Scientific Research

Effect of Biejiajian Oral Liquid on the Expression of RAAS in Hepatic Fibrosis Rats

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ABSTRACT

Objective: To study the effect of Biejiajian Oral Liquid (BOL) on the rennin angiotensin aldosterone system (RAAS) in plasma of hepatic fibrosis rats and in hepatic stellate cell (HSC) of normal rats. We explore the mechanism of BOL on inhibiting the activation of HSC and illustrate its mechanism of anti-hepatic fibrosis further. **Methods:** SD Rats were divided into 5 groups randomly: normal control group, model group, Enalapril group and BOL groups with different concentration (2.0 g/ml or 1.0 g/ml). Every group was administered with CCl_4 and olive oil solution to induce hepatic fibrosis except normal one. Each group was treated with the respective drug for 5 weeks and then got the blood. The level of renin, angiotensin II and aldosterone in the plasma of liver fibrosis rats were detected by the radioimmunoassay. By using reverse transcription-polymerase chain reaction (RT-PCR) to measure the gene expression of the rennin, ACE, angiotensinogen, AT1R and ALD. The AT1R gene expression in normal HSC was determined by the immunohistochemical measurement. **Results:** BOL could effectively reduce the activity of the PRA, AngIIand ALD, which showed a significant effect on the inhibition of the AngII ($P < 0.01$). Meanwhile, compared with the normal control group, there was a notable inhibitory action on the PRA of HSC which was administrated by serum containing BOL ($P < 0.05$). And yet, drug applied group showed no difference with the model group for other factors of the RAAS. **Conclusion:** BOL can inhibit the expression of RAAS in the rat plasma and can inhibit the expression of the mRNA of renin in the normal HSC, which could be the mechanism of anti-hepatic fibrosis.

Keywords: Hepatic Fibrosis; RAAS; Biejiajian Oral Liquid; Serum Pharmacology; Rat

1. Introduction

Hepatic fibrosis is a common pathological process of chronic liver diseases, which character is the excess accumulation of the extracellularmatrix (ECM) with the main part of collagen in the liver. It's the early and necessary stage of cirrhosis and can be reversed at certain conditions [1].

The occurrence of liver fibrosis has a close relationship with the hepatic stellate cell (HSC). The activation and proliferation of HSC as well as the increase of synthesis of ECM will eventually lead to the hepatic fibrosis [2]. Recent studies have shown that there is reninangiotensin- aldosterone system (RAAS) in HSC. The appearance and development of hepatic fibrosis is closely related to the RAAS [3]. The local liver RAAS participates in the development of hepatic fibrosis [4]. Angiotensin II (Ang II) is one of the most important bioactive peptides, which is able to induce the activation of HSC, promoting the secretion of transforming growth factor β

(TGF-β), platelet-derived growth factor (PDGF) and other cytokines and increasing the expression of collagens in type I and II, and then to promote the formation of hepatic fibrosis [5-7]. Some studies have also shown that angiotensin-convertase inhibitor (ACEI) and Ang II receptor type 1 blocker (ARB) can significantly attenuated the development of hepatic fibrosis, and possibly become a potential new potent drug for hepatic fibrosis [8-10].

Our previous research had proved that BOL could anti-hepatic fibrosis effectively. Being observed under the electronic microscope, the fat droplets in liver cell of the high-dose BOL group decreased, the structure of nucleus was clear and the mitochondria were tumid lightly compared with the model group [11,12]. A further study is necessary on whether the action of BOL inhibiting the proliferation of HSC is concerned with the expression of RAAS in HSC. Our research aims to study the effect of BOL on the expression of RAAS in the plasma of rats with hepatic fibrosis and in normal HSC so as to illustrate its mechanism of anti-hepatic fibrosis further.

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2. Materials and Methods

2.1. Animal Modeling and Samples Preparation

2.1.1. Animal Modeling

Sixty male Sprague-Dawley (SD) rats (provided by the Zhejiang Academy of Medical Sciences SCXKC-Zhe-2003-0001, Zhejiang, China) were used in this study. Liver fibrosis was induced in rats using a protocol described previously [12].

