920-8. http://10.1002/hep.23085.
21. Hang TC, Lauffenburger DA, Griffith LG, Stolz DB. Lipids promote survival, proliferation, and maintenance of differentiation of rat liver sinusoidal endothelial cells in vitro. American journal of physiology. Gastrointestinal and liver physiology, 2012; 302(3): G375–88. http://10.1152/ajpgi.00288.2011.

22. Xu M, Wang X, Zou Y, Zhong Y. Key role of liver sinusoidal endothelial cells in liver fibrosis. Biosci Trends, 2017; 11(2): 163-8. http://10.5582/bst.2017.01007.
23. Schaffner F, Poper H. Capillarization of hepatic sinusoids in man. Gastroenterology. 1963; 44(6): 239-42.
24. Narita M, Oussoultzoglou E, Chenard MP, Fuchshuber P, Rather M, Rosso E, Addeo P, Jaeck D, Bachellier P. Liver injury due to chemotherapy-induced sinusoidal obstruction syndrome is associated with sinusoidal capillarization. Ann Surg Oncol. 2012; 19(7): 2230-7. http://10.1245/s10434-011-2112-6.
25. Xie G, Wang X, Wang L, Wang L, Atkinson RD, Kanel GC, Gaarde WA, Deleve LD. Role of differentiation of liver sinusoidal endothelial cells in progression and regression of hepatic fibrosis in rats. Gastroenterology. 2012; 142(4): 918-27.e6. http://10.1053/j.gastro.2011.12.017.
26. Yao Q, Lin Y, Li X, Shen X, Wang J, Tu C. Curcumin ameliorates intrahepatic angiogenesis and capillarization of the sinusoids in carbon tetrachloride-induced rat liver fibrosis. Toxicol Lett. 2013; 222(1): 72-82. http://10.1016/j.toxlet.2013.06.240.
27. Natarajan V, Harris EN, Kidambi S. SECs (Sinusoidal Endothelial Cells), Liver Microenvironment, and Fibrosis. Biomed Res Int, 2017; 1-9. http://10.1155/2017/4097205.
28. Lafoz E, Ruart M, Anton A, Hernández-Gea V. The Endothelium as a Driver of Liver Fibrosis and Regeneration. Cells.2020; 9(4). http://10.3390/cells9040929.
29. Novo E, Cannito S, Valfrè di Bonzo L, Caligiuri A, Cravanzola C, Compagnone A, Colombatto S, Marra F, Pinzani M, Parola M. Proangiogenic cytokines as hypoxia-dependent factors stimulating migration of human hepatic stellate cells. Am J Pathol. 2007; 170(6): 1942-1953. http://10.2353/ajpath.2007.060887.
30. Lai WK, Adams, David H. Angiogenesis and chronic inflammation; the potential for novel therapeutic approaches in chronic liver disease. J Hepatol. 2005; 42(1): 7-11. http://10.1016/j.jhep.2004.11.008.
31. Wheaton AK, Agarwal M, Jia S, Kim KK. Lung Epithelial Cell Focal Adhesion Kinase Signaling Inhibits Lung Injury and Fibrosis.Am J Physiol Lung Cell Mol Physiol. 2017; 312(5): 722-730. http://10.1152/ajplung.00478.2016.
32. Zhang F, Kong, D, Chen L, Zhang X, Lian N, Zhu X, Lu Y, Zheng S. Peroxisome proliferator-activated receptor-γ interrupts angiogenic signal transduction by transrepression of platelet-derived growth factor-β receptor in hepatic stellate cells. J Cell Sci. 127(Pt2), 2014; 305-314.http://10.1242/jcs.128306.
33. Zhang K, Jiang MN, Zhang CH, Jia YJ. Effects of Ganfukang on expression of connective tissue growth factor and focal adhesion kinase/protein kinase B signal pathway in hepatic fibrosis rats. Chin J Integr Med.2014; 20(6): 438-444.http://10.1007/s11655-013-1597-1.

