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# Traditional Chinese Medicine Can Improve Liver Microcirculation and Reduce Portal Hypertension in Liver Cirrhosis

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## 1. Introduction

Liver microcirculation means that the blood circulation is sinusoids as a center, from the end branch of portal vein and hepatic artery through sinusoids to central vein in the liver. Liver microcirculation has two characteristics. One character is there are two import systems from portal vein and hepatic artery. Another is sinusoids with unique structure. All blood vessels which diameters are less 300 $\mu$ m are part of microcirculation system. These vessels are able to regulate the blood flow and distribution.<sup>1</sup>

Sinusoids are a capillary bed which is major place for regulation of blood and exchange of material in the liver. Sinusoids are constituted by microvasculature, sinusoid endothelial cells (SEC), Kupffer cells (KC) and hepatic stellate cells (HSC).<sup>2</sup> SEC and HSC are considered as cellular factors for regulation of sinusoids blood flow. HSC is located in perisinusoids with functions include storing Vitamin A, and controlling the diameter and blood flow of sinusoids by its pseudopods around sinusoids.<sup>3</sup> SEC, KC and HSC all have contractility because they contain fiber, microvasculature and contractile protein.<sup>2</sup>

The liver complex functions, which are biological synthesis, metabolism, detoxify and host defense, are tightly dependent perfect liver microcirculation.<sup>4</sup> It is showed that failure of liver microcirculation is a general pathological alteration in chronic hepatitis and liver cirrhosis because changed construction of microvasculature, capillarization of sinusoid (the collagen is filled in perisinusoids) and compression of fibrous tissue. Generally, stenosis of sinusoid will lead to increase of resistance, decrease of blood flow rate and flow volume, and formation of portal hypertension. It is indicated that after hepatitis B virus infection, platelets were recruited to the liver, and their activation correlated with severely reduced sinusoidal microcirculation, delayed virus elimination and increased immunopathological liver cell damage.<sup>5</sup>

In normal condition, liver microcirculation is regulated by 3 ways. (1) SEC is a regulator of blood flow in the liver. The liver injury will lead to injury of SEC, reduction of fenestrae in SEC, straitens of sinusoid within accumulation of erythrocytes and formation of microthrombus. Injury and necrosis of SEC are major causes of disorder of liver microcirculation. (2) KC can also regulate the liver blood flow by its pseudopod inner of sinusoid. Activated KC may lead stenosis of sinusoid due to conglutination of leukocytes.<sup>6</sup> (3) HSC is able to directly regulate contraction of sinusoids in normal liver. Active HSC has stronger character of contraction with increased concentration of  $Ca^{2+}$ . HSC is just located in the site of sinusoid contraction. Proportional relationship has been proved between contraction of HSC and activation of HSC both *in vivo* and *in vitro*.<sup>7,8</sup> Many chemical compounds are as inductors to induce contraction of HSC. They are included Endothelin-1 (ET-1), Angiotensin II and vasopressin. ET-1 is concerned a strongest cytokine to induce contraction of HSC. Perfusion of ET-1 through portal vein leads to contraction of sinusoids. However, the compounds of NO, CO and adrenal medullar hormone have the functions to against ET-1-stimulated contraction of HSC. Contraction of active HSC (myofibroblast cell), which locates in fibrous septum in cirrhotic liver, can change construction of hepatic lobule and lead to increase resistens of microcirculation in the liver.

Once failure of microcirculation in the liver, some symptoms and signs of patient are similar “blood stasis” in Traditional Chinese Medicine. Those are twinge in inside right flank or rib, hepatomegaly or splenomegaly, dim complexion, accompanied with spider nevi and liver palms, deep-red tongue, ecchymosis, taut or thready pulse. Some Chinese herb medicine, which is able to promote blood circulation to remove blood stasis, can prevent esophageal variceal bleeding on cirrhotic patients with portal hypertension, improve liver microcirculation and decrease portal hypertension through treating cirrhotic rats *in vivo* and inhibit contraction of active HSC *in vitro* in rescent 10 years.<sup>9</sup>

Fuzheng Huayu Capsule (FZHYC), a traditional Chinese medicine recipe, has demonstrated the effect of anti-fibrosis and reduced portal vein pressure in patients with chronic hepatitis B.<sup>10</sup> Fuzheng means supporting the healthy energy. Huayu means dispersing blood stasis. The recipe is composed by 6 herbs those are Dan Shen (*Radix Salviae Miltiorrhizae*), Chong Cao (*Paecilomyces hepiali* Chen & Dai), Tao Ren (*Semen Persicae*), Jiao Gu Lan (*Gynostemma pentaphyllum*), Song Hua Fen (*Pinus armandii* Franch) and Wu Wei Zi (*Fructus Schisandrae Chinensis*). FZHYC has been used in numerous studies in China and has been found to have a satisfactory prophylaxis effect on the chronic liver injury and hepatic fibrosis in rats and humans. In addition, it enhances hepatic fibrolysis.<sup>11</sup>

## 2. The clinical research

### 2.1 Aims

To elucidate the role of FZHYC in the prevention of esophageal variceal bleeding on cirrhotic patients with portal hypertension.

### 2.2 Methods

In a multi-center randomized and placebo-controlled trial, 146 cirrhotic patients, whose age range was between 18 and 70 years old, with esophageal varices were enrolled. According to the degree of esophageal and gastric varices, patients were stratified as 2 levels: small

level, and moderate and severe level. The randomization code was generated by SAS software for each subject. For patients with small varices, the trial was double blinded. The patients were randomly assigned to 2 groups: Patients were treated with FZHYC in FZHYC group or received placebo in control group. For patients with moderate and severe varices, the trial was single blind. They were divided into 3 groups: FZHYC group in which patients were treated with FZHYC, propranolol group in which patients were treated with Propranolol and combination group in which patients received both FZHYC and propranolol. The dose of FZHYC was 15 capsules (0.3g per capsule) per day and orally administrated at 3 times. The placebo was composed of stir-fried wheat powder with an identical-appearance in the aspects of dosage, color of the contents and packaging. The clinical intervention period lasts 2 years and the follow up would stop once the end points occur. The primary end point was esophageal or gastric variceal bleeding.

## 2.3 Results

### 2.3.1 In patients with small varices level

A total of 6 patients reached the primary end point of esophageal variceal bleeding: 1 of 29 patients in the Fuzheng Huayu group and 5 of 27 patients in the control group. The actuarial probability of remaining free of esophageal variceal bleeding was a significant different between 2 groups (96.3% vs. 77.01%,  $P=0.0422$ ) (Fig. 1). Some patients with small varices were willing to be re-examined by endoscopy after treatment and small varices was dispared in several patients. There was a significant difference of esophageal varices degree in FZHYC group compared to the control group ( $P=0.014$ ). (Tab. 1 and Fig. 2).

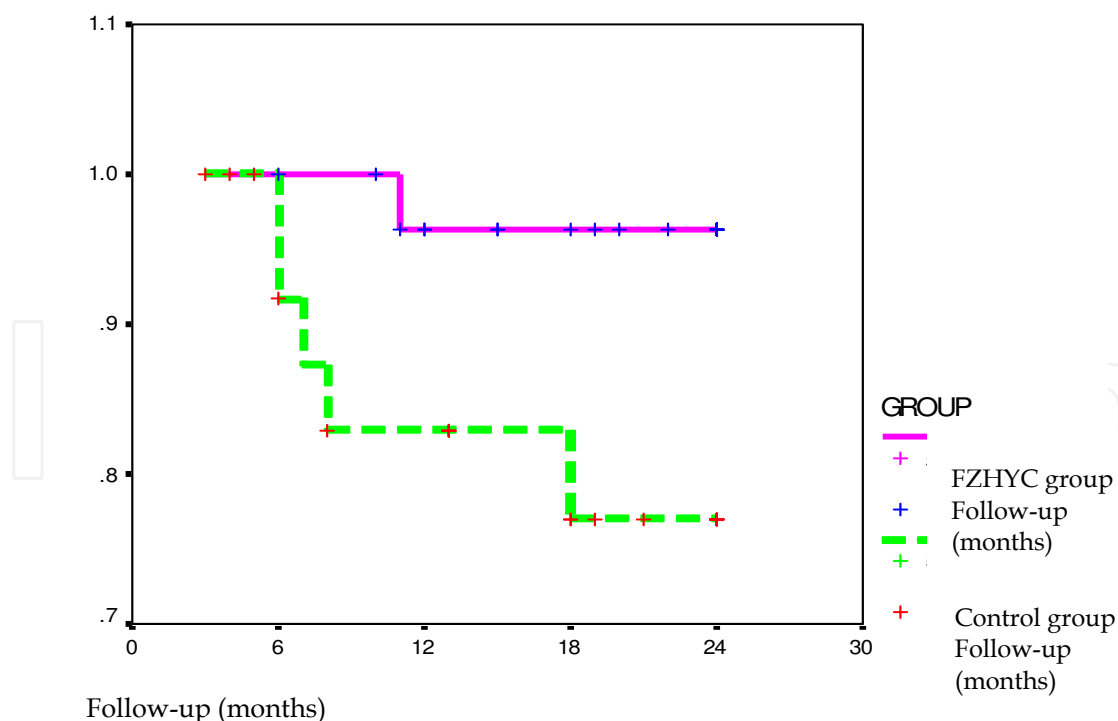


Fig. 1. Actuarial probability of remaining free of esophageal variceal bleeding in cirrhotic patients with small varices level. The patients were treated with Fuzheng Huayu Capsule in FZHYC group or placebo in control group for 24 months. Actuarial probability of remaining free of esophageal variceal bleeding was higher in FZHYC group than in controls ( $P<0.05$ ).

Groups	n	No varices (n)	No changes (n)	Enlarged varices (n)
FZHYC *	15	8	5	2
control	9	1	3	5

Note: compared with control group, \*:  $P=0.014$

Table 1. Difference of developing small varices in FZHYC group and in controls after 24 months

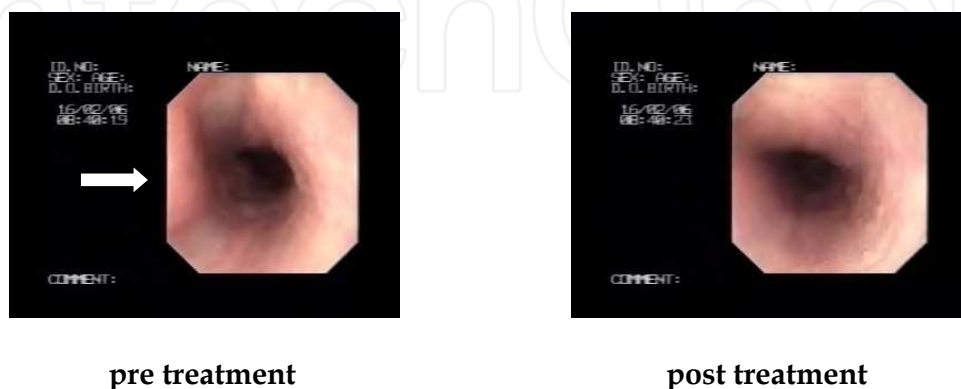


Fig. 2. Alteration of small varices by endoscopy examination at pre and post treatment of FZHYC. Arrow shows the small varices in a cirrhotic patient at pre treatment (left). The small varices, however, was disappeared in same patient at post treatment (right).

### 2.3.2 In patients with moderate and severe varices level

5 patients in FZHYC group ( $n=30$ ), 8 patients in Propranolol group ( $n=30$ ) and 3 patients in the combination group ( $n=30$ ) had esophageal variceal bleeding during their follow-up. Compared to the Propranolol group (56.99%), there were significant differences in the actuarial probability of remaining free of esophageal variceal bleeding in FZHYC group (76.13%,  $P=0.0131$ ) and the combination group (87.55%,  $P=0.0086$ ). Remaining free of variceal bleeding has a higher trend in combination group, but there was no significant difference between combination group and FZHYC group ( $P=0.3876$ ) (Fig.3).

### 2.4 Summary

FZHYC could effectively reduce the risk of esophageal variceal bleeding for cirrhotic patients with varices; especially the combination of the capsule and Propranolol delivered a better effect. The capsule could reduce the varices size in patients with small ones.

## 3. The experiment *in vivo*

For studying the mechanism of Fuzheng Huayu Capsule on prevention of esophageal variceal bleeding, we performed several experiments. *Radix Salviae Miltiorrhizae* is a major herb medicine in Fuzheng Huayu Recipe. Salvianolic-acid B (SA-B), which was extracted from *Radix Salviae Miltiorrhizae*, has been demonstrated to inhibit proliferation and functions of HSC *in vitro*<sup>12</sup> and anti-fibrosis *in vivo*.<sup>13</sup> We did lot of work to deal with how Fuzheng Huayu Recipe and SA-B relieves portal hypertension.