The hepatic fibrosis rats were randomly divided into 4 groups *i.e.* model group (n = 12), Enalapril group (n = 12), BOL high dosage group (n = 13) and low dosage group (n = 13). The normal group and the model group ig 0.9% NaCl (NS) 10 ml/kg. The Enalapril group ig Enalapril 10 mg/kg. BOL low and high dosage group ig BOL 10 ml/kg (containing raw drug 1.0 and 2.0 g/ml respectively). All groups had been treated once a day for 5 weeks and took water and fodder ad lib. Venous blood was collected from the abdominal cavity vein of anesthetized rat intraperitoneal injection of 3% pentobarbital sodium at 40 mg/kg. Separated plasma was used to detect renin activity, level of Ang II and aldosterone by radioimmunoassay. Serum was separated to detect the activity of ACE.

2.1.2. Preparation of Different Concentration BOL

BOL was an improved preparation from Biejiajian Pill that was recorded in *Jinkui Yaolue* (Zhang Zhongjing, Eastern Han Dynasy). It contained 2 g/ml raw drug. The dosage for rats was 20 g/kg for high dosage and 10 g/kg for low dosage respectively.

2.2. The Preparation of the Serum Containing BOL

Thirty two male SD rats of 280 - 320 g were randomly divided into 4 groups *i.e.* NS group, Enalapril (10 mg/kg) group, groups receiving high (20 g/kg) and low (10 g/kg) dosages of BOL. The rat was ig twice a day and last for 7 days. Venous blood was collected from inferior vena cava under asepsis 1 h after the last ig, then serum was separated (3000 r/min, 20 min, 4°C), inactivated at 56°C for 30 min, and stored at −70°C for use.

2.3. The Culture of HSC

The HSC was provided by Professor Zhishan Ding of College of Life Science at Zhejiang Chinese Medical University. The maintenance of HSC had been described previously [13].

2.4. Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

A total of $2 \times 10^5/cm^2$ HSC were inoculated into culture flasks and incubated in DEME containing relative drug serum for 48 h. Total RNAs from HSC were isolated. Semi-Quantitative RT-PCR was performed using an oligo dT primer and SuperScript One-Step RT-PCR system (Invitrogen Corp), according to the manufacturer's instructions. The following primer sets were used in the RT-PCR:

Agt (F 5'-GCACGACTTCCTGACTT-3', R 5'-TG-ACGCTGGCCTTGT-3'); ACE (F 5'-AAACATTGGC-TACACTGTAAGT-3', R 5'-TTCGTGGAGGAGTAT-GACC-3'); Agtr1 (F 5'-GAGGCGGACTACTACGCC-AA-3', R 5'-TAGCGCCAGGAATTGTTGCT-3'); Ren (F 5'-AAAGTTGCCCTGGTAATG-3', R 5'-GTGCTA-AAGGAGGAAGTGTT-3'); Cyp11b (F 5'-CTCCTCGT-CATATCCATCTG-3', R 5'-GCAGCCAATCAGAAA-TGTGG-3'); Actb (F 5'-ACTGCCGCATCCTCTTCC-TC-3', R 5'-ACTCCTGCTTGCTGATCCACAT-3'). The mRNA levels of genes of interest were normalized to that of Actb mRNA expression.

2.5. Statistical Analysis

Data was reported as mean ± standard deviation (SD). Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) with the post-hoc Bonferroni adjustment was used for comparisons between multiple groups. In cases when there were only two groups for comparison, the two-tailed Student's t test was used. All P values were two-sided and the level of statistical significance was set $P < 0.05$.

3. Results

3.1. The Effect of BOL on RAAS in Plasma of Hepatic Fibrosis Rats

Compared with normal control group, ACE ($P < 0.05$), PRA and Ang II ($P < 0.01$) increased significantly in the model group. It indicated that liver fibrosis model was established successfully. Compared with model group, after being treated once a day for 5 weeks, PRA, Ang II and ACE ($P < 0.01$) decreased significantly in Enalapril group. Ang II and ACE ($P < 0.01$), PRA and ALD ($P < 0.05$) decreased significantly in BOL high dosage group. PRA and Ang II ($P < 0.05$) also decreased significantly in BOL low dosage group. But ALD and ACE ($P > 0.05$) of BOL low dosage group didn't decrease significantly (**Table 1**).