Figures

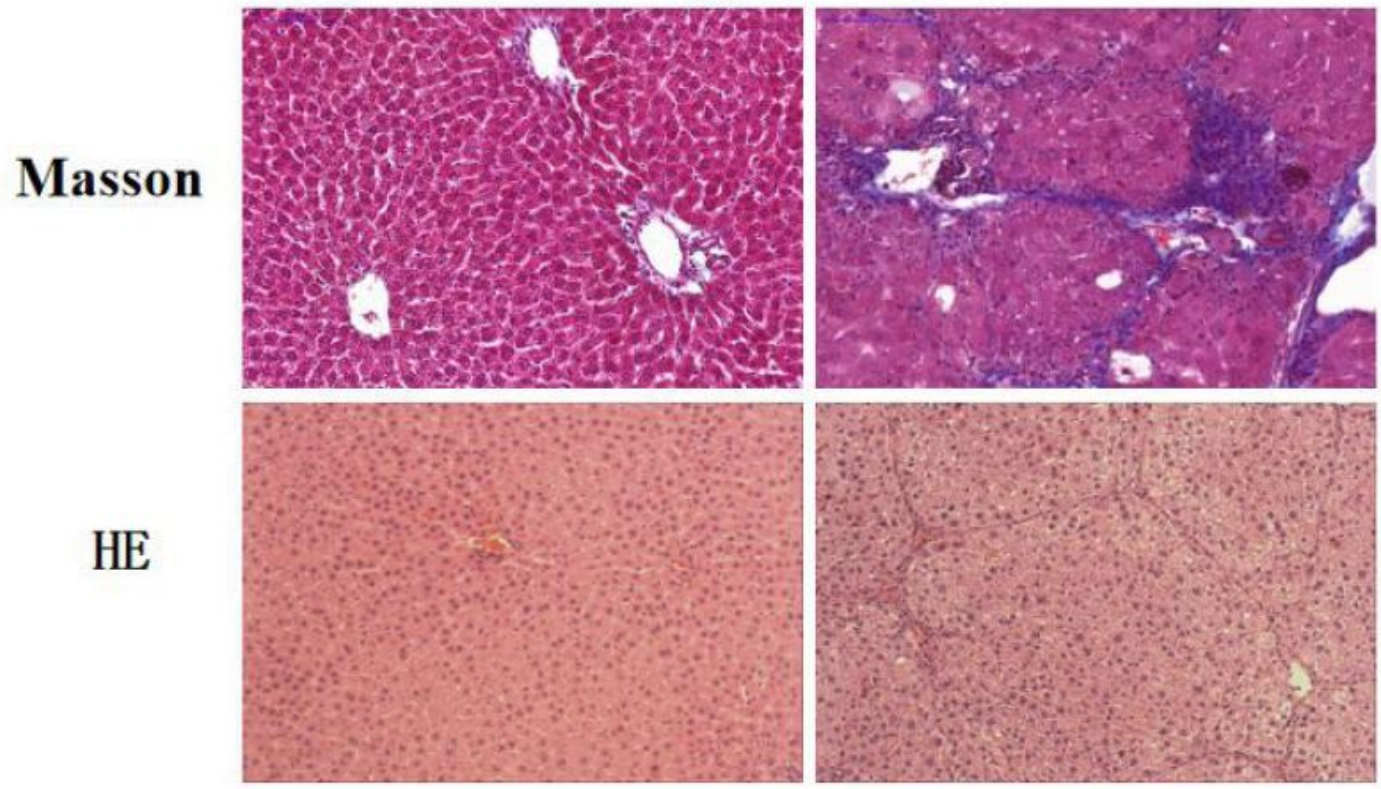

Figure 1

The rat liver tissue, Stain, Masson; scale bar, 2 μm. HE stain,×200

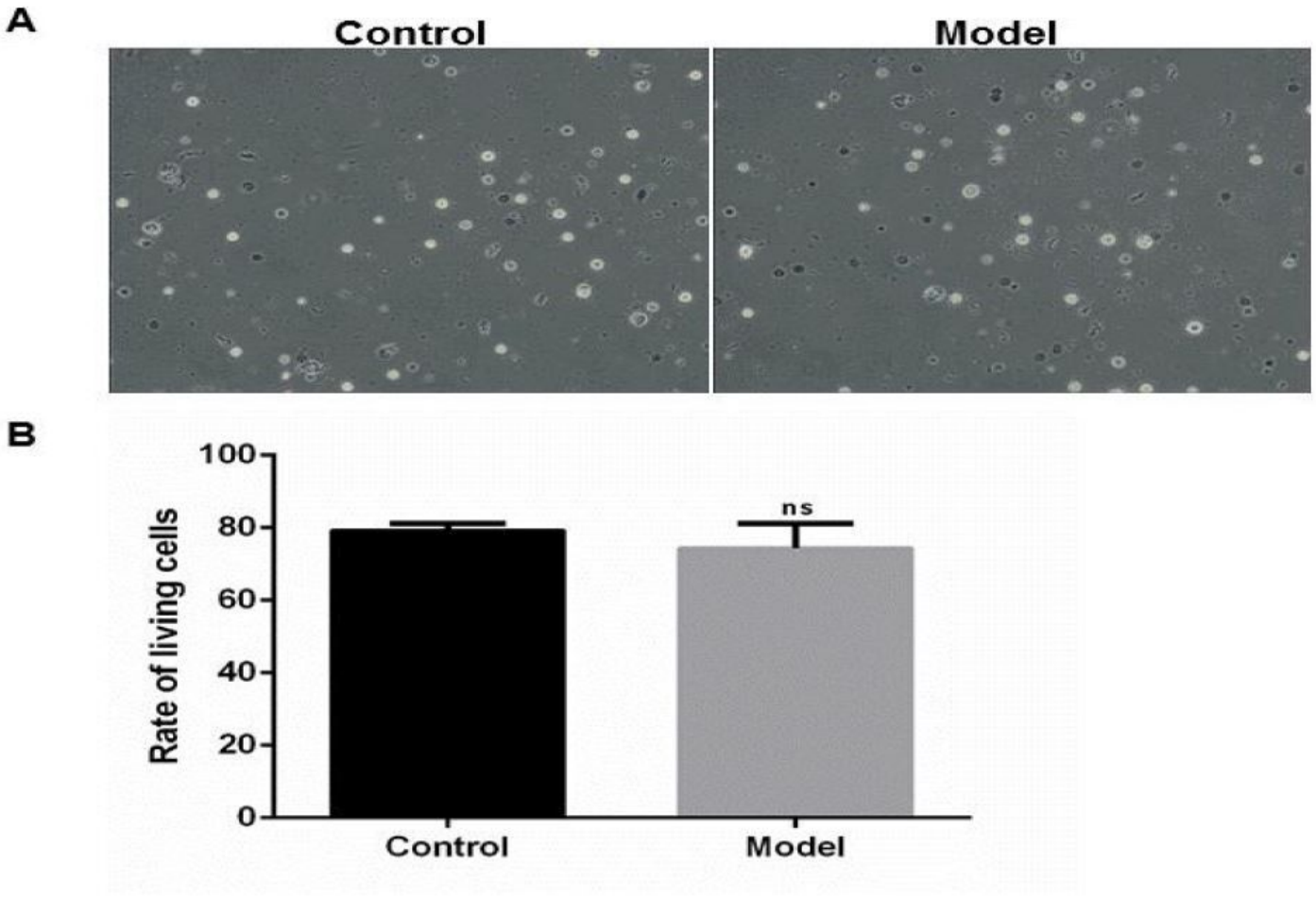

Figure 2

A. Trypan blue staining of hepatic sinus endothelial cells. A. Trypan blue staining of hepatic sinus ndothelial cells in each group,image magnification: 400 times. The Control group represented the normal Control group, and the Model group represented the liver fibrosis Model group. B. Comparison of cell survival rate between the two groups, and T-test was used for data analysis, with no statistical significance.

