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Original Article

Balancing Effect of Biejiajian Oral Liquid (鳖甲煎口服液) on ACE-Ang Ⅱ-AT1R Axis and ACE2-Ang-(1–7)-Mas Axis in Rats with CCl_4-Induced Hepatic Fibrosis*

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ABSTRACT **Objective**: To explore the effect of Biejiajian Oral Liquid (鳖甲煎口服液, BOL) on CCl_4-induced hepatic fibrosis in rats by detecting the changes in the levels of angiotensin Ⅱ (Ang Ⅱ), angiotensin-(1–7) [Ang-(1–7)], angiotensin-converting enzyme (ACE), ACE2, angiotensin Ⅱ type 1 receptor (AT1R), Mas, etc. **Methods**: A total of 180 Wistar rats were randomly divided into two groups by random digital table method: prevention experiment and treatment experiment. Each group was further subdivided into the following 6 subgroups: normal control group, model group, vitamin E [100 mg/(kg·d), VE] group, enalapril [10 mg/(kg·g), Ena] group, high-dosage [20 g/(kg·d)] BOL group, and low-dosage [10 g/(kg·d)] BOL group. The hepatic fibrosis rat model was established by subcutaneous injection of CCl_4 for 6 weeks. Prevention experiment and treatment experiment were administered with specific drugs at different times. At the end of treatment experiment, the pathological changes of liver were observed after hematoxylin-eosin staining. The expressions of ingredients in renin-angiotensin-aldosterone system (RAAS) such as Ang Ⅱ, Ang-(1–7), ACE, ACE2, AT1R, Mas, renin, CYP11B2 and angen in liver were detected by enzyme linked immunosorbent assay, immunohistochemistry method or reverse transcription-polymerase chain reaction, respectively. **Results**: The levels of Ang Ⅱ and Ang-(1–7) at the 6th week increased by 496.10% and 73.64%, respectively, compared with those at the 2nd week in the model group (P<0.01). With prevention or treatment with high-dosage BOL, there was an evident reduction of Ang Ⅱ level but an improvement of Ang-(1–7) level. Specifically, Ang Ⅱ level of high-dosage group decreased by 77.50% in prevention experiment (P=0.000) and by 76.93% in treatment experiment (P=0.002) compared with that in the model group. Ang-(1–7) level increased by 91.69% in prevention experiment (P=0.006) and by 70.77% in the treatment experiment (P=0.010) compared with that in the model group. The expression levels of mRNA of renin, ACE, CYP11B2, angen and AT1R decreased by 58.15%, 99.90%, 99.84%, 99.99% and 99.99% (all P<0.01), respectively. **Conclusions**: BOL could help resist liver fibrosis in rats by enhancing the level of each ingredient in ACE2-Ang-(1–7)-Mas axis, while decreasing the level of each ingredient in ACE-Ang Ⅱ-AT1R axis. To some extent, BOL could enhance the regulation of RAAS in rats with CCl_4-induced hepatic fibrosis.

KEYWORDS hepatic fibrosis, Biejiajian Oral Liquid, renin-angiotensin-aldosterone system, carbon tetrachloride, Chinese medicine

Hepatic fibrosis is characterized by the activation and proliferation of hepatic stellate cells (HSCs), extracellular matrix hyperplasia and abnormal precipitation. It is a process in the middle of chronic hepatitis turning into cirrhosis.[1,2] Since hepatic fibrosis is a reversible process, it is necessary and crucial to block the development of this process. Recently, research has shown that the repair process after tissue injury is accompanied with the activation of the renin-angiotensin-aldosterone system (RAAS), which could promote the formation of hepatic fibrosis in partial liver.[3] Angiotensin-converting enzyme (ACE)-angiotensin Ⅱ (Ang Ⅱ)-type 1 receptor (AT1R) axis can promote the process of hepatic fibrosis, and Ang Ⅱ especially advances the activation of HSCs. Notably, there also exists the ACE2-Ang-(1–7)-mas