3.2. The Effect of BOL on RAAS in HSC

Compared with normal control group, expressions of mRNA of PRA, Prehypertensin, ACE, CYP ($P < 0.05$) and AT1R ($P < 0.01$) were down regulated significantly by administering with Enalapril. Expressions of mRNA

of PRA were down regulated significantly both in BOL high and low dosage groups (P < 0.05), but there was no significant difference between them. Expressions of mRNA of Prehypertensin, ACE, CYP and AT1R in BOL high and low dosage groups showed no significant difference (P > 0.05) (**Table 2**).

3.3. Expression of AT1 Receptor in HSC

Immunohistochemical detection demonstrated that the expression of AT1R in HSC could be found. According to our result, there is no significant difference between normal control group and other administration groups (P > 0.05) (**Table 3**).

4. Discussion

4.1. The Effect of BOL on RAAS of the Hepatic Fibrosis Rats Plasma and the Normal HSC

4.1.1. The Effect of BOL on RAAS of the Hepatic Fibrosis Rats Plasma

Recent studies had demonstrated that there was independent RAAS in the liver, which could be abnormally stimulated during the hepatic fibrosis. There had positive correlation between Ang II level and the fibrotic degree [14]. This study demonstrated that in vivo experiment, the content of PRA, Ang II and ACE in plasma were obviously increased as the hepatic fibrosis forming, which showed a dramatic difference compared with the normal group (P < 0.01).

The above-mentioned indexes in positive control group were obviously reduced (P < 0.01), which was

consistent with relative literature. In BOL high concentration group, the content of PRA, Ang II, ALD and ACE were all decreased, and PRA and ACE showed significant difference (P < 0.01). The content of PRA and Ang II in BOL low concentration group showed difference significantly (P < 0.05). These results hinted that inhibiting the expression of RAAS in plasma of rats was probably the mechanism of BOL to prevent the denaturation and necrosis of hepatic cells as well as anti-hepatic fibrosis.

BOL could significantly down regulate the RAAS and effect on multi-links in RAAS, blocking various links of RAAS activation effectively, which showed advantages of multitarget compared with AT-1 receptor antagonist Candesartan and ALD antagonist Antisterone [15,16].

4.1.2. The Inhibition Effect of BOL on Local RAAS in HSC of Normal Rats

HSC occupied for about 5 percent of the entire hepatic cells, which existed in Space of Disse. Under normal condition, HSC could promote the split and hyperplasia of hepatic cells and synthesize extracellular matrix [17-19]. HSC could also express CYPII β2, which was the key enzyme for synthesizing aldosterone and increases gradually in the formation of hepatic fibrosis [20].

This study had shown that there was an obvious inhibitive effect of serum containing BOL on the expression of rennin mRNA of normal HSC (P < 0.05), meanwhile, showing no significant difference on the expression of other genes of RAAS compared with normal group.

Table 1. The effect of BOL on PRA, Ang II, ALD and ACE in plasma of hepatic fibrosis rats (X ± S).

groups	n	PRA (ng/ml)	Ang II (pg/ml)	ALD (pg/ml)	ACE (ng/ml)
Normal control	8	10.86 ± 4.08	250.73 ± 76.85	124.60 ± 37.90	68.80 ± 6.78
model	11	21.13 ± 3.89$^{\Delta\Delta}$	736.51 ± 411.94$^{\Delta\Delta}$	147.01 ± 43.05	80.98 ± 9.44$^{\Delta}$
Enalapril	12	14.11 ± 8.31**	377.18 ± 106.25**	111.97 ± 23.02	69.55 ± 7.67**
High dosage	12	15.75 ± 2.63*	480.30 ± 208.91**	88.91 ± 18.13*	70.04 ± 6.09**
Low dosage	12	16.55 ± 5.09*	540.37 ± 135.1*	121.43 ± 45.31	80.00 ± 4.62

Note: $^{\Delta}$P < 0.05, $^{\Delta\Delta}$P < 0.01 vs. normal control group; *P < 0.05, **P < 0.01 vs. model group.