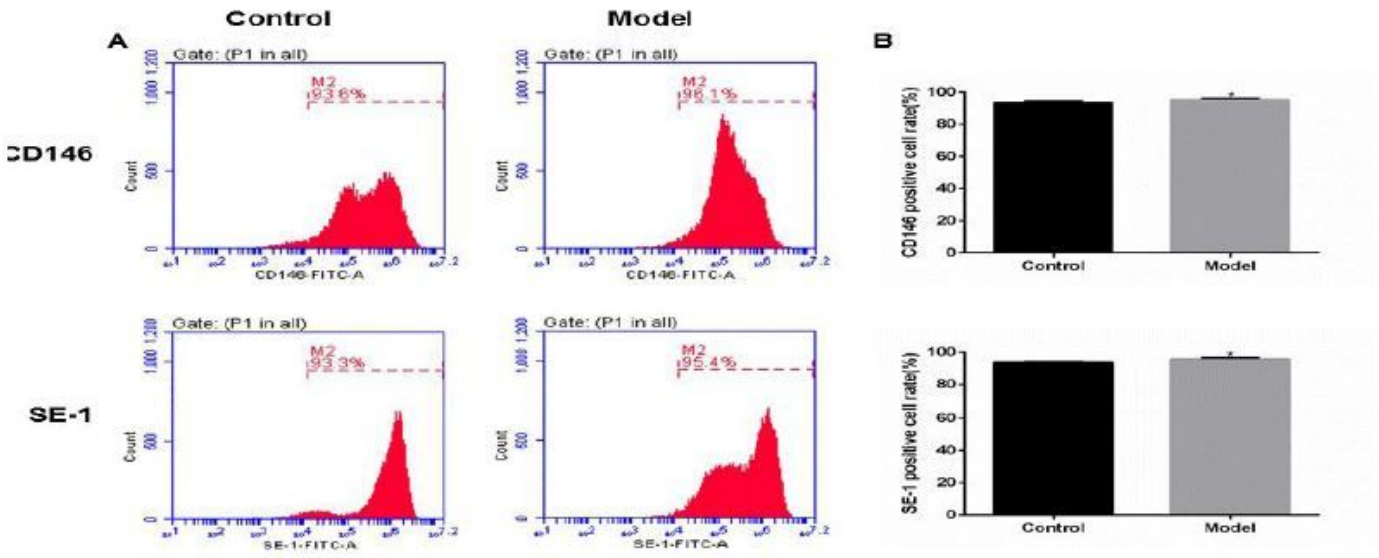

Figure 3

Identification of LSECs by flow cytometry. A. LSECs were identified by flow cytometry. B. The LSECs flow test results of each group were analyzed, and the data were analyzed by t-test, * denoting $p<0.05$ (vs Control).

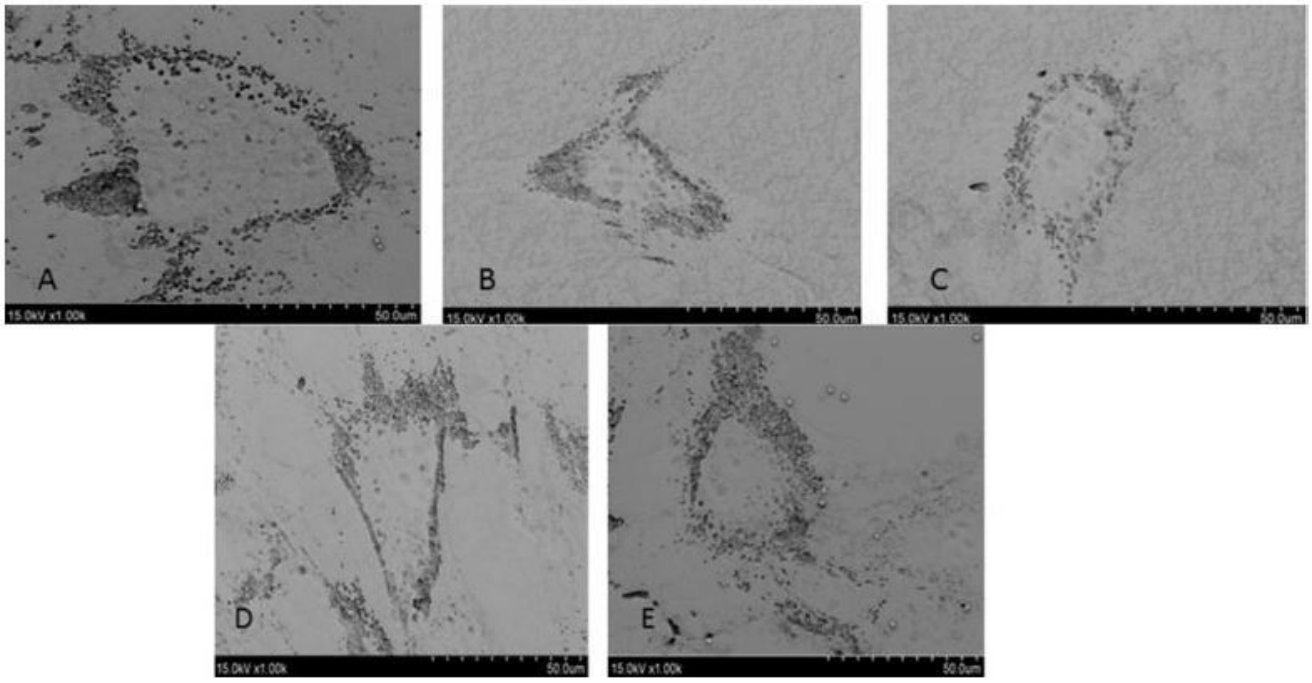

Figure 4

The fenestration on LSECs in each group was observed by scanning electron microscopy(scale bar = 50 μm). A. LSECs in normal group,plused normal serum without drugs. B. Model group,model SECs,plused normal drug-free serum. C. Qizhu granules low-dose group: model LSECs and added low-dose Qizhu granule-containing serum for treatment. D. Qizhu granules middle-dose group: model LSECs, plused medium-dose Qizhu granule-containing serum treatment. E. Qizhu granules high-dose group: model LSECs and added high-dose Qizhu granule-containing serum for treatment.