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axis, which can prevent liver from being injured.[4] These results indicate that regulating RAAS maybe a new therapeutic strategy to treat and prevent liver fibrosis, which deserves to be explored more.[5,6]

Many experiments have proven that Biejiajian Pills (鳖甲煎丸) can significantly reduce the levels of aspartate transaminase (AST), alanine aminotransferase (ALT), hyaluronic acid (HA), laminin (LN) and collagens in serums of rats with hepatic fibrosis. The levels of these indicators often increase during the process of fibrosis, which then aggravate fibrosis. Biejiajian Pills could treat liver fibrosis induced by complex factors such as pig serum, alcohol and carbon tetrachloride (CCl_4), etc.[7-11] Also, Biejiajian Pills have been proven to have treatment effects in clinical practice.[12]

Carapax trionycis, containing oligo peptides, amino acids and a variety of trace elements, can inhibit proliferation of HSCs and hyperplasia of connective tissue, promote reabsorption of fibrous tissue and degradation of HAs, and reduce lipid peroxidation.[12-14] Many prescriptions with *Carapax trionycis* as the main component can be used to treat liver fibrosis. The Biejiajian Oral Liquid (鳖甲煎口服液, BOL) in this study is also one of such prescriptions.[15] BOL is an improved preparation from Biejiajian Pills, which was recorded in an ancient medical book called *Synopsis of the Golden* (Jin Kui Yao Lue). BOL can treat hepatic fibrosis by inhibiting HSCs from synthesizing and secreting extracellular matrix (ECM), improving liver microcirculation and regulating immune function.[16] In this study, we studied the influence of BOL on RAAS in rats with CCl_4-induced hepatic fibrosis. Specifically, we detected changes in the levels of AngⅡ, Ang-(1–7), ACE, ACE2, AT1R and Mas in rats, on which the mechanism of BOL against hepatic fibrosis might be revealed.

METHODS

Materials

AngⅡ enzyme-linked immunosorbent assay (ELISA) kit and Ang-(1–7) ELISA kit were obtained from Shanghai WestangBio-Tech Co., Ltd. (China, 1041489 and 1041490, respectively). Immunohistochemistry (IHC) kit was obtained from Wuhan Boster Biological Technology Co., Ltd. (China). Blood total RNA extraction kit, reverse transcription kit and fluorescent quantification kit were obtained from Hangzhou Biosci Biotech Co., Ltd. (China, RE10050, RT02020 and PM10003, respectively). DNase Ⅰ was obtained from Promega Co., Ltd. (Madison, USA, M6101). Vitamin E was obtained from Xinchang Pharmaceutical Factory of Zhejiang Pharmaceutical Co., Ltd. (China, batch No. 130215). Enalapril was obtained from Jiangsu Yangzi Pharmaceutical Co., Ltd. (China, batch No. 12091703).

Animals

All animal experiments were approved by the Medical Ethics Committee of Zhejiang Chinese Medical University. A total of 180 male Wistar rats weighing 150±20 g were used in this study [Certification No. SCXK (Hu) 2013-0016]. The animals of specific pathogen free grade were raised in an air-conditioned room at temperature (25±3 ℃) and humidity (50%±5%) with a 12-h dark/light cycle. All experiments were conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and the guidelines of Zhejiang Chinese Medical University Committee on Animals.