Table 2. The effect of BOL on expressions of RAAS mRNA in HSC (X ± S, n = 6).

groups	PRA	Prehypertensin	ACE	AT1R	CYP
Normal	1.05 ± 0.18	0.73 ± 0.16	0.29 ± .011	0.41 ± 0.18	0.57 ± 0.06
Enalapril	0.70 ± 0.23$^{\Delta}$	0.49 ± 0.22$^{\Delta}$	0.18 ± 0.17$^{\Delta}$	0.24 ± 0.04$^{\Delta\Delta}$	0.46 ± 0.07$^{\Delta}$
High dosage	0.65 ± 0.13$^{\Delta}$	0.59 ± 0.13	0.32 ± 0.04	0.41 ± 0.09	0.55 ± 0.06
Low dosage	0.70 ± 0.19$^{\Delta}$	0.51 ± 0.11	0.28 ± 0.07	0.37 ± 0.93	0.53 ± 0.08

Note: $^{\Delta}$P < 0 .05, $^{\Delta\Delta}$P < 0 .01 vs. normal group.

Table 3. Expression of AT1 receptor in HSC (X ± S, n = 6).

groups	normal	Enalapril	High dosage	Low dosage
AT1R	2.25 ± 082	2.33 ± 0.52	3.50 ± 1.10	3.50 ± 1.64

According to our study, the inhibitive effect of BOL on the signal pathway of RAAS of HSC was mainly focused on the process of synthesizing the rennin, thus blocking the development of hepatic fibrosis further [21-23]. BOL serum reduced the synthesis of rennin of RAAS in HSC, inhibiting the transformation of angiotensinogen induced by rennin, and might make further influence on the Ang II level, which ultimately inhibited the activation of HSC [24,25]. The effect of supporting the healthy energy of Traditional Chinese Medicine aimed to recover benign intracellular communication. For instance, any abnormity occurring in certain link of signal pathways might cause the uncontrollable development during the process of tumor generation. Traditional Chinese Medicine had the anti-tumor ability by influencing the cytokine and growth factor. This experiment revealed the target of BOL on depressing RAAS in HSC, which provided a novel way to explore theeffect of anti-hepatic fibrosis for Traditional Chinese Medicine [26].

However, BOL displayed an insignificant effect on other links of RAAS in HSC. The reasons might be interpreted as the following two aspects [27-30].

1) It was concerned with the two-way regulation effect of Traditional Chinese Medicine. The two-way regulation effect of Traditional Chinese Medicine could be considered as an integration role for multitarget in cellular signal pathways of multi-component in Chinese medicinal herbs, which mainly displayed in the aspects on immunization, endocrine, intestinal tract and temperature. Consequently, for HSC in various functional statuses, BOL might show different terminal effect. According to this experiment, the genes in positive control group were obviously inhibited (P < 0.05) while the genes in Chinese medicinal herbs groups were not obviously inhibited, which suggested that BOL had no effect on RAAS of normal HSC.

2) It was concerned with the effect time of BOL on HSC. *In vivo* study, the time for treatment of BOL was 5 weeks, which showed different down regulation effect on the expression of PRA, Ang II, ALD and ACE in plasma. Meanwhile, only the expression of rennin mRNA in normal HSC had been reduced by serum containing BOL. Thus, it was presumed that the regulation effect of BOL on expressions of indexes in RAAS of HSC was concerned with the effect time of serum containing BOL. Further experiment should be launched to explore whether serum containing BOL had the effect on regulating mRNA expression of angiotensinogen, ACE and

AT1R in HSC as well as the influence from the drug action time.