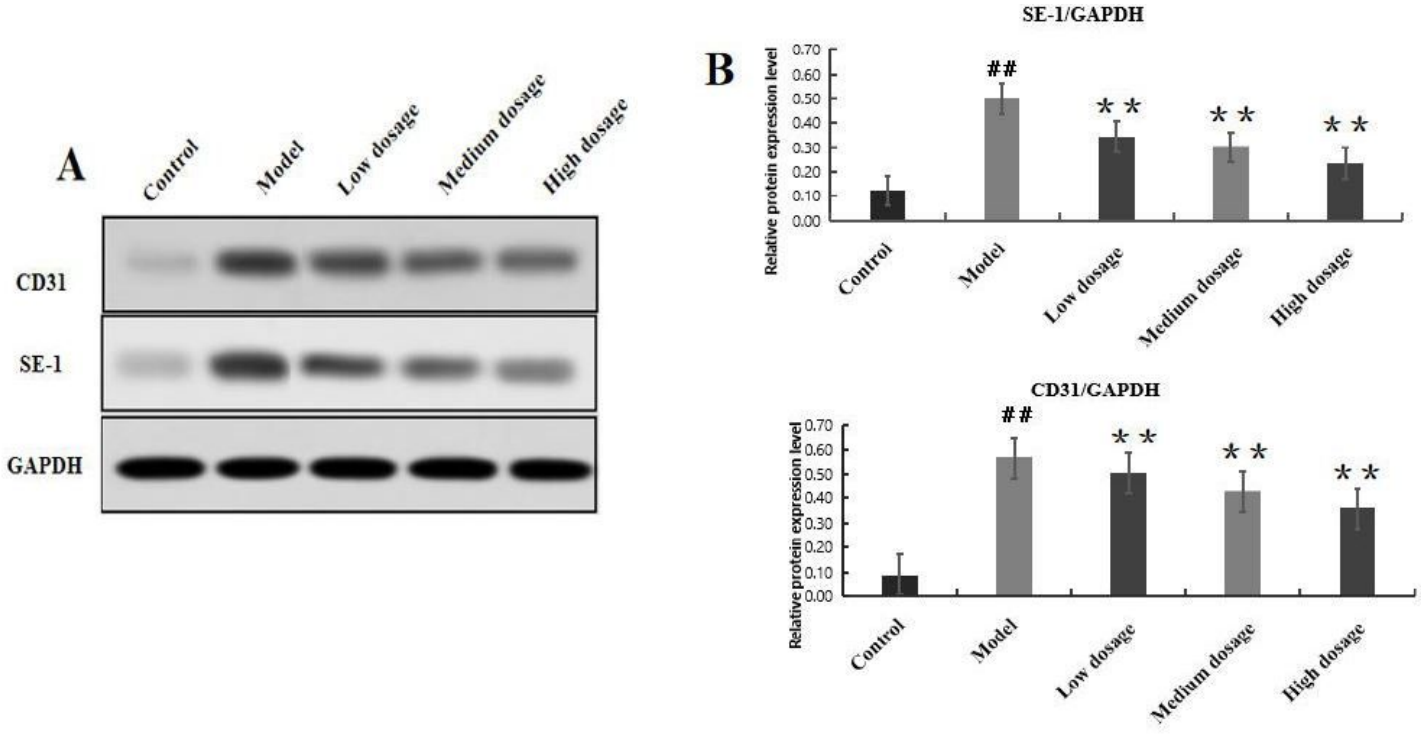

Figure 5

Expression of SE-1 and CD31 in LSECs of each group. A: SE-1 and CD31 expression in each group. Control group: normal control group LSECs, plused normal drug-free serum. Model group: Model group LSECs, plused normal drug-free serum. Low dosage: model LSECs, plused low-dose drug serum of Qishu granules. Medium dosage: LSECs for modeling, plused medicinal serum for Qishu granules dose. High dosage: model LSECs, plused high-dose drug serum of Qishu granules. B: Analysis of the expression levels of SE-1, CD31 in each group.Compared with the normal control group ##P<0.05, compared with the model group□□P<0.05.