Preparation of BOL

BOL was prepared by the Pharmacy College of Zhejiang Chinese Medical University, consisting of the following herbs: *Carapax Trionycis* 30 g, *Radix Scutellariae* 7.5 g, *Radix Bupleuri* 15 g, *Radix et Rhizoma Rhei* 7.5 g, *Ramulus Cinnamomi* 7.5 g, *Cortex Moutan* 12 g, *Semen Lepidii* 2.5 g, *Folium Fructus* 7.5 g, *Rhizoma Belamcandae* 7.5 g, *Cortex Magnoliae Officinalis* 7.5 g, *Dungbeetle* 15 g, *Rhizoma Zingiberis* 7.5 g, *Radix Paeoniae Rubra* 12 g, *Dianthus Superbus* 7.5 g, *Flos Campsis* 7.5 g, *Pinellia Ternata* 2.5 g, *Ginseng* 2.5 g, *Eupolyphaga seu Steleophaga* 12 g, *Colla Corii Asini* 7.5 g, *Semen Persicae* 5 g, *Nidus Vespae* 10 g and *Pillbug* 7.5 g. These herbs were made into 100 mL of BOL, in which the crude concentration of Chinese medicine was 2 g/mL. The high dosage of BOL for rats was set to 20 g/(kg·d) and the low dosage was set to 10 g/(kg·d).[17]

Establishment of Animal Model

Twenty rats were used as normal controls, and the remaining rats were induced with liver fibrosis according to the protocol described previously.[16] The time required for liver fibrosis induction was 6 weeks.

Groups and Administration

Treatment experiment: 90 rats were randomly

divided into 6 groups by random digital table method: normal control group (*n*=10), model group (*n*=10); vitamin E [100 mg/(kg·d), VE] group (*n*=15), enalapril [10 mg/(kg·d), Ena] group (*n*=15); high-dosage BOL group [20 g/(kg·d), *n*=20], and low-dosage BOL group [10 g/(kg·d), *n*=20]. After liver fibrosis induction, rats were treated for 6 weeks respectively. Rats in the normal and model groups were given normal saline instead. Rats were executed in batches at the end of the 2nd, 4th, and 6th weeks, meanwhile with administration.

Prevention experiment: the remaining 90 rats were included and the grouping and administration was the same as above except that all the rats were administered with corresponding drugs till the end of the induction.

Histological Analysis

At the end of the experiment, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (3%) at a dose of 40 mg/kg. The livers were taken out from the abdominal cavity aseptically. After their shape and texture were observed, liver tissues were washed with saline solution, fixed in 10% neutral-buffered formalin, embedded in paraffin, and finally cut into 4-μm sections. The sections were stained using hematoxylin and eosin (HE), and then the degree of pathological progression in the liver was evaluated under a light microscope.

IHC Examinations

Immunohistochemistry kit was used to detect the expression of ACE, ACE2, AT1R and Mas. The processes of dewaxing and hydration were in accordance with preceding descriptions. Antigen retrieval, antigen antibody reaction and dianlino benzidine coloration were performed according to the manufacturer's instructions.

Detection of Ang Ⅱ and Ang-(1–7) Levels

Freshly harvested liver tissues were immediately flash frozen in liquid nitrogen and stored at –70 ℃ until further processing. The frozen tissues were weighed using a balance on dry ice. Ice-cold buffer was added to liver tissue (10 mL per gram of liver) and the tissues were minced in beaker with scissors. Tissues were homogenized at 700 r/min for 20 s using a glass homogenizer on ice. Ang Ⅱ and Ang-(1–7) were determined by ELISA.

Reverse Transcription-Polymerase Chain Reaction Analysis of RAAS

Total RNA was extracted using total RNA extraction kit, chloroform and isopropanol. Then, cDNA was synthesized using reverse transcription kit. Reverse transcription-polymerase chain reaction (RT-PCR) amplification was implemented with a PCR amplifier. The primer sets used in the RT-PCR are listed in Table 1. In addition, the equation $2^{-\triangle\triangle Ct}$ was used to determine the relative amount of mRNA.

