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RAPID COMMUNICATION

Effect of Oxymatrine on the TGFbeta-Smad signaling pathway in rats with CCl_4-induced hepatic fibrosis

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Abstract

AIM: To explore the anti-fibrotic effect of Oxymatrine on CCl_4-induced liver fibrosis in rats and its modulation on the TGFbeta-Smad signaling pathway.

METHODS: One hundred healthy male SD rats were randomly divided into three groups: normal group (n = 20), treatment group of Oxymatrine (n = 40) and CCl_4-induced fibrosis group (n = 40). Experimental hepatic fibrosis was induced by subcutaneous injection of carbon tetrachloride (CCl_4 soluted in liquid paraffin with the concentration of 300 g/L, the dosage of injection was 3 mL/kg, twice per week for 8 wk). The treated rats received Oxymatrine *via* celiac injection at a dosage of 10 mg/kg twice a week at the same time. The deposition of collagen was observed with H&E and Masson staining. The concentration of serum TGF-β1 was assayed with ELISA. The gene expression of Smads and CBP (CREB binding protein) was detected with *in situ* hybridization (ISH) and immunohistochemistry (IH), respectively. All the experimental figures were scanned and analyzed with special figure-analysis software.

RESULTS: A significant reduction of collagen deposition and rearrangement of the parenchyma was noted in the liver tissue of Oxymatrine-treated rats. The semi-quantitative histological scores (2.43 ± 0.47 μm^2 *vs* 3.76 ± 0.68 μm^2, $P < 0.05$) and average area of collagen in those rats were significantly decreased when compared with hepatic cirrhosis model rats (94.41 ± 37.26 μm^2 *vs* 290.86 ± 89.37 μm^2, $P < 0.05$). The gene expression of Smad 3 mRNA was considerably decreased in the treated animals. The A value of Smad 3 mRNA was lower in the treated rats than the model rats (0.034 ± 0.090 *vs* 0.167 ± 0.092, $P < 0.05$). Contrarily, the A value of Smad 7 mRNA was increased considerably in the treated animals (0.175 ± 0.065 *vs* 0.074 ± 0.012, $P < 0.05$). There was an obvious decrease in the expression of CBP mRNA in treated rats as illuminated by a reduction of its A value when compared with model rats (0.065 ± 0.049 *vs* 0.235 ± 0.025, $P < 0.001$).

CONCLUSION: Oxymatrine is effective in reducing the production and deposition of collagen in the liver tissue of experimental rats. Oxymatrine could promote the expression of Smad 7 and inhibit the expression of Smad 3 and CBP in CCl_4-induced hepatic fibrosis in SD rats, could modulate the fibrogenic signal transduction of TGFβ-Smad pathway.

Key words: Oxymatrine; Hepatic fibrosis; TGF-Smad signaling

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INTRODUCTION

Several studies have shown that hepatic fibrosis is a reversible disease, therefore an effective treatment would probably prevent or reverse the fibrotic process in the liver[1]. In the long pathological period of hepatic fibrosis to cirrhosis, transforming growth factor beta 1 (TGFβ1) is one of the strongest pro-fibrotic cytokine[2,3], and TGFβ-Smad signaling is the cardinal signal transduction pathway[4] which has been verified by several related studies. The down regulation of TGFβ expression and modulation of TGFβ-Smad signaling may be effective in preventing liver fibrosis[5]. This study is aimed to explore the anti-fibrotic effect and the probable mechanisms of the extraction of the traditional Chinese medicine, Oxymatrine, in experimental hepatic fibrosis of rats. By examining histopathological changes and deposition of collagen protein in the liver tissue with H&E and Masson

staining, detecting the expression of Smads and CBP with *in situ* hybridization (ISH) and immunohistochemistry (IH), assaying the concentration of serum TGFβ1 with ELISA, we present anti-fibrotic effects of Oxymatrine and discuss the molecular mechanism in an experimental model of carbon tetrachloride (CCl_4)-induced hepatic fibrosis in rats.

MATERIALS AND METHODS

Animals

One hundred healthy male SD rats (weight 140-160 g) were obtained from the Experimental Animal Center of Sichuan University (Chengdu, Sichuan Province, China).