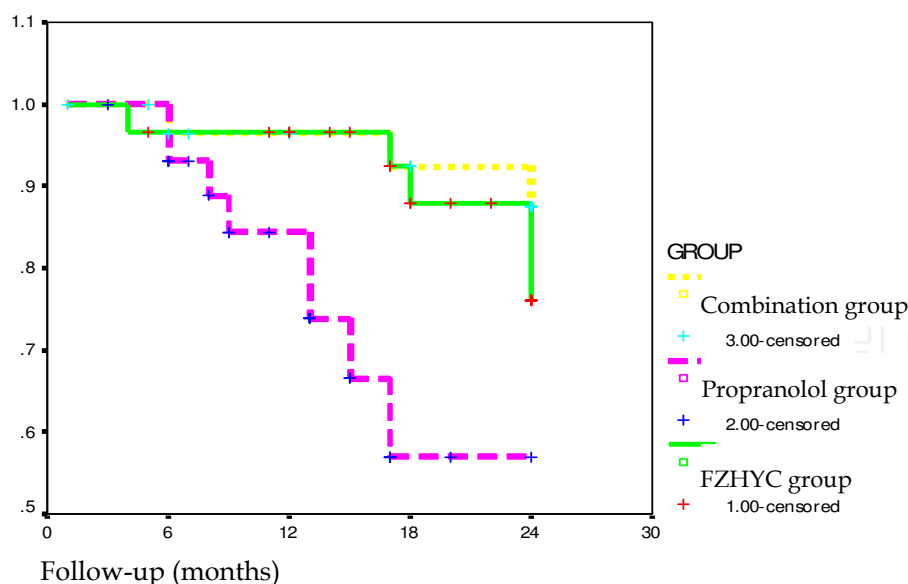


Fig. 3. Actuarial probability of remaining free of esophageal variceal bleeding in cirrhotic patients with moderate and severe varices level. The patients were treated with Fuzheng Huayu Capsule in FZHYC group, Propranolol in Propranolol group, or both in combination group for 24 months. Actuarial probability of remaining free of esophageal variceal bleeding was higher in combination group and FZHYC group than in Propranolol group. Compared with Propranolol group, the difference was significant in combination group ( $P < 0.01$ ) and in FZHYC group ( $P < 0.05$ ). Although the probability was higher in combination group than in FZHYC group, but the difference was not significant.

### 3.1 Experiment 1: Chinese herb medicine can decrease portal vein pressure in cirrhotic rats

#### 3.1.1 Aims

To investigate effect of Fuzheng Huayu Recipe and SA-B on decreasing portal vein pressure in cirrhotic rats.

#### 3.1.2 Methods

##### 3.1.2.1 Establishment of liver cirrhosis model

The Sprague-Dawley male rats were randomly divided into model group ( $n=46$ ) and normal group ( $n=14$ ). The rats in model group were administered intraperitoneally with 0.5% dimethylnitrosamine (DMN) at a dose of 2ml/kg body weight for 3 consecutive days and interval 4 days per week. The treatment was for 4 weeks. The rats in normal group were administered intraperitoneally with physiological saline at a dose of 2ml/kg body weight for same duration.

##### 3.1.2.2 Grouping animals and drug administration

After molding, two rats were randomly sacrificed to observe pathological changes of the liver tissue. The rats in model group were randomly more divided into 3 groups, control

group (n=13), Fuzheng Huayu (FZHY) group (n=15) and the SA-B group (n=16), once liver cirrhosis was confirmed. The rats in the FZHY group were gavaged with extractor of Fuzheng Huayu Recipe. The content of extractor equals 36.9g crude drug per 100ml. The rats in the SA-B group were gavaged with SA-B solution (125mg per 100ml). Simultaneously, the rats in control group and normal group were gavaged with water. All dose of gavage was 1ml/100g body weight, once per day, for 3 weeks.

### 3.1.2.3 Measurement of portal vein pressure

The rats were anesthetized with 2% sodium pentobarbital (2ml/kg body weight) in the end of experiment. One PE-10 tube filling with heparin was inserted from superior mesenteric vein into portal vein and then connected with pressotransducer to measure pressure. Enterocoelia was exposed totally after measurement. If ascites was found, dry cotton ball was used to blot liquid and then it was weighted for measurement of ascites. Serum separated from blood which was taken in the vena cava was used for liver function detection. The sample of liver tissue, which was 1.0cm×0.8cm×0.3cm, was fixed by formalin, embedded by paraffin, and cutted for 4μm thick for HE and collagen staining. Moreover, 100mg liver tissue was used to detect hydroxyproline (Hyp).

## 3.1.3 Result

### 3.1.3.1 Investigating death and ascites in the rats

During making hepatic cirrhosis model, there were no dead rats in week 4 but the death started in week 5. Until the end of week 7, there was still no death in normal group but there were 4 dead rats in control group, 2 dead rats both in FZHY group and in SA-B group, respectively. The reason that leads to death was liver failure. The number of the rats with ascites was most in control group than in other groups. The difference was significantly. (Tab. 2)

Groups	n	Death (n)	Ascites (n)
normal	14	0	0
control	13	4	6
FZHY	15	2	1*
SA-B	16	2	0*

Note: compared with control group, \*:  $P < 0.05$

Table 2. The situation of death and ascites in cirrhotic rats in each group

### 3.1.3.2 Observation of Pathology of liver in rats

#### 3.1.3.2.1 Inflammation was reduced by Chinese herb medicine in the liver of cirrhotic rats

The structure of liver lobule was clear, hepatocytes arranged as cords radially from central vein to the periphery and a few connective tissue was observed in the portal area in normal group. At the end of treatment, pathological alteration was observed in the liver that was

large bleeding area, a lot of swell or necrotic hepatocytes, a lot of infiltrating inflammatory cells including lymphocytes and mononuclears, distorted sinusoids and obviously widened portal areas in controls. They were varying decreased that was degeneration or necrosis of hepatocytes, and intrahepatic hemorrhage both in FZHY group and in SA-B group compared with controls. (Fig. 4)

### 3.1.3.2.2 Hepatic fibrosis was reversed by Chinese herb medicine

A little collagen fibers located only in portal areas or surrounded central veins in normal rat liver. Increased fibrous tissue formed thick interval that invaded into the hepatic lobules and moved round to form pseudolobules with different size in controls' liver. The fibrous intervals were thinner both in FZHY group and SA-B group than in controls. It means that hepatic fibrosis was reversed by FZHY recipe and SA-B. (Fig. 5)

### 3.1.3.2.3 The liver function of cirrhotic rats can be improved by Chinese herb medicine

Compared with normal rats, it was significantly increased in controls that are the activity of serum ALT and AST, serum level of TBiL ( $P < 0.01$  and  $P < 0.05$ ) but meanwhile the concentration of serum Alb were significantly decreased ( $P < 0.05$ ). However, they were obviously lower both in FZHY group and SA-B group than in controls that are the activity of serum ALT and AST ( $P < 0.01$ ), and serum level of TBiL ( $P < 0.05$ ). The concentration of serum Alb were back to normal level with significantly difference compared with controls ( $P < 0.05$ ). (Fig. 6)

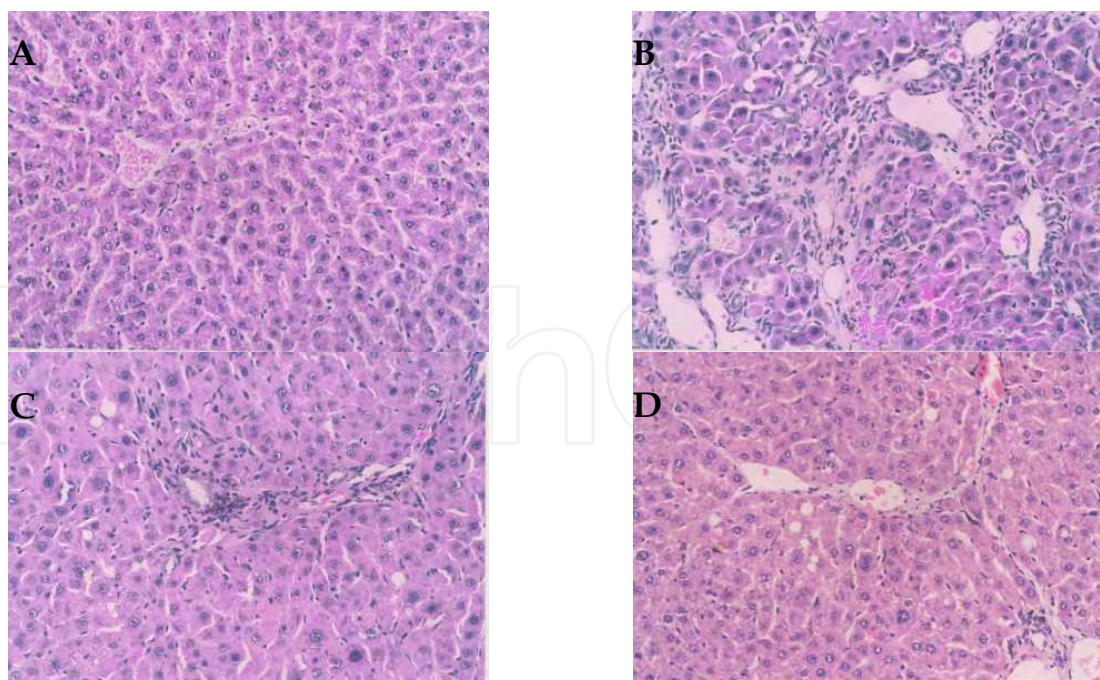


Fig. 4. Fuzheng Huayu Recipe and SA-B could relieve inflammation and structure damage in the liver tissue of DMN model rats A: normal group, B: control group, C: FZHY group and D: SA-B group. The staining was by HE (200×).