Table 1. RT-PCR Primers

Primers	Sequence 5'–3'	Product size (bp)
Angen	Forward: AGCACGGACAGCACCCTATT	125
	Reverse: ATGGGCACAGACACTGAGGT	
AT1R	Forward: CAACTGCCTGAACCCTCTGT	138
	Reverse: CGGTAAGAAAGCGTGCTCATT	
ACE	Forward: CTGGGACTTCTACAACGGCA	88
	Reverse: CATTTCGTGGTGGGCTATCA	
Renin	Forward: GCCCTGGGAGTCAAAGAGAA	112
	Reverse: TGCTGAGAGTGTAGGTCCTGC	
CYP11B2	Forward: TCAGACCTACAGTGGCATTGT	102
	Reverse: GCTCCATAGAGTTGGCTTTGA	
β-actin	Forward: CAGCCTTCCTTCTTGGGTAT	105
	Reverse: CTGTGTTGGCATAGAGGTCTT	

Statistical Analysis

Data were presented as mean ± standard deviation ($\bar{x} \pm s$). Statistical analysis was performed with ANOVA using SPSS 13.0 software and *t*-test was adopted between the groups. Results were considered statistically significant when $P<0.05$.

RESULTS

Visual Inspection after Treatment Administration

Visual inspection was performed to investigate the general status of the liver. The liver of rats in the normal group had dark red color and pliable, smooth and elastic texture. In contrast, the liver of rats in the model group showed slightly reduced volume and brown color. Moreover, it had grainy surface and hard texture. There were varying degrees of improvement in liver conditions of rats in the positive control groups (VE and Ena groups), as well as in the low- and high-dosage BOL groups. The liver of rats in these groups had deep red color and smooth surface with only a few granular nodules.

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Hepatic Histopathological Changes after Treatment Administration

Liver tissues in the normal group showed normal lobular architecture with central vein and radiating hepatic cords. No degeneration, inflammation or necrosis of hepatocytes was observed. In the model group, histopathological lesions were observed, hepatocytes were swollen severely, and hepatic cords were disordered. The normal lobular architecture was destroyed and maintained in different sizes. Moreover, there was infiltration of inflammatory cells. Compared with the model group, VE and Ena groups showed improved liver conditions, except the infiltration of sinusoid by inflammatory cells. The high-dosage BOL group also showed better liver conditions, with mild lobular disorder, clear lobular structure, and infiltration with only a small number of inflammatory cells (Figure 1).

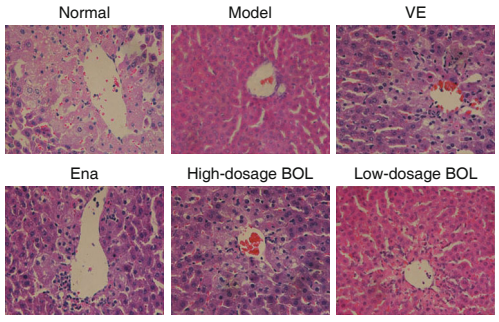

Figure 1. Pathological Morphology (HE staining, ×400)

Effects of BOL on Expressions of ACE, ACE2, AT1R and Mas after Treatment Administration

IHC analysis demonstrated that the expressions of ACE, ACE2, AT1R and Mas were significantly increased and mainly distributed in cytoplasm and nucleus in the model group compared with those in normal group. After BOL was administered, the expression of ACE2 and Mas receptors was decreased obviously, especially in cytoplasm and nucleus, compared with that in the model group. However, the expressions of ACE and AT1R was decreased only slightly compared with that in the model group. Compared with the positive control groups, BOL group showed increased expressions of ACE2 and Mas but decreased expressions of ACE and AT1R (Figure 2).

Dynamic Changes of Ang Ⅱ and Ang-(1–7) Levels

Dynamic Changes during Formation of Hepatic Fibrosis in Rats

During the formation of hepatic fibrosis in rats, ELISA was performed to detect the levels of Ang Ⅱ and Ang-(1–7) in liver homogenate at the 2nd, 4th and 6th weeks, respectively. With the development of hepatic fibrosis, the levels of Ang Ⅱ and Ang-(1–7) at the 6th week were gradually increased by 496.10% and 73.64%, respectively, compared with those at the 2nd week in the model group (P=0.001, P=0.002, Figure 3).