Reagents

Carbon tetrachloride (CCl_4) was obtained from the Chemical and Industrial Reagent Institute in Chengdu. Oxymatrine was from Green Valley Pharmaceutical Co. Ltd., Shanghai, China. Commercial Rat TGFβ1 ELISA Kit and Smad 7 IH Kit were obtained from Boster Biotechnology Co. Ltd., Wuhan, China. The oligonucleotide probe of Smad 3 mRNA (5'→3'): Biotin-GAAGGCCGGCTCACAGTAGGTGACTGGCTG (981-1010 bp, GC% = 63.33), Smad 7 mRNA (5'→3'): Biotin-GAGCTGTCCGAGGCAAAAGCCATTCCCCTG (2310-2339 bp, GC% = 60.00), and CBP mRNA (5'→3'): Dig-TGACAGTTGTTTATGTTTGGACGC (371-394 bp, GC% = 41.67) were obtained from Shanghai Shenergy Biotechnology Co. Ltd.

Methods

The experimental rats were housed in a room with controlled temperature (15℃-20℃) and lighting (10 h light, 14 h dark). Free access of water and food was allowed during the experimental period. All 100 rats were randomly divided into three groups: Control (n = 20), Treatment (n = 40) and Model group (n = 40). For the model group, 300 g/L CCl_4 soluted in liquid paraffin was injected subcutaneously at a dosage of 3 mL/kg twice per week[6]. The treated rats received Oxymatrine celiac injections at 10 mg/kg twice a week besides the injection of CCl_4. The injection of CCl_4 and Oxymatrine were without anesthesia. There were no bleeding and other complications after injection. The control group was given normal food and water and received the same dosage and duration of liquid paraffin only as the model group. At the end of the 8-wk experimental period, all the rats were anaesthetized by an intra-muscular injection of sodium pentobarbital (30 mg/kg) before being put to death. Blood was collected from the heart and serum was obtained through centrifugation. The liver was removed rapidly and conserved in 40% neutral formalin for further examination.

Serum concentration of TGFβ1 was detected with enzyme-linked immunoadsorbent assay (ELISA). Liver samples were embedded in paraffin and stained with hematoxylin-eosin (H&E) and Masson collagen staining[7]. A total of five sections for each liver tissue sample were observed under an optical microscope. The semi-quantitative fibrosis staging scores were acquired according to the HAI[8] (histological action index, Table 1): 0: no fibrosis; 1: slight fibrosis, fibrosis located in the central liver lobule; 2: moderate fibrosis, fibrous space formation, but the structure of liver lobule reserved; 3: severe fibrosis, fibrous space enlarged and lobular structure distortion; 4: early cirrhosis or certain cirrhosis, pseudolobule formation.

Each embedded liver sample in paraffin was sliced and fixed onto a poly-L-Lysine covered glass. The gene expression of Smad 3, Smad 7 and CBP mRNA in liver tissues were evaluated with *in situ* hybridization (ISH). The Oligonucleotide probes of Smad 3 and Smad 7 mRNA were marked with biotin at their 5' ends. The Oligonucleotide probe of CBP mRNA was marked with digoxin at its 5' end. The procedure of *in situ* hybridization (ISH) consisted of de-waxing, digesting, pre-hybridizing, hybridizing, coloring, and fixing[9]. For the color reaction of Smad 3 and Smad 7 mRNA, the NBT/BCIP method was used, and CBP mRNA was detected using DAB. Positive colors of these two methods were purple and brown, respectively. The expression of Smad 7 protein was detected with immunohistochemistry (IH). The procedure of immunohistochemistry (IH) consisted of de-waxing, exposing antigen, repairing antigen, blocking irrespective antigen, combining antigen and antibody, and coloring, respectively. For the color reaction DAB was used, and the positive result were brown particles in the cytoplasm[10].

Statistical analysis

The quantified markers were counted with mean ± SD. Slices were scanned under the inverted microscope attached to a computer. The figures were collected and quantified with the software of statistics system. (type TE2000-H, Nikon Ltd, Japan). Random analysis of variance was adopted in the comparison among the different groups, and t-test was used in the comparison between different groups.

RESULTS

At the end of experimental period, there were only eighty five rats remained. Five rats in treated group and ten in model group died. Most of the fifteen rats died from injury of biting or being poisoned by Carbon tetrachloride.