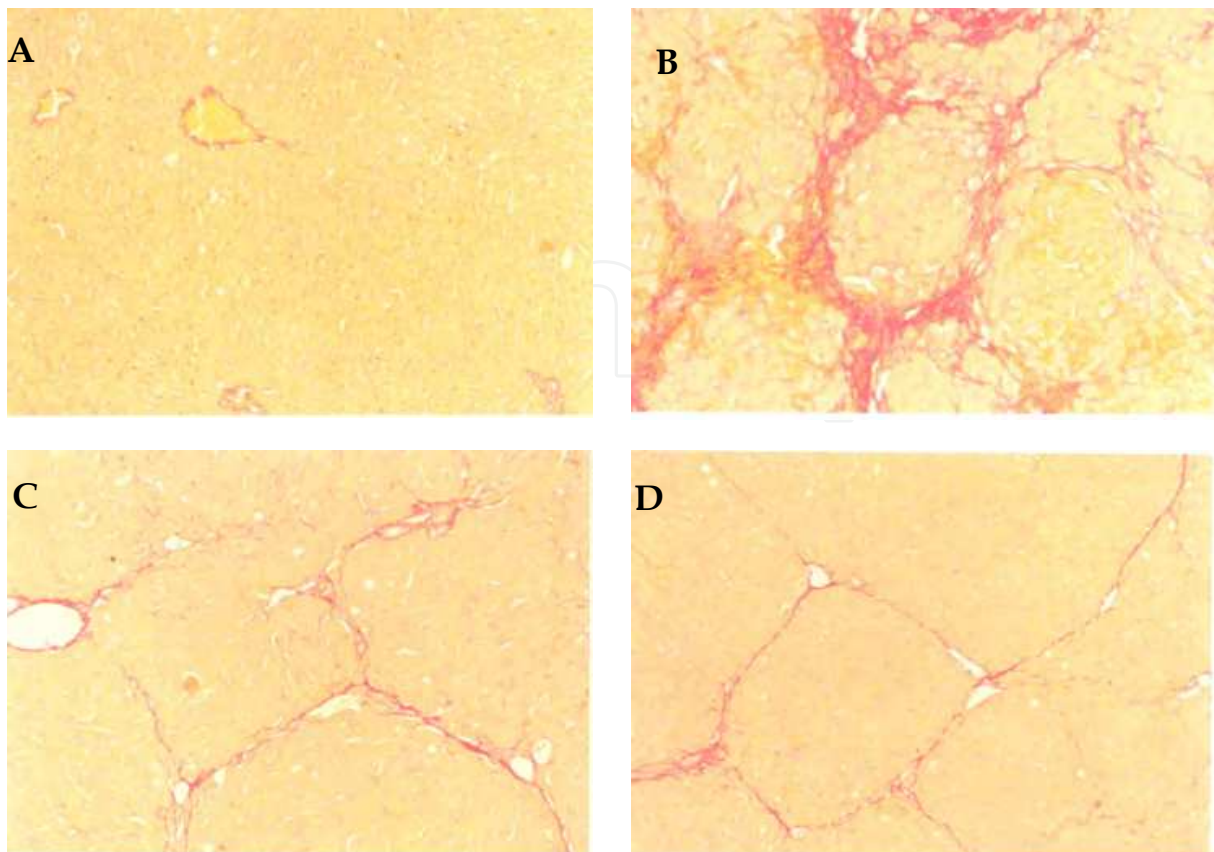
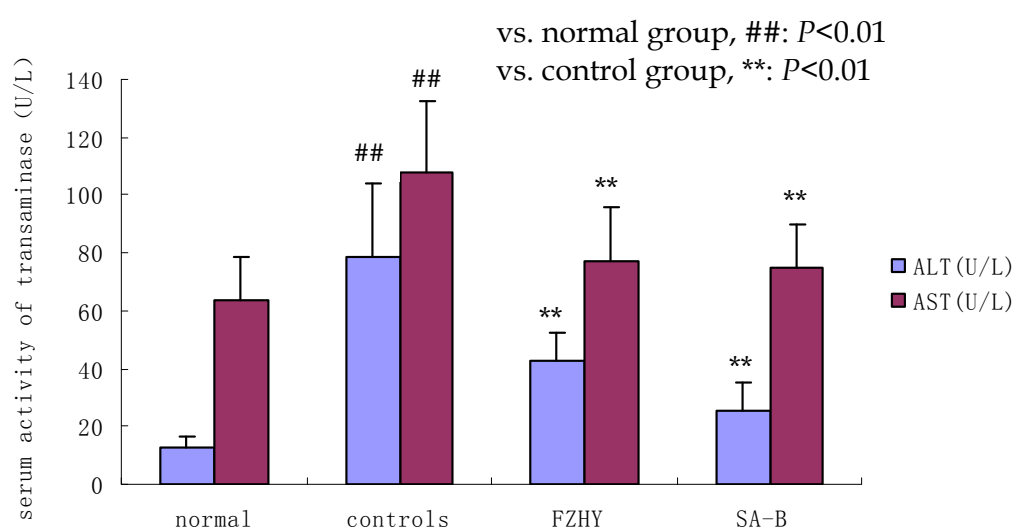


Fig. 5. Fuzheng Huayu Recipe and SA-B could relieve collagen fiber deposition in the liver tissue of DMN model rats A: normal group, B: control group, C: FZHY group, and D: SA-B group. The staining was by Sirius red (200 $\times$ )



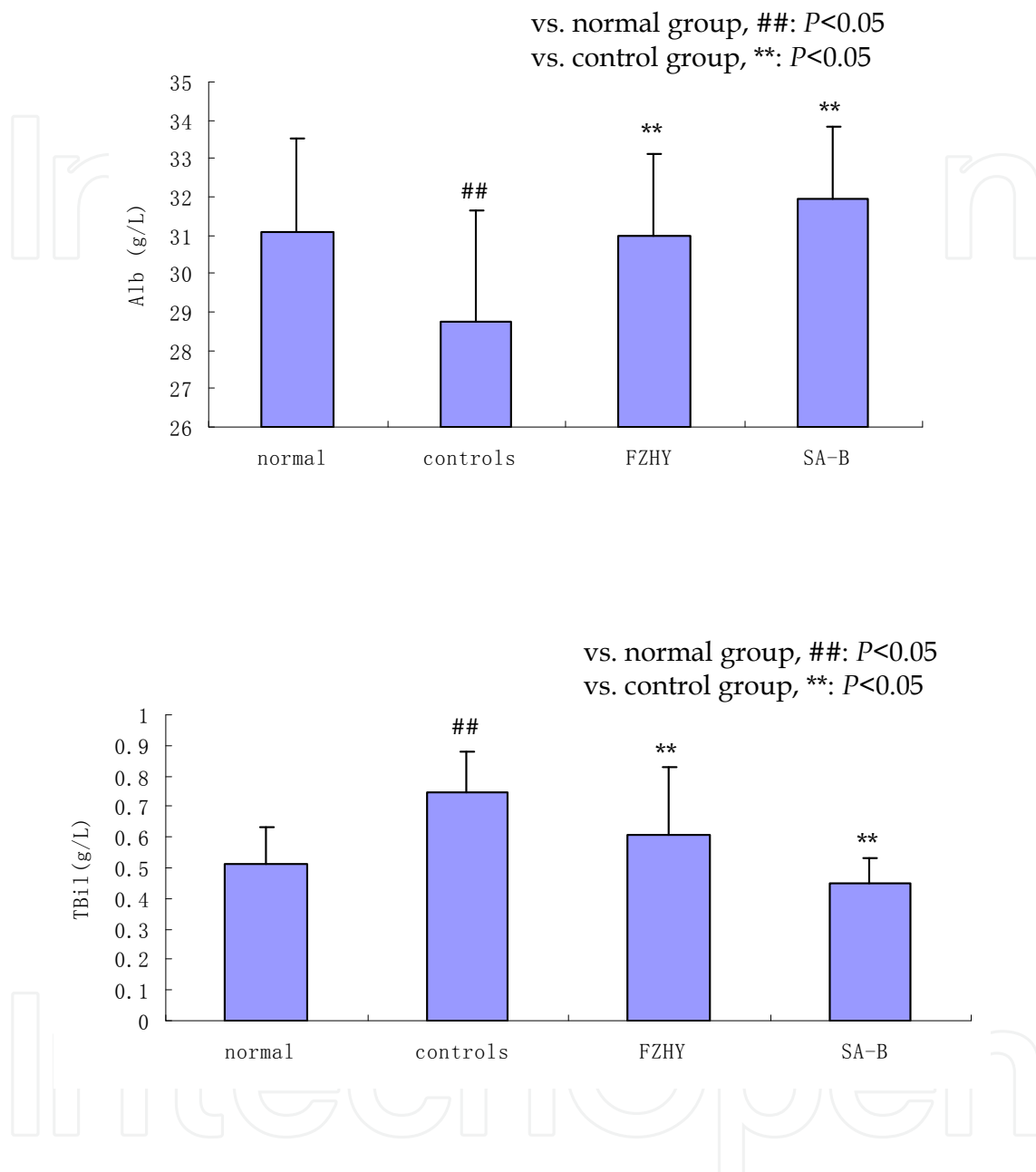


Fig. 6. Fuzheng Huayu Recipe and SA-B could decrease the activity of serum ALT and AST, and serum level of TBiL, but increase serum concentration of Alb in DMN model rats normal group, n=14; control group, n=9; FZHY group, n=13; SA-B group, n=14.

### 3.1.3.2.4 The concentration of Hyp in the liver tissue of cirrhotic rats was decreased by Chinese herb medicine

Compared with normal group, concentration of Hyp in the liver tissue was significantly increased at the end of experiment in controls ( $P < 0.01$ ). The concentration of Hyp, however, was significantly lower both in FZHY group and SA-B group than in controls ( $P < 0.05$ ). (Fig. 7)

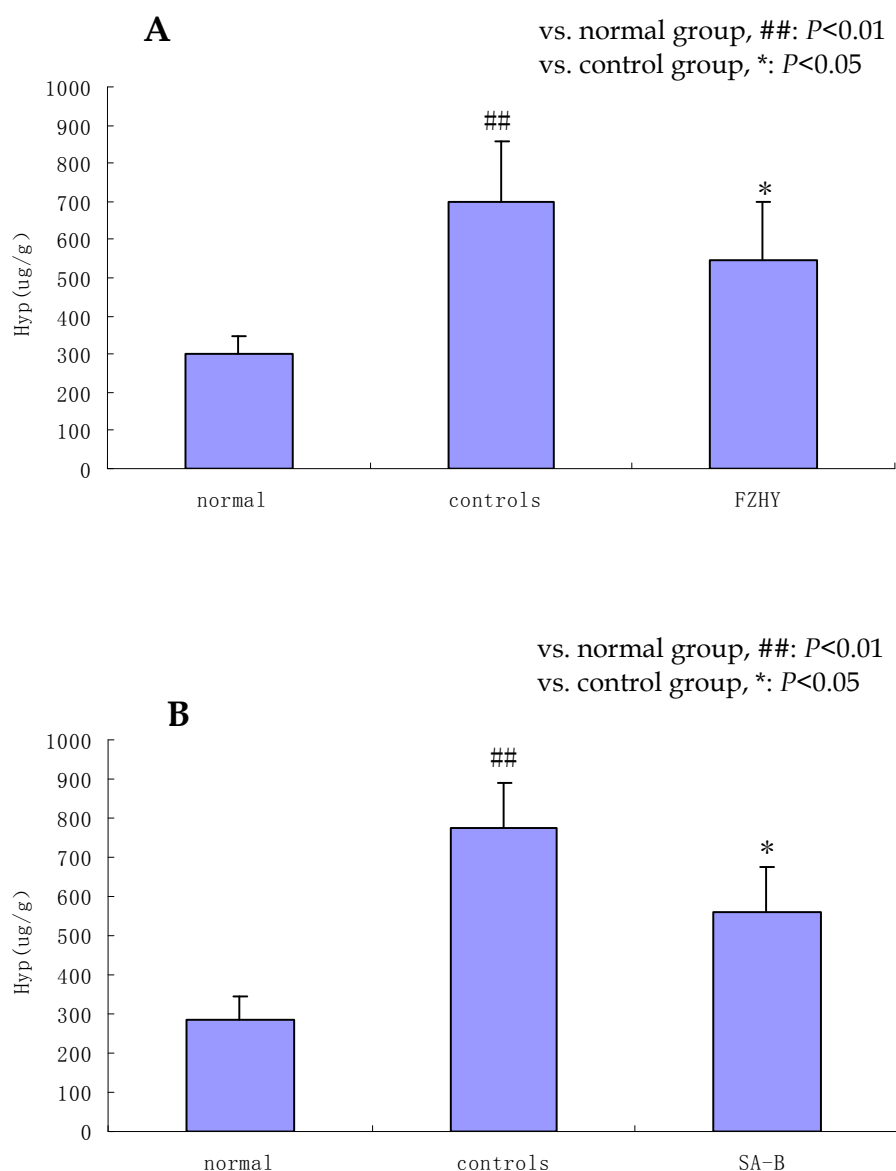


Fig. 7. Fuzheng Huayu Recipe and SA-B could decrease concentration of Hyp in the liver tissue of DMN model rats A. normal group,  $n=12$ ; control group,  $n=9$ ; FZHY group,  $n=12$ . B. normal group,  $n=13$ ; control group,  $n=9$ ; SA-B group,  $n=12$ .

### 3.1.3.2.5 Portal vein pressure of the cirrhotic rats was declined and the tissue concentration of ET-1 was dropped by Chinese herb medicine

The portal vein pressure of control rats was significantly increased in the end of experiment compared with normal rats ( $P < 0.01$ ). The increased range was up to 8mmHg. The portal pressure, however, was significantly lower in the rats treated with FZHY Recipe or SA-B than controls ( $P < 0.05$ ). (Fig. 8) The hepatic tissue concentration of ET-1 was obviously higher in controls than in normal group ( $P < 0.01$ ). But concentration of ET-1 was remarkably declined in FZHY group and SA-B group compared with controls ( $P < 0.05$ ). (Fig. 9.)