Dynamic Changes during Prevention Administration

In prevention experiment, groups were administered with corresponding drugs till the end of induction. It was found that high-dosage BOL could decrease the level of liver Ang Ⅱ by 77.50% while increase the level of Ang-(1–7) by 91.69%. The results

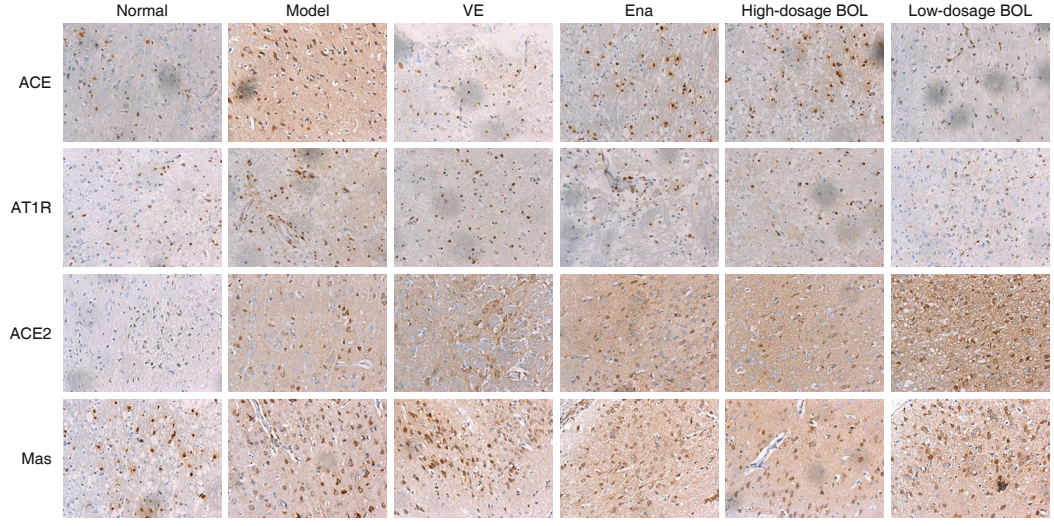

Figure 2. Expressions of ACE, ATIR, ACE2 and Mas in Rats with Hepatic Fibrosis (HE staining, ×200)

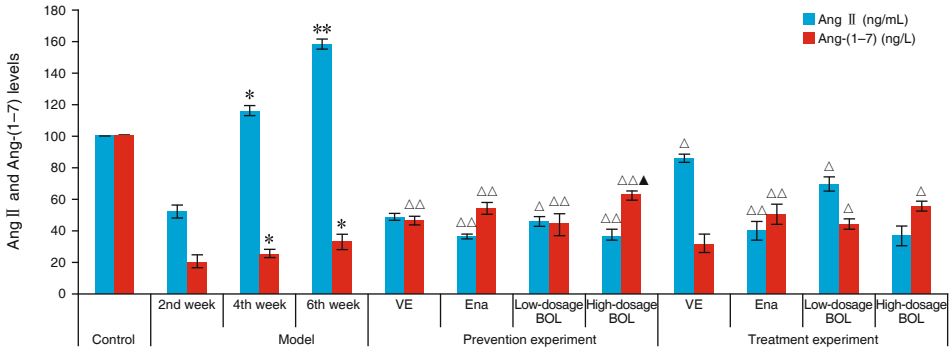

Figure 3. Ang Ⅱ and Ang-(1–7) Levels in Liver Homogenate of Rats

Notes: $^{*}P<0.05$, $^{**}P<0.01$, *vs*. the control group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$, *vs*. the model group; $^{\blacktriangle}P<0.05$, *vs*. the Ena group