4.2. The Mechanism of BOL on Anti-Hepatic Fibrosis by Regulating RAAS

RAAS performed a key role in formation of high blood pressure of human and animal model. Lately, the focus of interest on the role of RAAS in the pathophysiology of hypertension and organ injury had changed to a major emphasis on the role of the local RAS in specific tissues [31,32]. It was thought previously that RAAS belonged to endocrine system. As the development of molecular biology, rennin and some other compounds of RAAS had been discovered in extra-renal tissues and organs, which suggested that RAAS was not only a cyclical endocrine system, but also probably a general distributed paracrine, autocrine or endocrine system [33]. Recently, some studies on the endogenous RAAS had shown that angiotensin had direct effect on the proliferation of cells in artery and heart, which was achieved by different angiotensin in different tissues and organs [34]. Ang II receptor was the major receptor of angiotensin, which was classified into AT1 and AT2. AT1 performed a critical role in a variety of physiological functions and pathophysiological state [35-37]. It was considered that after binding with AT1 receptor, angiotensin regulated the phospholipase through GTP binding protein, hydrolyzing phosphatidylinositol-4,5-bisphosphate into diacylglycerol (DG) and inositol triphosphate (IP3), thus forming a set of second messenger system based on phosphoinositide, which showed close relationship with the regulation of cell proliferation [38]. Recently, it had been reported that the endogenous RAAS promoted fibrosis of myocardium, vessel and renal interstitium. It was proved that Ang II could stimulate the synthesis and deposit of extracellular matrix of vascular smooth muscle cell, resulting in vessel wall fibrosis, which inferred to the similar function in other organs [39]. Lim *et al.* got obvious effect by using AT-1 receptor antagonist Losartan to treat hypertrophic cardio myopathy rats. The expressions of mRNA of type I collagen and TGF-β were decreased more than 50% [40]. By now, the effect of RAAS on hepatic fibrosis had been paid more attention. Yoshiji *et al.*, found that AT-1 receptor antagonist Candesartan could alleviate hepatic fibrosis of rats, reducing the expressions of serum markers of hepatic fibrosis and TGF-β mRNA [41]. Xishan Yang, *et al.*, proved that the expression of aldosterone synthase gene CYPIIβ2 mRNA was enhanced during the formation of hepatic fibrosis [42]. Aldosterone antagonist Spironolactone had inhibitive effect on the early stage of hepatic fibrosis. AT-1 receptor was found on HSC, which had been confirmed by sequencing.

This study found that BOL could inhibit the expres-

sion of rennin mRNA in RAAS of normal HSC. Besides, it had inhibitive effect on several links of cyclical RAAS, inhibiting hepatic fibrosis through multitarget [34]. Consequently, the mechanism of BOL on inhibiting the expression of RAAS and anti-hepatic fibrosis was probably to reduce the activity of rennin in organs, blocking AT1 receptor and ALD receptor simultaneously, inhibiting the activity of ACE and reducing the Ang II level in liver, so as to inhibit the activation of HSC. BOL could decrease the level of Ang II and ALD in circulatory system. The reduction of Ang II and block of AT-1 receptor could effectively inhibit the secretion of TGF-β. TGF-β was the key factor for HSC on promoting proliferation and transforming into fibroblasts, which performed vital role in preventing and treating hepatic fibrosis and regulating the activity and function of target cells.

BOL was an improved formulation of Biejiajian pills recorded in Jinkui Yaolue (Zhang Zhongjing, Eastern Han Dynasy), having ability to reduce the content of Laminin (LN) and Hyaluronic Acid (HA) in hepatic fibrosis serum [11]. This study showed that RAAS in liver was significantly activated during the formation of hepatic fibrosis. After being administrated with BOL, the activity of RAAS in rat serum and the degree of hepatic fibrosis were obviously reduced; this indicated that local RAAS in liver participated in the occurrence of hepatic fibrosis.

BOL could effect on several links of RAAS. Compared with the single target drug AT-1 receptor antagonist Candesartan and ALD antagonist Spironolactone, BOL owned multitarget advantage, which showed better prospect for application and more suitable for hepatopath.

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