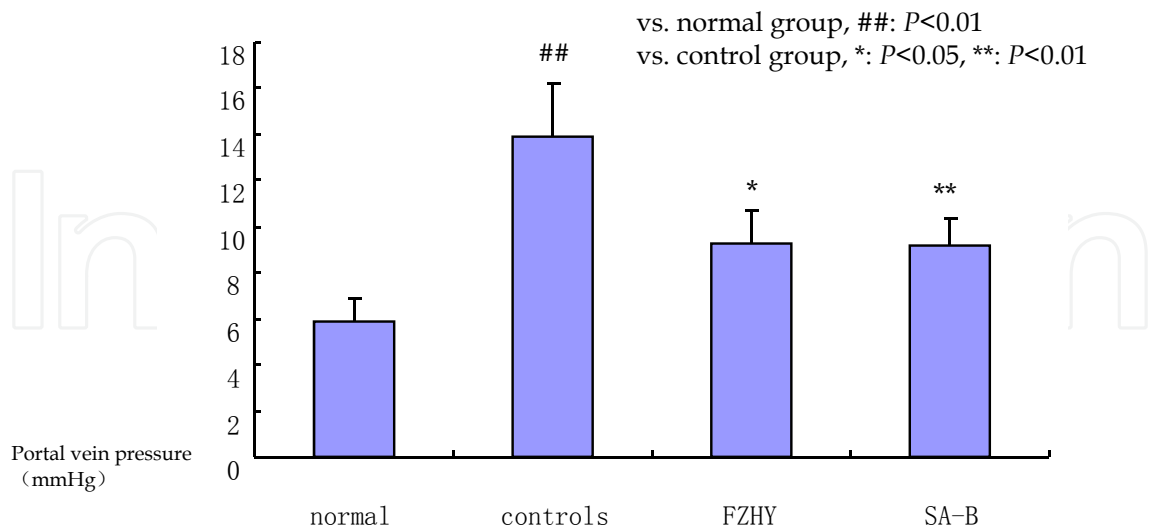


Fig. 8. Fuzheng Huayu Recipe and SA-B could decrease portal vein pressure in DMN model rats normal group, n=12; control group, n=9; FZHY group, n=13; SA-B group, n=14.

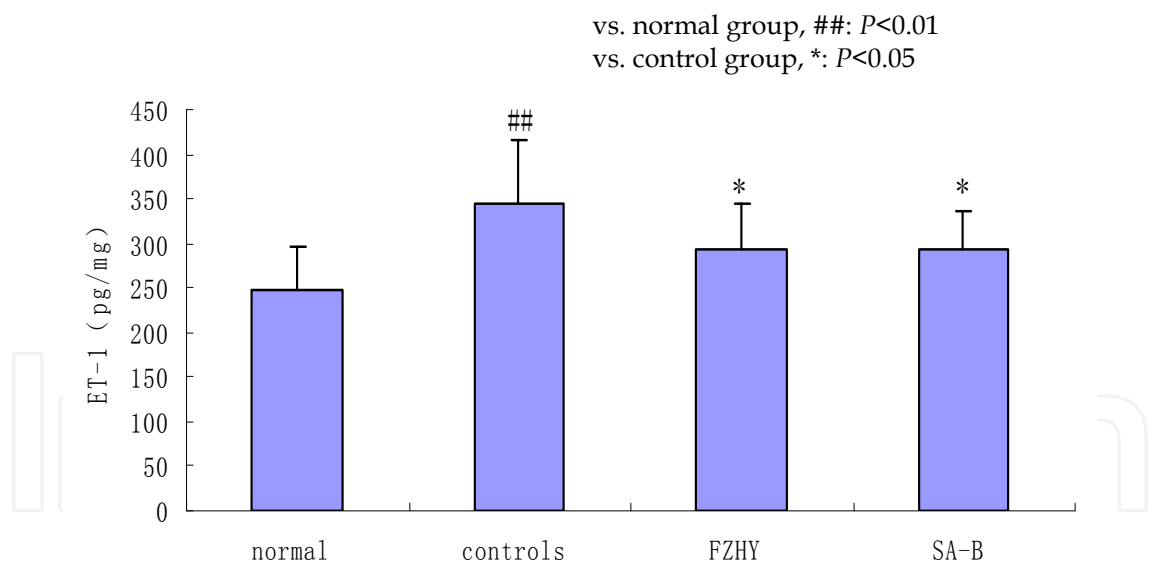


Fig. 9. Fuzheng Huayu Recipe and SA-B could decrease content of ET-1 in the liver tissue of DMN model rats normal group, n=14; control group, n=9; FZHY group, n=13; SA-B group, n=14.

### 3.1.4 Summary

The elevation of portal vein pressure is positively correlated with the increase of ET-1 concentration in the liver tissue during the process of liver cirrhosis. FZHY Recipe and SA-B

can dramatically decrease the cirrhosis-induced elevation of portal vein pressure and concentration of ET-1 in the liver tissue through their effect on anti-fibrosis in the liver.

### 3.2 Experiment 2: Chinese herb medicine can relieve capillarization of sinusoid in cirrhotic rats

#### 3.2.1 Aims

To investigate the effects of FZHY Recipe on capillarization of hepatic sinusoids.

#### 3.2.2 Methods

32 Sprague-Dawley male rats, weighing about 150g, were randomly divided into normal group (n=7) and model group (n=25). The modeling method was the same as Experiment 1 for making cirrhosis. The cirrhotic rats were more randomly divided into control group (n=11), and FZHY group (n=14). The drug, dose and method of administration for each group were the same as Experiment 1, too. But the duration of treatment was for 4 weeks.

Rat's blood, under etherization condition, was collected from inferior vena cava, and was separated to serum. The sample of liver (1.0cm×0.8cm×0.3cm) was immediately taken and fixed in neutral formalin, embedded in paraffin, sectioned at 5 μm thick. The sections of liver were stained with HE, Masson and immunohistochemistry. Three rats were randomly selected from each group respectively for observation of hepatic microstructure. The each liver sample was cut to a 1 mm<sup>3</sup> cube, fixed with 2.5% glutaraldehyde and 1% osmium tetroxide for 2h, embedded with araldite 618, and cut to ultrathin sections. The sections were observed by the electron microscope (H-600). 100mg liver tissue was used to detect Hyp.

#### 3.2.3 Results

##### 3.2.3.1 Fuzheng Huayu Recipe had the potent effect against hepatic fibrosis of the model rat

It was observed that hemorrhage, necrosis and extensive connective tissue hyperplasia in the liver tissues of model rats. The fiber septa reached out its branches towards the liver lobule. (Fig. 10 and Tab. 3) The level of hydroxyproline was significantly increased. Compared with controls, liver inflammation, necrosis of hepatocytes and hepatic fibrosis was reduced in FZHY group. The concentration of liver hydroxyproline was significantly lower in FZHY group than in controls ( $P<0.05$ ). (Tab. 4)

Groups	n	Hyperplasia degree of collagenous fibers				
		0	+	++	+++	++++
normal	7	7	0	0	0	0
controls	11	0	1	1	4	5
FZHY *	14	0	0	8	4	2

Note: compared with controls, \*:  $U=4.06$ ,  $P<0.01$

Table 3. Hyperplasia degree of collagenous fibers in each group

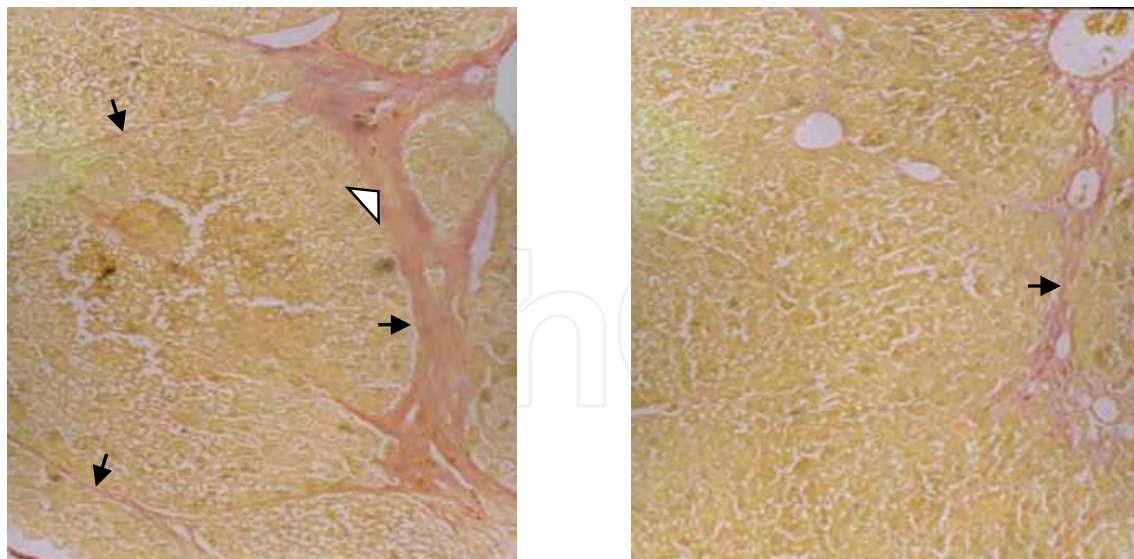


Fig. 10. Fuzheng Huayu Recipe inhibited hepatic fibrosis The liver sections were stained with Masson and showed hepatic fibrosis. A: Fibrous septa (arrows indicated) and pseudolobule (arrow's head indicated) have formed in controls. B: Fibrous septa (arrow indicated) is less and pseudolobule is absent in FZHY group (100×).

Groups	n	Hyp( $\mu\text{g/g}$ liver wet weight)
normal	7	112.37 $\pm$ 11.93
controls	11	292.83 $\pm$ 36.78*
FZHY	14	215.03 $\pm$ 75.46#

Note: compared with normal, \*:  $P < 0.05$ ; compared with controls, #:  $P < 0.05$

Table 4. Liver Hydroxyproline level in liver tissues ( $\bar{x} \pm s$ )

### 3.2.3.2 Fuzheng Huayu Recipe could effectively inhibit sinusoid capillarization in fibrotic rats

It was observed that the structure of sinusoids is clearly and the phenotype of SEC is thin by microscopy in normal group. There were many fenestrae in SEC cytoplasm and no basement membrane covered on SEC in perisinusoid side. In controls, the sinusoids were twisted and narrow, the fenestrae in SEC were reduced or disappeared, and basement membrane was observed. (Fig.11)Factor VIII related antigen (VIII R·Ag),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and laminin (LM), the important indexes for hepatic sinusoid capillarization, were positive staining in liver tissue. But collagen type IV (Col IV), which is a composition of functional membrane but not a composition of basement membrane, expressed majorly in fibrous septa and HSC but less and discontinuously in perisinusoids in controls. Compared with controls, however, the narrow sinusoids were reduced and some sinusoids returned to normal phenotype, basement membrane looked discontinuous thinner, and the positive degree of VIII R·Ag,  $\alpha$ -SMA and LM were lower and their positive area was significantly smaller by image analysis in the liver of FZHY group than in that of controls ( $P < 0.05$ ). Col IV expressed still continuously in perisinusoids in FZHY group. It indicated that the normal structure of sinusoids was most kept and it meanted that the alteration of hepatic sinusoid capillarization was reversed. (Fig.12)