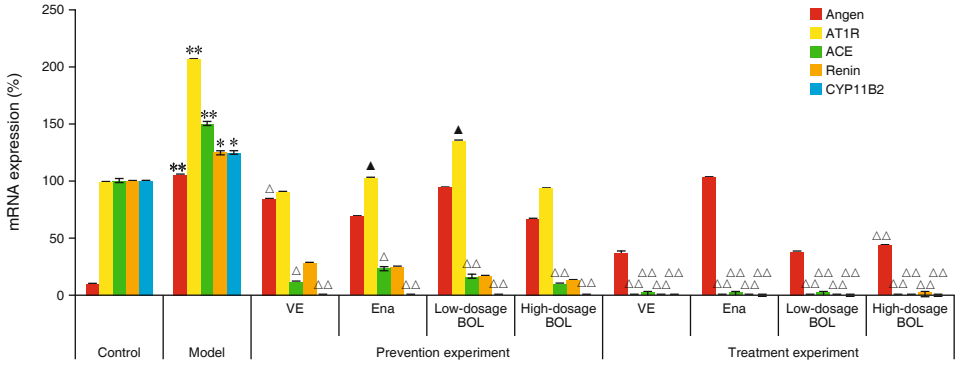

Figure 4. Expressions of Angen, AT1R, ACE, Renin, and CYBP11B2 mRNA in Rat Liver

Notes: $^{*}P<0.05$, $^{**}P<0.01$ *vs*. the control group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ *vs*. the model group; $^{\blacktriangle}P<0.05$ *vs*. the Vit E group

of Ena group and high-dosage BOL group showed significant difference compared with that of the model group (P=0.000; P=0.006, Figure 3).

Dynamic Changes after Treatment

In treatment experiment, groups were administered with drugs for six weeks after induction. It was found that high-dosage BOL could decrease the level of liver Ang Ⅱ by 76.93% and increase the level of Ang-(1–7) by 70.77%. The Ena group and the high-dosage BOL group showed better effects than other groups, with significant differences compared with the model group (P=0.002; P=0.010, Figure 3).

Effects of BOL on Expressions of Angen, Renin, ACE, CYP11B2, and AT1R mRNA

With the development of CCl_4-induecd hepatic fibrosis, the expression of mRNA of Renin, ACE, CYP11B2, AT1R and Angen were increased. After high-dosage BOL treatment, expression levels of renin, ACE, CYP11B2, angen and AT1R were decreased by 58.15%, 99.90%, 99.84%, 99.99% and 99.99% respectively, compared with those in model group (all P<0.01; Figure 4).

DISCUSSION

Hepatic fibrosis is a complex disease provoked by a range of wound-healing responses to chronic injuries, and is a result of a substantial accumulation of ECM.[17-19] Hepatocytes are stimulated by cytokines, which lead to an abundant deposition and proliferation of ECM. Accumulated evidence indicates that there exists RAAS in partial heart, kidney, lung and other tissues. It plays a vital role in fibrotic process.[20,21] RAAS is an endocrine system, which performs an important role in controlling blood pressure and regulating electrolytes. It was reported that activated HSCs are associated with a marked over expression of the components of RAAS and a production of Ang Ⅱ.[3] There also exists independent RAAS in liver tissues.[4]

Tissues could express partial ingredients of RAAS, including Angen, renin, ACE and AT1R. Locally generated Ang Ⅱ could participate in tissue remodeling in human liver.[4,21] Additionally, it can stimulate the proliferation and migration of ECM as well as up-regulate cytokines, leading to hepatic

fibrosis. ACE level is obviously increased in the plasma of patients with hepatic cirrhosis, indicating that RAAS plays a crucial role in the processes of hepatic fibrosis.

The study reveals that liver injuries could lead to the up-regulation of the components of RAAS, causing free radical formation and oxidative stress, and stimulating redox-sensitive intracellular pathways.[22] It was found that, in cultured HSCs of rats, Ang Ⅱ could stimulate the formation of reactive oxygen species, cell proliferation and secretion of pro-inflammatory cytokines.[22] Moreover, Ang Ⅱ could stimulate the procoagulant activity of HSCs, a newly described biological function for these cells.