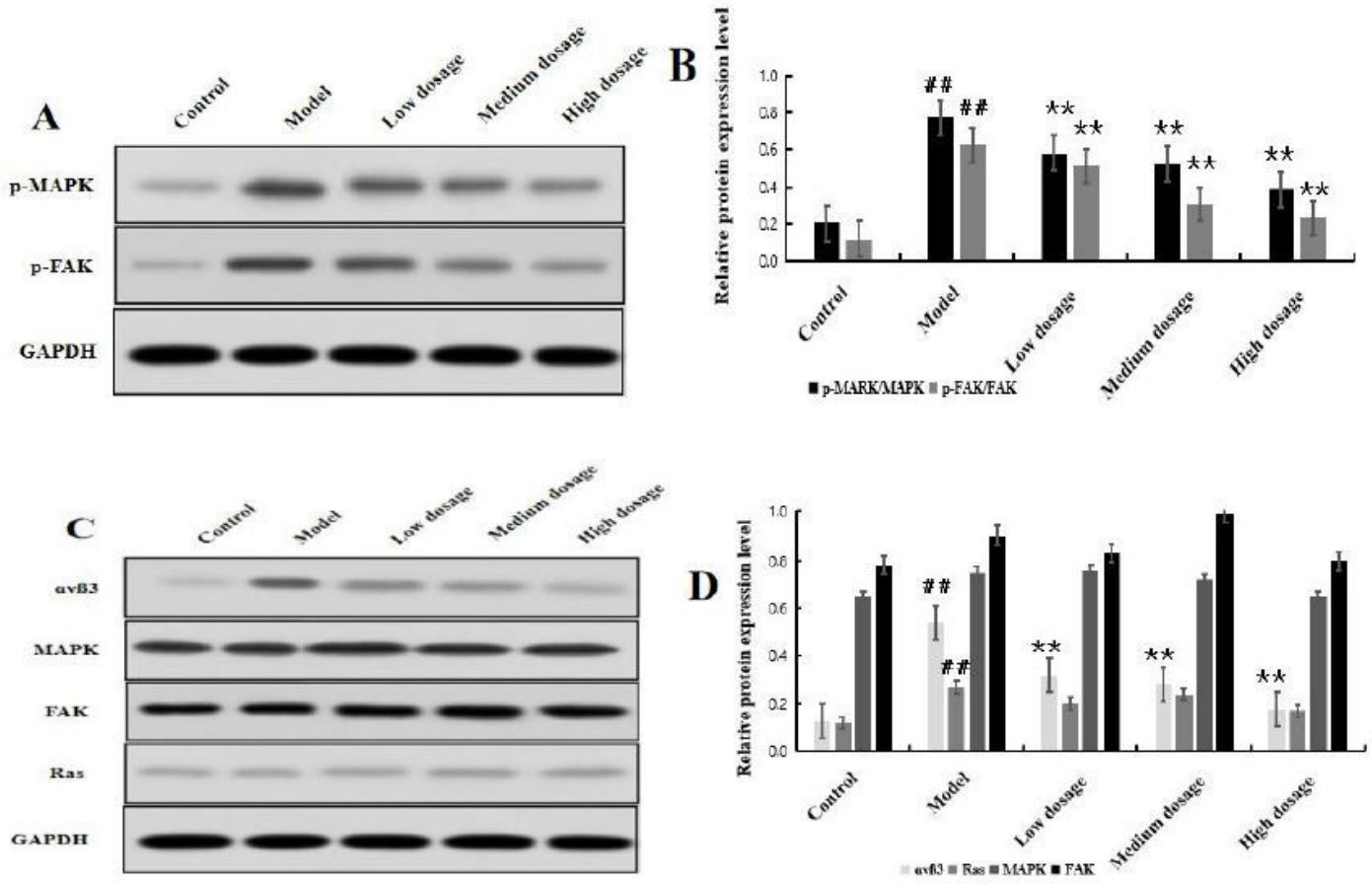

Figure 6

Qizhu Granules suppressed angiogenesis via inhibiting αVβ3-FAK-Ras / MAPK signaling pathway. Western blot for FAK and MAPK phosphorylated (A)and total integrin αVβ3, FAK, Ras and MAPK expression (C) were shown.GAPDH was used as loading control. The experiments were repeated at least three times. Data are presented as means±SEM (B, D). Compared with the control group ##P<0.05, compared with the model group**P<0.05.