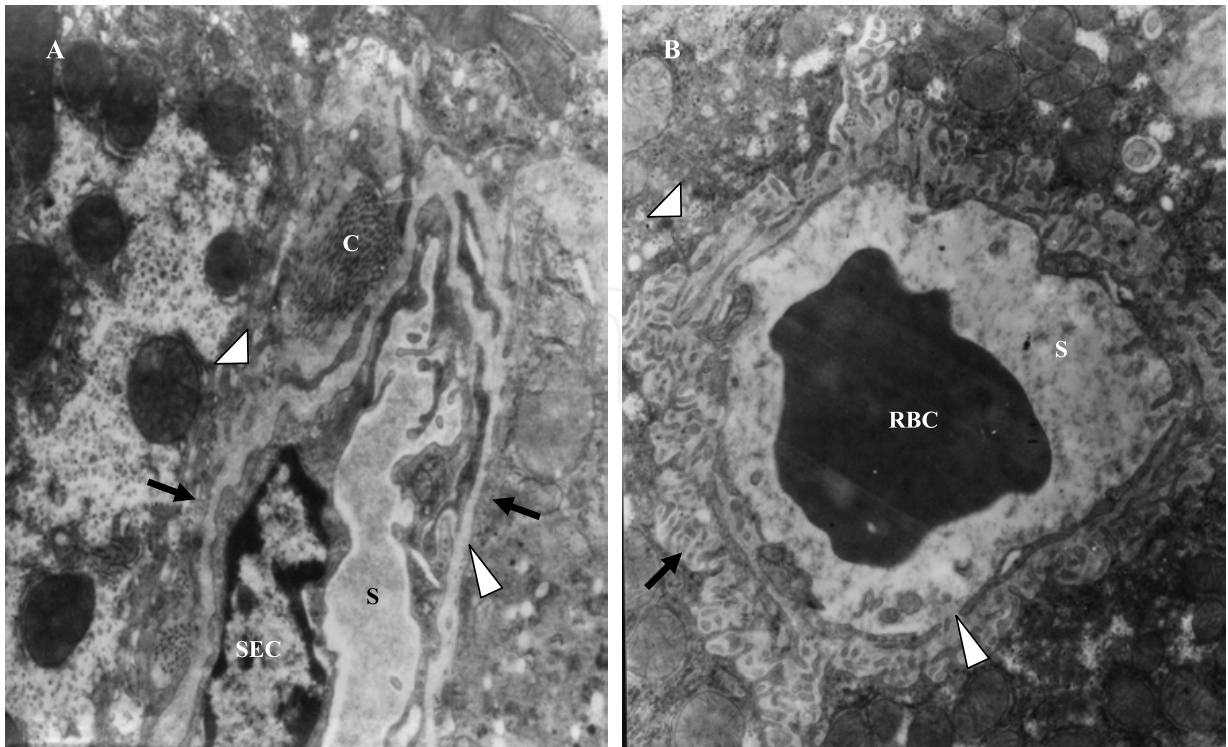
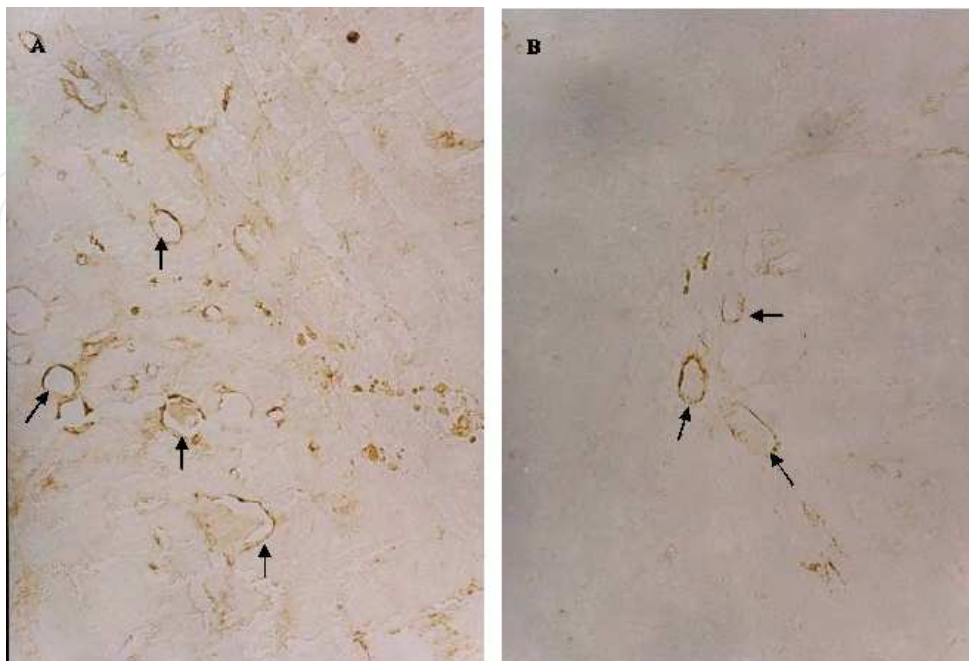
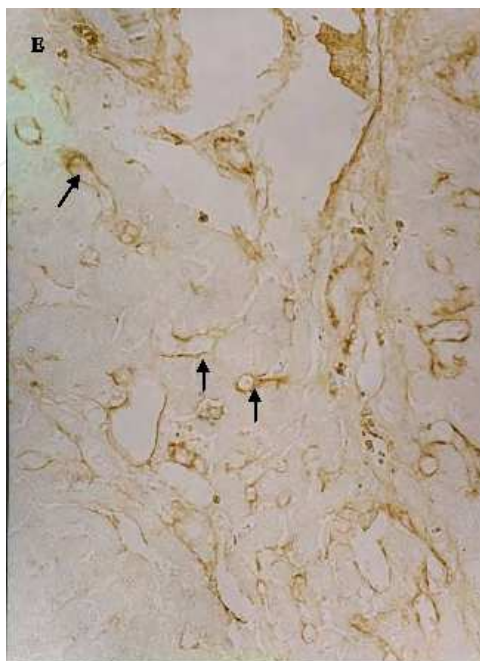
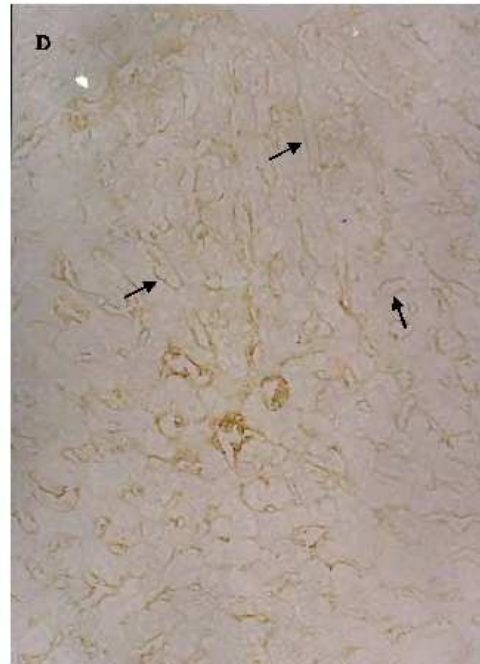
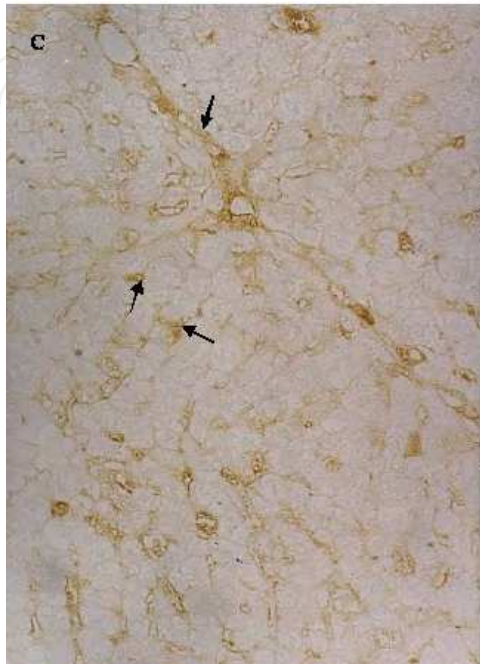


Fig. 11. Fuzheng Huayu Recipe could inhibit sinusoid capillarization by observation of electron micrographs S: sinusoid; C: collagen. A: Hepatic sinusoid appear as a typical capillary surrounded by continuous basement membrane. (arrow's heads indicated) in the liver of fibrotic rat. The perisinusoid is stenosis (arrows indicated) and microvilli of hepatocyte are almost absent in the space (15000 $\times$ ). B: Sinusoid capillarization is not formed in the liver of rat treated with Fuzheng Huayu Recipe. The fenestrae are observed (arrow's heads indicated) in cytoplasm of SEC. A mass of microvilli of hepatocyte stretches into perisinusoid is exist (arrow indicated) but basement membrane is absent (10000 $\times$ ).







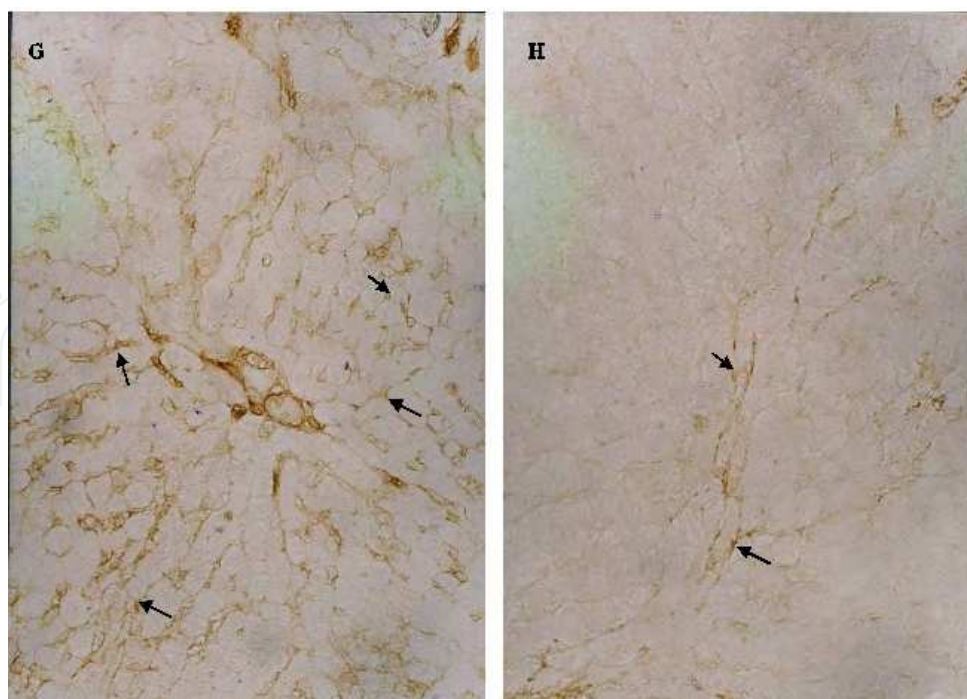


Fig. 12. Fuzheng Huayu Recipe could alter the express of indexes for hepatic sinusoid capillarization by immunohistochemistry staining in fibrotic liver of rats Arrows show the positive area. A: VIII R·Ag is around the vessels with strong staining in controls. B: The positive area of VIII R·Ag is decreased in FZHY group. C: Col IV expresses majorly in fibrous septa and HSC but less and discontinuously in perisinusoids in controls. D: Col IV expresses almost continuously in perisinusoids in FZHY group. E: The express of LM is observed on the wall of vessels and sinusoids in the liver of controls. F: The express of LM is only observed on the wall of vessels in the liver of FZHY group. G:  $\alpha$ -SMA expresses strongly in perisinusoids, HSC and fibous septa in controls. H:  $\alpha$ -SMA expresses dramatically less in FZHY group (100 $\times$ ).

### 3.2.4 Summary

Fuzheng Huayu Recipe had the potent effect on against liver fibrosis. The one of mechanisms of the recipe perhaps is associated with inhibition of hepatic sinusoid capillarization.

## 3.3 Experiment 3: SA-B could decrease portal vein pressure in rats induced by ET-1

### 3.3.1 Aims

To establish a rat model of portal hypertension induced by ET-1 and investigate the effect of SA-B on decreasing portal vein pressure in the model.

### 3.3.2 Methods

#### 3.3.2.1 To establish portal hypertension model of rats induced by ET-1

48 Sprague-Dawley male rats were randomly divided into four groups: the NS solution group, the ET-1 low-dose group (0.3 $\mu$ g/kg body weight), the ET-1 medium-dose group

(1.0 $\mu$ g/kg body weight) and the ET-1 high-dose group (3.0 $\mu$ g/kg body weight). Rats were injected with 200 $\mu$ l NS solution into rat via femoral vein by a pump with a rate of 80 $\mu$ l/min in NS solution group. As the same method, rats were injected with 200 $\mu$ l ET-1 solution with the dose of 0.3 $\mu$ g/kg body weight, 1.0 $\mu$ g/kg body weight or 3.0 $\mu$ g/kg body weight respectively in 3 ET-1 groups. Then, monitored the changes of carotid pressure and portal vein pressure in each group at 2min, 4min, 6min, 8min, 10min, 15min, 20min, 25min and 30min after injection.

### 3.3.2.2 To treat rats with drugs in portal hypertension models

20 Sprague-Dawley male rats with about 330g body weight were randomly divided into four groups: normal group, SA-B group, BQ-123 (ET<sub>A</sub>R antagonist) group and BQ-788 (ET<sub>B</sub>R antagonist) group (n=5 in each group). In normal group, BQ-123 group and BQ-788 group, rats were gavaged with water at a dose of 1ml/100g body weight and rats in SA-B group were gavaged with SA-B solution (containing 125mg/100ml) at the same dose. The gavage was performed twice per day for 5 days. The experiment started at 1 hour after the final gavage. First, via rat's femoral vein, rats was injected with BQ-123 (12.5 $\mu$ g/kg body weight) in BQ-123 group and with BQ-788 (15 $\mu$ g/kg body weight) in BQ-788 group. 30min later, rats were injected with a dose (3 $\mu$ g/kg body weight) of ET-1 solution by a pump with rate of 80 $\mu$ l/min in each group. Then, the portal vein pressure was measured before and after injection of ET-1.

### 3.3.2.3 To measure carotid pressure, heart rate and portal vein pressure

A piece of PE-50 tube, which full with heparin solution, was inserted into the left carotid artery of the rat. Following that, connect the tube to a pressure transducer in order to measure carotid pressure and heart rate. Then a piece of PE-10 tube, which full with heparin solution, was inserted from superior mesenteric vein to the middle of portal vein. Connect the tube to a pressure transducer for measurement of portal vein pressure.