There are two axes in RAAS: ACE-Ang Ⅱ-AT1R axis and ACE2-Ang-(1–7)-Mas axis. Balancing the ACE-Ang Ⅱ-AT1R axis and the ACE2-Ang-(1–7)-Mas axis is a new strategy in anti-hepatic fibrosis. According to our study, Ang Ⅱ level was gradually increased in the process of liver fibrosis in rats and remained significantly higher than that in the normal group. Meanwhile, the expression of mRNA of Angen, Renin, ACE and AT1R in liver tissue were enhanced. This suggested that renin could promote Angen into Ang Ⅰ, and increased expression of ACE can improve the production of Ang Ⅱ. In addition, Ang-(1–7) level was also increased. Both the expression of ACE2 and Mas receptors in the liver were enhanced. Ang Ⅱ could transform into Ang-(1–7) under catalysis by ACE2.

Our results indicated that in both prevention and treatment experiments BOL reduced the level of Ang Ⅱ while increasing the level of Ang-(1–7). The effect after 2 weeks of prevention was not evident, which might be due to the short damage time and drug prevention. Notably, the effects after six weeks of prevention and treatment were evident. With the extension of time, liver damage became more serious.

The protective effects of the drugs were also highlighted by comparison with the model group. In all administration groups, Ena and BOL showed better effects. Although the VE group showed decreased Ang Ⅱ, VE had little influence on Ang-(1–7) level. However, BOL could inhibit the expression of all ingredients of ACE-Ang Ⅱ-AT1R axis in liver RAAS, blocking the transformation of Angen into Ang Ⅰ and decreasing the expression of ACE to inhibit the generation of Ang Ⅱ. Meanwhile, the decreased expression of ACE2 promoted the transformation of Ang Ⅱ into Ang-(1–7), while inhibiting the transformation of Ang-(1–7) into Ang-(1–5) under the effect of ACE. Thus, the expression of Ang-(1–7) was increased in rats.

Furthermore, the expression of Mas receptors was increased in rat livers. Ang-(1–7) could act on Mas receptors and then resist liver fibrosis. Although Ena had better antifibrotic effects, it is an angiotensin-converting enzyme inhibitor. It could directly affect RAAS, strongly inhibiting the angiotensin-converting enzyme, directly reducing Ang Ⅱ, inducing vasodilation, decreasing the blood pressure and leading to dry cough, angioneurotic edema, hyperkalemia, etc. These make it difficult to control the dose of Ena. BOL could enhance the animal's appetite, improve the body's condition, repair injured liver cells, and mitigate liver fibrosis.[23] The result proves that RAAS is activated in liver fibrosis.

Also, BOL could enhance the effects of the receptors of Ang-(1–7), Ang Ⅱ and Mas, while weakening the functions of ACE, Ang Ⅱ and AT1R to resist liver fibrosis. The inhibition effect is consistent with the conclusions of our previous study.[24] According to the results, the long-time drug prevention could lead to recovery from liver fibrosis. Because of the complex pathogenesis of liver fibrosis, further study needs to be done in the future.

During the progression of liver fibrosis in rats, RAAS in the liver was activated, with increased expressions of its ingredients. ACE2-Ang-(1–7)-Mas axis could protect the liver and inhibit the formation of liver fibrosis. BOL exerts antifibrotic effects by increasing the level of each ingredient of the ACE2-Ang-(1–7)-Mas axis while decreasing the level of each ingredient of the ACE-Ang Ⅱ-AT1R axis. Both the prevention and treatment experiments showed BOL had protective effects against liver fibrosis, but long-term preventive medication showed better effects.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Yao L conceived the study and established the initial design of the study. Peng Y and Bu XW conducted the

experiments. Yao J provided the technological guidance. Peng Y and Li XY wrote the manuscript. All authors have read and approved the final manuscript.

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