## 3.3.3 Results

### 3.3.3.1 A kind of model of rat with portal hypertension was successfully established by injection of ET-1

There was no obvious effect of solution volume on carotid pressure and portal vein pressure after injection of NS solution. ET-1 solution with equal volume of NS solution was injected into the rats in low- and medium-dose groups. The carotid pressure was not noticeable rise or fall, only minor fluctuations within 20 min after injection. The carotid pressure, however, was increased slightly after injection of ET-1 in high-dose group but the alteration was not significant (Fig.13). Portal vein pressure was increased in short time after injection of ET-1 although the alteration was not same in three different dose groups. The alteration of pressure was most remarkable in high-dose group than in others. After reaching to peak level, portal vein pressure was stable for 4min, 4min and 10min in low-, medium- and high-dose groups, respectively (Fig.14 and Tab. 5). The results indicated that the increase of portal vein pressure was solely caused by the injection of ET-1 and without related with the volume of solution in this kind of animal model. This inducing method would not cause a significant increase of systemic blood pressure. Compared with the existing portal vein perfusion model, this kind of portal hypertension model is more similar to the "living" state. It was also avoided that increase of portal vein pressure caused by sudden increase of partial

blood volume by quickly direct injection of ET-1 in portal vein. This kind of model would better reflect the true effect of ET-1 compared with other kind of model. Therefore, this kind of model could use not only for investigation of efficacy and mechanism of drug, but also provided an ideal experimental subject for screening drugs to treat portal hypertension.

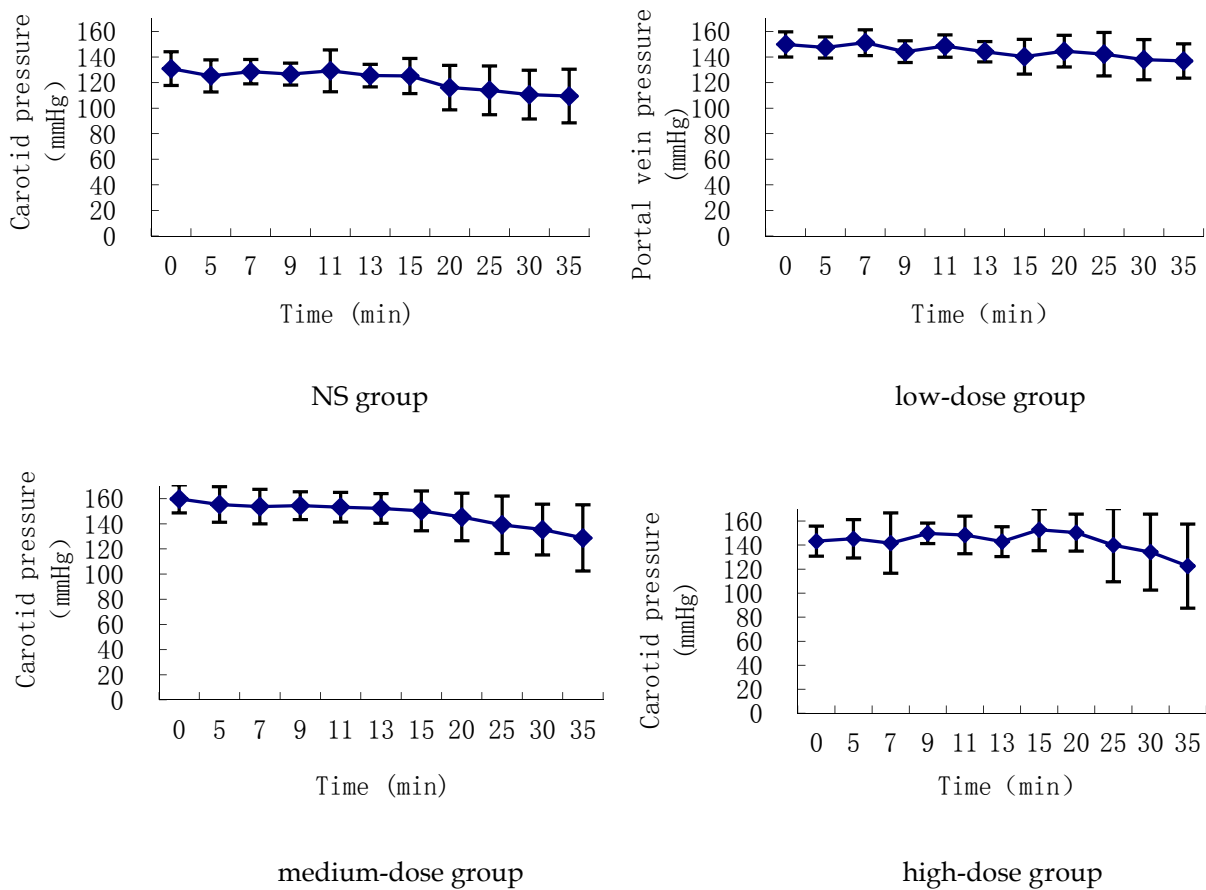
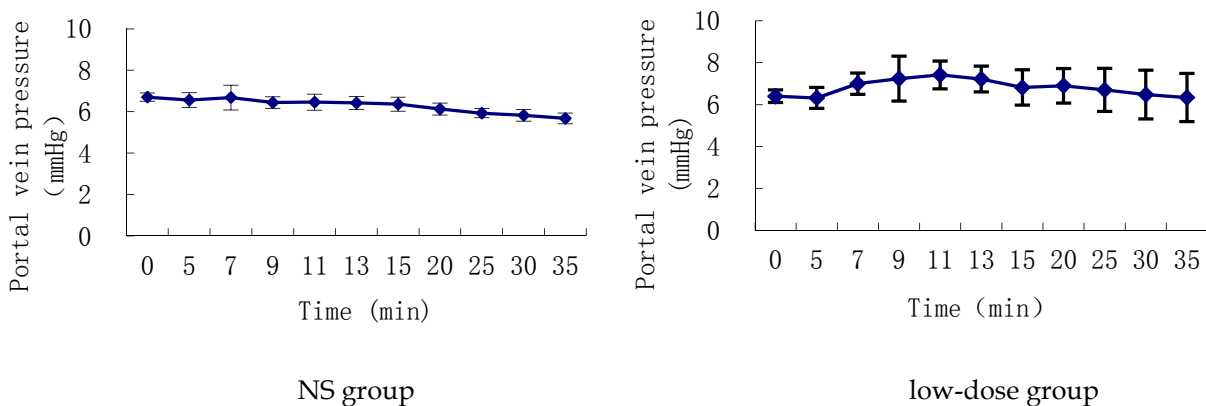


Fig. 13. Effect of ET-1 with different doses on rat carotid pressures After detecting carotid pressure for 5min, NS solution or ET-1 with same volume but different doses were constantly injected and carotid pressure was detected for 30min. The pressure was not increased during detected time in NS group, and low- and medium-dose groups. But the carotid pressure was increased slightly in high-dose group.



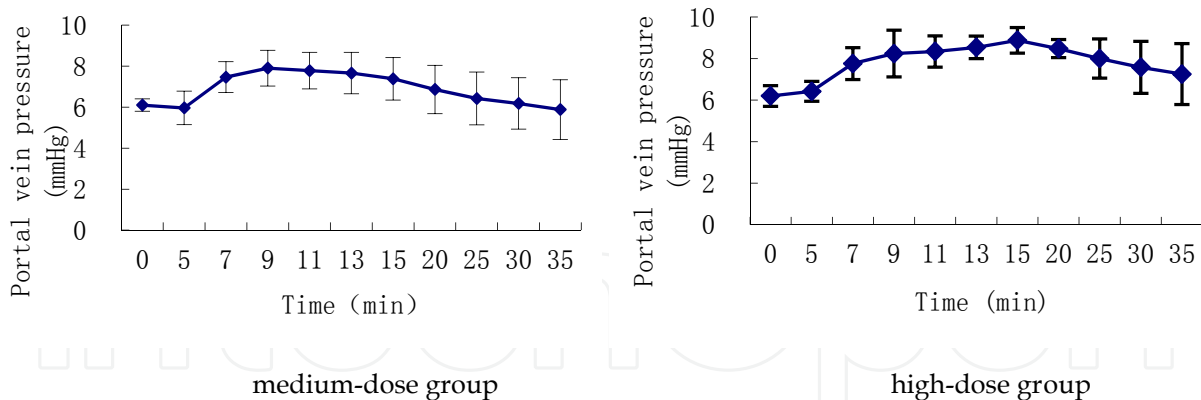


Fig. 14. Effect of ET-1 with different doses on rat portal vein pressure After detecting portal vein pressure for 5min, NS solution and ET-1 with same volume but different doses were constantly injected and portal vein pressure was detected for 30min. There was no obvious effect of NS solution on portal vein pressure. The pressure was increased at 5min when starting injection of ET-1 and kept high level for 4min, 4min and 10min in low-, medium- and high-dose groups after the pressure reaching to a peak level at 9 min, respectively.

Groups	n	Initial pressure (mmHg)	Highest pressure (mmHg)	Increased range (mmHg)
NS	8	6.6±0.3	6.5±0.4	-0.1±0.2
low-dose	10	6.1±0.8	7.5±0.8**	1.3±0.3▲▲▲
medium-dose	10	6.3±0.5	8.1±0.6**	1.8±0.5▲▲▲##
high-dose	9	6.0±0.6	8.5±0.9**	2.5±0.5▲▲▲##☆☆

Note: compared with the initial pressure in the interior-group, \*\*:  $P < 0.01$ ; compared with NS group, ▲▲▲:  $P < 0.001$ ; compared with low-dose group, ##:  $P < 0.01$ ; compared with medium-dose group, ☆☆☆:  $P < 0.01$

Table 5. Effect of ET-1 with different doses on portal vein pressure of rats ( $\bar{X} \pm S$ )

Groups	n	Initial pressure (mmHg)	Highest pressure (mmHg)	Increased range (mmHg)
control	5	6.1±0.7	8.6±1.1***	2.5±0.4
SA-B	5	6.1±0.7	7.8±1.6**	1.6±0.5##
BQ-123	5	5.9±1.1	7.1±1.0	1.2±0.2###
BQ-788	5	5.9±0.9	6.9±1.1	0.98±0.5###

Note: compared with initial pressure interior-group, \*\*:  $P < 0.01$ , \*\*\*:  $P < 0.001$ ; compared with the controls, ##:  $P < 0.01$ , ###:  $P < 0.001$

Table 6. Effect of SA-B, BQ-123 or BQ-788 on reducing portal vein pressure of rats induced by ET-1 ( $\bar{X} \pm S$ )

### 3.3.3.2 SA-B could inhibit the rise of portal vein pressure in rats induced by ET-1

Portal vein pressure significantly increased after ET-1 solution (3µg/kg body weight) was injected to rats pretreated with water by gavage. Although portal vein pressure of the rats,

which were pretreated with SA-B solution by gavage or with BQ-123 or BQ-788 by injection, was also increased after ET-1 injection, however, the range-increased was obviously less than that in controls ( $P<0.01$ ,  $P<0.001$ ) (Tab.6). Furthermore, the stable time, when portal vein pressure kept high level after reaching peak level, was short in SA-B group. It was just 6 min in SA-B group but was 10min in controls. (Fig. 15)

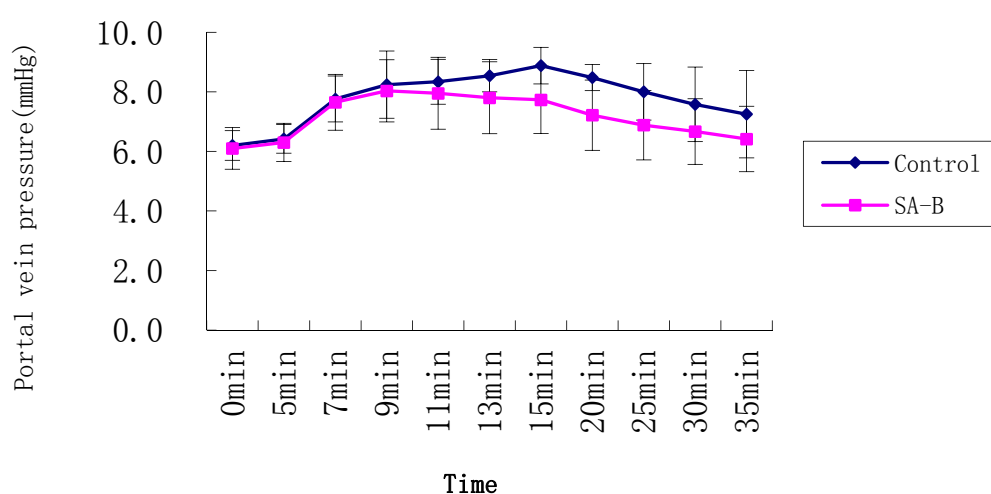


Fig. 15. SA-B could inhibit portal vein pressure of rats induced by ET-1 Portal vein pressure began increase at 5min when starting injection of ET-1. The peak level of pressure was at 9min in SA-B group. Then the pressure kept high level until to at 15min. In controls, however, the pressure continuously increased until to highest peak level at 15min and began to decrease. The pressure level was still higher (a little bit higher than a peak level in SA-B group) until to at 25min.

Groups	n	Carotid artery pressure (mmHg)	Heart rate (Beats/min)
control	3	130.9±13.2	439±21
SA-B	5	145.6±10.1	439±21

Table 7. Compare of the carotid artery pressure and heart rate in SA-B group and controls

### 3.3.3.3 SA-B did not affect on carotid pressure and heart rate in normal rat

Rats were pretreated with SA-B solution by gavage for 5d, but the carotid pressure and heart rate were no significant different between in SA-B group and controls. (Tab.7)

### 3.3.4 Summary

A kind of rat model of portal hypertension is successfully established by injection of ET-1. SA-B can down regulate the raised portal vein pressure by antagonizing the effect of extraneous ET-1.

### **3.4 Experiment 4: SA-B can decrease portal vein pressure in mice induced by ET-1**

#### **3.4.1 Aims**

To establish portal hypertension model of mice induced by ET-1 and investigate the effect of SA-B on decreasing portal vein pressure in this kind of model.

#### **3.4.2 Methods**

##### **3.4.2.1 To establish a kind of portal hypertension model mice induced by ET-1**

36 male Kunming mice,  $30 \pm 5$ g body weight, were randomly divided into three groups: control group (n=12), SA-B group (n=12) and blocker group (n=12). Mice were gavaged with SA-B (1mg/ml) in SA-B group and gavaged with water in control group and blocker group. The volume of gavage was 0.5 ml per mouse once a day for 3 days. Mice were injected with BQ-123 ( $2 \mu\text{g}/\text{kg}$  body weight) by tail vein at 0.5h before start of below experiment in blocker group.

Mice were injected with ET-1 ( $1.6 \mu\text{g}/\text{kg}$  body weight) at constant velocity by tail vein. 6 mice were randomly taken out from each group to measure blood flow volume in liver microcirculation using a laser-Doppler flow instrument before and after injection of ET-1. Other 6 mice in each group were measured the blood flow rate in liver microcirculation by an inverted fluorescence microscope with microscopic live video technology before and after injection of ET-1.

##### **3.4.2.2 To measure the average blood flow volume in the perfusion of liver microcirculation**

Each mouse was injected with 2% pentobarbital sodium solution at a dose of 2ml/kg body weight for anesthesia. After opening abdomen the liver was put on a detector connected with a laser-Doppler flow instrument to record the blood flow volume in the perfusion of liver microcirculation for 5min. After a pause, ET-1 was injected by a syringe pump at a constant rate of  $80 \mu\text{l}/\text{min}$  through tail vein. Then, the blood flow volume was recorded again for 5min. The average blood flow volume was calculated from the stable data during the former 5min record and late 5min record, respectively.

##### **3.4.2.3 To measure the blood flow rate in liver microcirculation**

Each mouse was injected with 2% pentobarbital sodium solution at a dose of 2ml/kg body weight for anesthesia. After opening abdomen the liver was put on a piece of glass plate which was on an inverted fluorescence microscope. After 0.05ml pre-labeled erythrocytes with FITC was injected into the inferior vena cava, a suitable site was focused to observe the liver microcirculation. Some FITC-RBCs were observed to be moving quickly in microvasculature or sinusoids and recorded with digital video. ET-1 ( $1.6 \mu\text{g}/\text{kg}$  body weight) was injected by a syringe pump at a constant rate of  $80 \mu\text{l}/\text{min}$  through tail vein of mouse then moving FITC-RBCs were immediately recorded again with digital video. The moving range of FITC-RBCs in record was calculated by the analytic software to compare the differences of blood flow rate in liver microcirculation in 3 groups.

### 3.4.3 Results

#### 3.4.3.1 SA-B reduced average blood flow volume in liver microcirculation

The blood flow volume in liver microcirculation was decreased in 3 groups after injection of ET-1. The reduced range was smaller in SA-B group and in block group than in controls ( $P<0.01$ ). (Tab. 8)

#### 3.4.3.2 SA-B reduced average blood flow rate in liver microcirculation

The blood flow rate in liver microcirculation was significantly decreased after injection of ET-1 ( $P<0.01$ ). But the reduced range was smaller in SA-B group and in blocker group than in controls ( $P<0.05$  and  $P<0.01$ ). (Tab. 9)

Average blood flow volume in liver microcirculation (BPU)				
Groups	n	Before ET-1 injection	After ET-1 injection	Reduced range
control	6	1186.83±41.14	1060.50±18.33	126.33±27.51
SA-B	6	1269.50±59.90	1189.33±40.33	80.17±20.30 **
blocker	6	202.00±36.54	1131.00±23.67	71.00±12.82 **

Note: compared with the controls, \*\*:  $P<0.01$

Table 8. SA-B and BQ-123 could inhibit reduction of average blood flow volume in mice induced by ET-1 ( $\bar{X} \pm S$ )

Blood flow rate in liver microcirculation (mm/s)				
Groups	n	Before ET-1 injection	After ET-1 injection	Reduced range
Control	6	0.60±0.01	0.36±0.02☆☆	0.23±0.02
SA-B	6	0.62±0.04	0.39±0.01☆☆	0.18±0.04 *
blocker	6	0.60±0.05	0.43±0.01☆☆	0.17±0.05 **

Note: compared with initial blood flow rate,☆☆:  $P<0.01$ ; compared with controls, \*:  $P<0.05$ , \*\*:  $P<0.01$

Table 9. SA-B and BQ-123 could inhibit reduction of the liver blood flow rate in mice induced by ET-1 ( $\bar{X} \pm S$ ).

### 3.4.4 Summary

The average blood flow volume and blood flow rate in mice liver microcirculation were reduced by extraneous ET-1. SA-B could inhibit the effect of ET-1 to improve liver microcirculation.

## 4. The experiment *in vitro*

It is believed that recipe of herb medicine is not suitable for research of mechanism *in vitro*. For more studying the mechanism of Fuzheng Huayu Recipe on reducing portal hypertension, we can only select SA-B as investigated subject *in vitro*.

## 4.1 Aims

To investigate the effects of SA-B on inhibiting contraction of human HSC induced by ET-1.

## 4.2 Methods

### 4.2.1 To observe contraction of HSC

Human HSC was isolated from the normal section of transplant patients' liver by enzymatic digestion and density-gradient centrifugation with Nycodenz solution.<sup>14</sup>. Passaged HSC was planted on the collagen gel which was pre-poured into 12-wells plates. After cells had adhered, HSC was divided into 5 groups: 1) serum-free group in which HSC was cultured with serum-free M199; 2) ET group in which HSC was treated with ET-1( $10^{-8}$  mol/L); 3) low-dose group in which HSC was treated with ET-1 and  $10^{-7}$ mol/L SA-B; 4) medium-dose group in which HSC was treated with ET-1 and  $10^{-6}$ mol/L SA-B; 5) high-dose group in which HSC was treated with ET-1 and  $10^{-5}$ mol/L SA-B. The edge of the gel was scraped out wall of the well by a syringe needle after adding drugs. The diameter of gel was observed at 2h, 4h, 6h, and 12 h during HSC culture and a gel image analysis system was used to calculate the area of gel. The area of gel can indicate the contractibility of HSC. The experiment was repeated for 3 times.

### 4.2.2 To detect the concentration of free calcium ( $[Ca^{2+}]_i$ ) in HSC

HSC was planted in 6-wells plates. Flu-3/AM was added into media with final concentration of  $5\mu\text{mol/L}$ . Then HSC was in a incubator with  $37^\circ\text{C}$ ,  $5\%\text{CO}_2$ - $95\%$  humid air and dark for 30min. HSC was washed twice with serum-free M199 for moving the dye and divided into 5 groups as above. HSC in each group was observed under a laser confocal microscope before and after adding relevant drugs. The observation conditions were Kr/Ar laser, excitation wavelength 488nm, emission wavelength 505-530nm. The experiment was repeated 2 times.

## 4.3 Results

### 4.3.1 SA-B inhibited contraction of HSC induced by ET-1

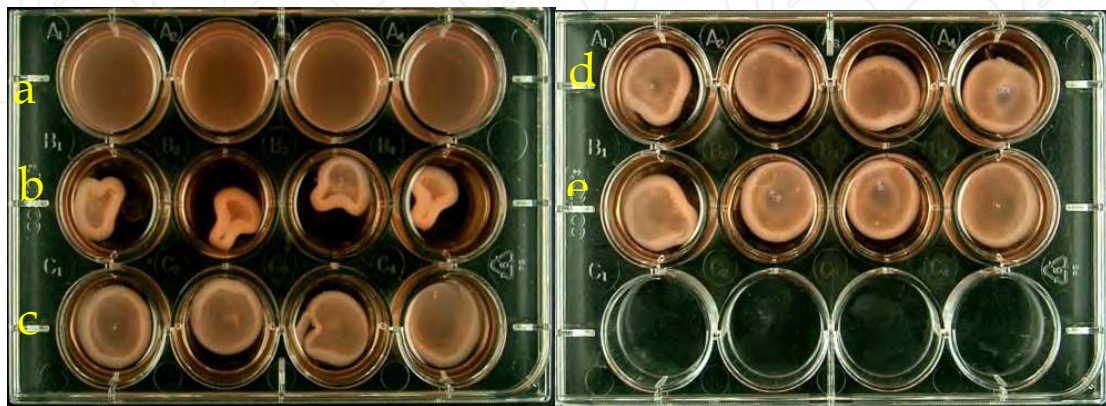
Area of gel was significantly bigger in low-dose group ( $2209.02\pm 177.96\mu\text{m}^2$ ), medium-dose group ( $2164.95\pm 111.84\mu\text{m}^2$ ) and high-dose group ( $2374.73\pm 218.38\mu\text{m}^2$ ) than in ET group ( $156.23\pm 102.16\mu\text{m}^2$ ). The statistical difference was dramatically ( $P<0.01$ ). But there was no obviously difference among in 3 SA-B treatment groups. (Fig. 16) The morphological changes of HSC showed dose-dependent after incubation with SA-B. The phenotype of HSC in low-dose group was similar to that in ET group. The phenotype of HSC in high-dose group was similar to that in serum-free group. The phenotype of HSC in medium-dose group was between the low- and high-dose groups. (Fig. 17)

### 4.3.2 SA-B suppressed the intracellular concentration of $[Ca^{2+}]_i$ in HSC induced by ET-1

Compared with ET group in which fluorescence of HSC was very strong,  $[Ca^{2+}]_i$  fluorescence image appeared the opposite phenomenon in 3 SA-B treatment groups. Only a few cells appeared slightly enhanced fluorescent and the intracellular concentration of



$[Ca^{2+}]_i$  was obviously lower in 3 SA-B treatment groups than in ET group. The intracellular concentration of  $[Ca^{2+}]_i$  was weak and only two relative low-lying peaks were in low-dose group within 120sec. The alteration of intracellular concentration of  $[Ca^{2+}]_i$  was smaller in medium-dose group and high-dose group during observation. (Fig.18). The results indicated that one of mechanisms of SA-B on inhibiting contraction of HSC may be related with that SA-B suppressed the intracellular concentration of  $[Ca^{2+}]_i$  in HSC.



★★VS model group  $P<0.01$

☆☆VS serum-free group  $P<0.01$

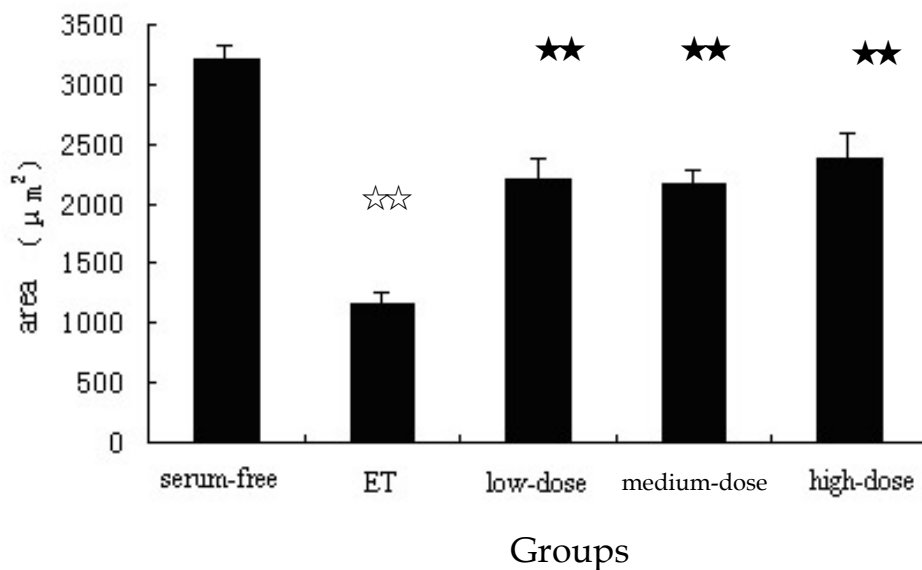


Fig. 16. SA-B could inhibit contraction of human HSC a: serum-free group; b: ET group; c: low-dose group; d: medium-dose group; e: high-dose group. Human HSC was cultured on collagen gel. Activated HSC are able to contract to lead to area of gel becoming small. In serum-free group, contraction capacity of HSC was weak so that the area of gel was nearly invariant. Active HSC induced by ET-1 contracted strongly. The area of gel was significantly smaller in ET group than in serum-free groups. SA-B could inhibit contraction of HSC induced by ET-1. The area of gel was bigger in 3 SA-B treatment groups than in ET group.

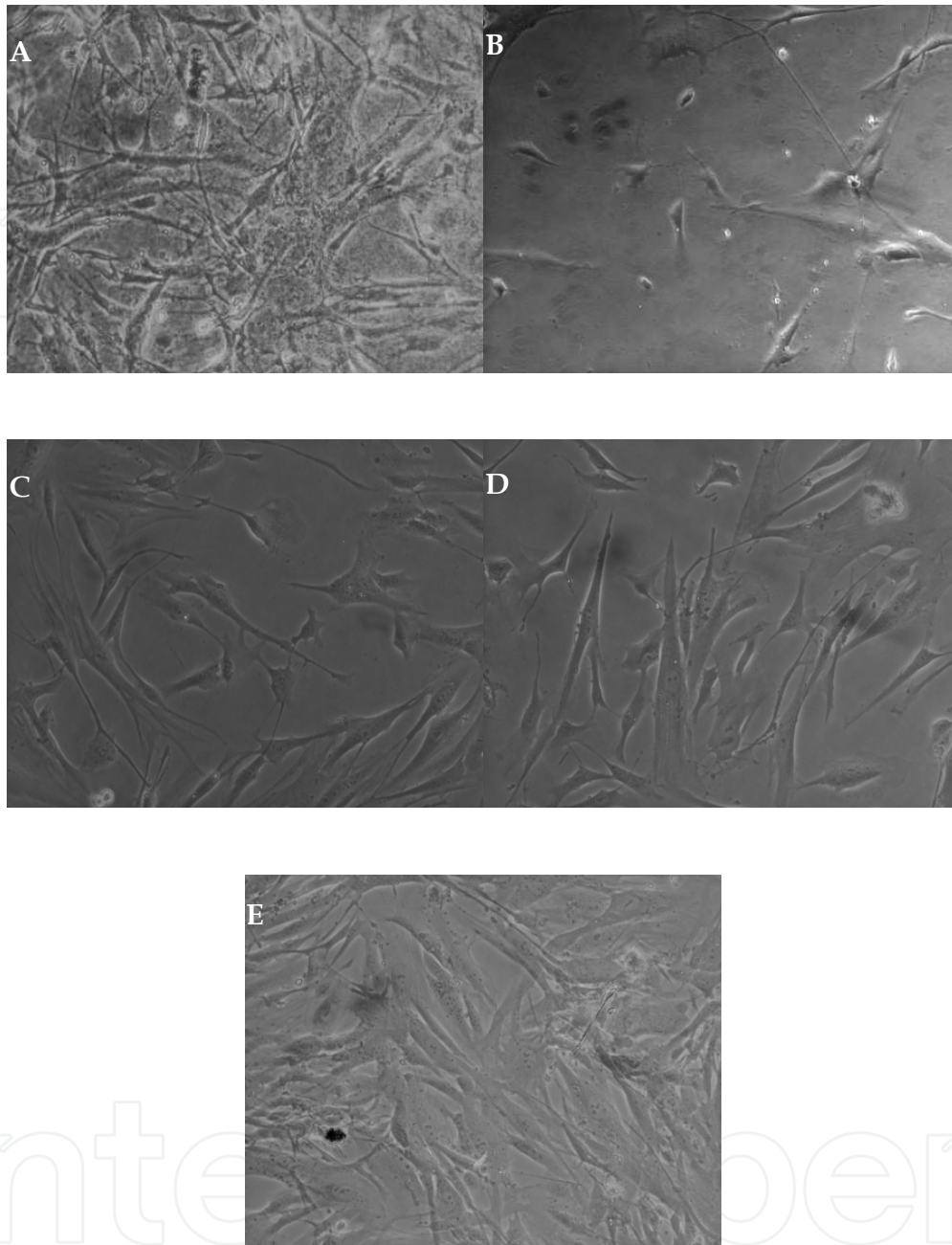


Fig. 17. The morphological changes of HSC in each group a: serum-free group; b: ET group; c: low-dose group; d: medium-dose group; e: high-dose group. HSC grew exuberantly on gel and the pseudopodium of HSC was a lot, thin and long in serum-free group. After induced by ET-1 for 12h, however, HSC became obviously small with contraction. The quantity and the pseudopodium of HSC was decreased in ET group. The phenotype of HSC in low-dose group was similar to that in ET group. The phenotype of HSC in high-dose group was similar to that in serum-free group. The phenotype of HSC in medium-dose group was between the low- and high-dose groups (By OLYMPUS I × 50 / I × 70 inverted system microscope, Japan, 200×).

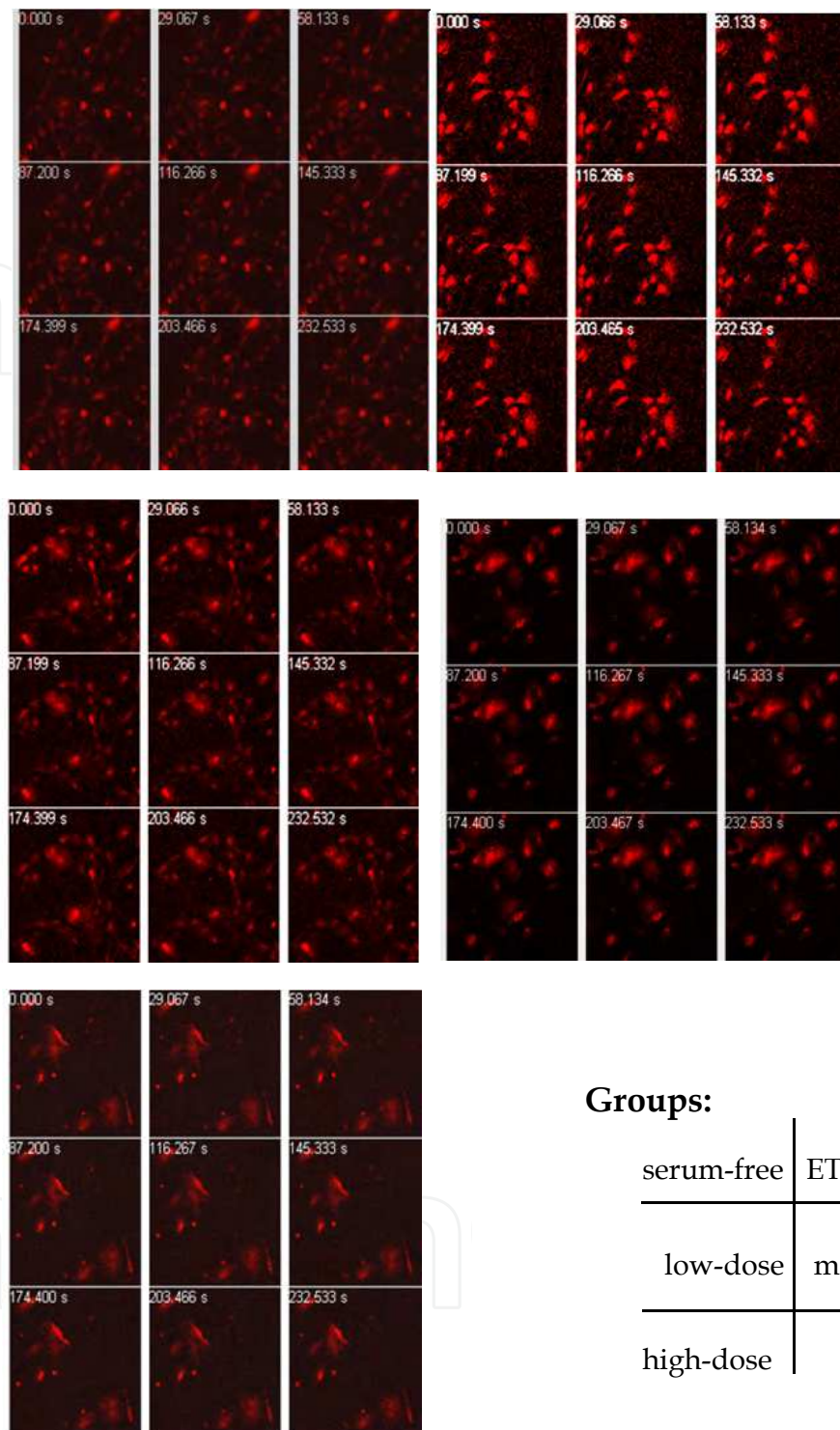


Fig. 18. SA-B suppressed the intracellular concentration of  $[Ca^{2+}]_i$  in HSC Fluorescent of Flu-3/AM which represents the intracellular concentration of  $[Ca^{2+}]_i$  was observed. The expression of fluorescent in HSC was very stronger in ET group than in serum-free group. But the expression of fluorescent in HSC was dose-dependent in 3 SA-B treatment groups. The higher the dose of SA-B was, the weaker expression of fluorescent was (By Zeiss510 laser confocal microscope, German, 200 $\times$ ).

## 5. Conclusion

The Chinese medicine, Fuzheng Huayu Recipe, is able to reduce portal hypertension in liver cirrhosis both in clinic and *in vivo*. The effect of Fuzheng Huayu Recipe is related with reversing hepatic fibrosis and capillarization, and decreasing concentration of ET-1 in the liver tissue. SA-B is an extracted component of Fuzheng Huayu Recipe. Several studies showed that the effect in SA-B was similar to Fuzheng Huayu Recipe on reversing hepatic fibrosis and decreasing concentration of ET-1 in the liver tissue. SA-B inhibited contraction of HSC through suppressing the intracellular concentration of  $[Ca^{2+}]_i$ . Based on above effects, intrahepatic vessel resistance was reduced which led to improve liver microcirculation. It has distinctively character that Chinese herb medicine is used to treat hepatic portal hypertension which is caused by failure of liver microcirculation in chronic liver diseases.

## 6. Acknowledgements

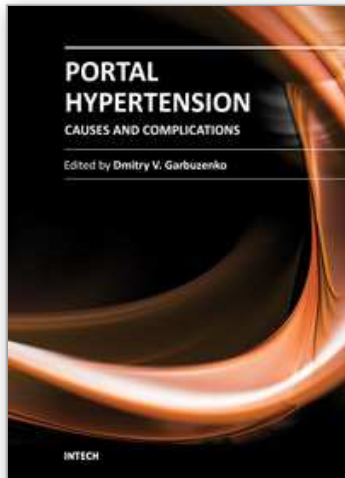
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## **Portal Hypertension - Causes and Complications**

Edited by Prof. Dmitry Garbuzenko

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Portal hypertension is a clinical syndrome defined by a portal venous pressure gradient, exceeding 5 mm Hg. In this book the causes of its development and complications are described. Authors have presented personal experiences on conducting patients with various displays of portal hypertension. Moreover, the book presents modern data about molecular mechanisms of pathogenesis of portal hypertension in liver cirrhosis, the information about the original predictor of risk of bleeding from gastro-esophageal varices and new methods for their conservative treatment.

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