Change of TGFβ1 serum concentration

In the control group, the serum concentration of TGFβ1 was only at 1.34 ± 0.25 μg/L. In the model group, the serum concentration of TGFβ1 was significantly elevated to 3.59 ± 1.23 μg/L (P = 0.004 *vs* control group). It correlated with the semi-quantitative scores of liver fibrosis (the correlation coefficient r was +0.59, $P < 0.05$). With the administration of Oxymatrine, the serum concentration of TGFβ1 was decreased significantly to 1.82 ± 0.61 μg/L (P = 0.023 *vs* model group), although it was still higher than the serum concentration of the control group (P = 0.069 *vs* control group) (Table 2).

Histopathological changes in the liver

In the control group, the livers showed normal lobular architecture with central veins and radiating hepatic

Table 1 Semi-quantitative scores of hepatic fibrosis staging

Score	Pathological description	Stage
0	No fibrosis	No fibrosis
1	Slight fibrosis	Periportal fibrosis
2	Medium fibrosis	Enlarged periportal fibrosis
3	Severe fibrosis	Bridging fibrosis
4	Cirrhosis	Cirrhosis

Table 2 Serum TGFβ1 concentration and collagen area in liver tissues (mean ± SD)

Group	TGFβ1 (μg/L)	Collagen area (μm^2)
Control	1.34 ± 0.25[b]	56.12 ± 21.45[a]
Model	3.59 ± 1.23	290.86 ± 89.37
Treat	1.82 ± 0.61[a]	94.41 ± 37.26[a]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* model group.

cords, with 0 staging score. Subcutaneous injection of CCl_4 caused severe hepatic pathological damages such as inflammation, significant hepatic cell necrosis and excessive collagen deposition. The semi-quantitative hepatic fibrosis staging score was raised to 3.76 ± 0.68 in the model group ($P < 0.01$ *vs* control group). The rats' livers in the Oxymatrine treated group showed less hepatic cells necrosis, less collagen deposition and a significantly decreased staging score of 2.43 ± 0.47 ($P < 0.05$ *vs* model group) (Table 3, Figure 1 A and B).

Molecular changes of Smad 3, Smad7 and CBP gene expression

There were less positive signals of Smad 3 and CBP mRNA detected with ISH (*in situ* hybridization) in the normal group. The *A* (optical density) value of CBP was nearly 0 (Table 2). In the model group, the positive expression of Smad 3 and CBP mRNA increased significantly. The *A* value of CBP and Smad 3 were increased to 0.235 ± 0.025 and 0.167 ± 0.092 respectively ($P < 0.01$ *vs* normal group). The treatment with Oxymatrine significantly reduced the *A* value to 0.065 ± 0.049 and 0.034 ± 0.090 ($P < 0.05$ *vs* model group). At the same time, the positive rate of Smad 7 protein expression was increased from 1.9% to 4.3% ($P < 0.05$ *vs* model group) (Table 2). Those results demonstrated that Oxymatrine was effective in inhibiting the expression of TGFβ1, Smad 3 and CBP, promoting expression of Smad 7 in the liver (Figures 2 and 3, Table 4).

DISCUSSION

Hepatic fibrosis is thought to be a reversible disease, however, there has not been a satisfied method in the clinical practice to reverse the pathological process yet. Several drugs, including antisense TGFβ receptor, cytokines[11], antioxidant, chemical drugs, soluble type Ⅱ receptor of TGFβ1, antibody of TGFβ1 have been used in research work to block experimental hepatic fibrosis, but their effects were not as prosperous as we had expected.

Table 3 Liver histopathological semi-quantitative scores (According to HAI)

Group	*n*	Scores					Staging scores
		0	+1	+2	+3	+4	
Control	20	20	0	0	0	0	0[b]
Model	30	0	0	2	3	25	3.76 ± 0.68
Treat	35	0	2	18	13	2	2.43 ± 0.47[a]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* model group.

Table 4 Expression of Smad 3, Smad 7 and CBP in liver tissues

Group	Smad 7 (IH, %)	mRNA (*A*)		
		Smad 7	Smad 3	CBP
Control	0[b]	12:00 AM	0[b]	0[b]
Model	0.019 ± 0.002	0.074 ± 0.012	0.167 ± 0.092	0.235 ± 0.025
Treat	0.043 ± 0.009[a]	0.175 ± 0.065[b]	0.034 ± 0.090[a]	0.065 ± 0.049[a]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* model group.

Besides, some traditional Chinese drugs have been found effective in preventing fibrogenesis and other causes of chronic liver injury[12,13], and this help to develop a more hopeful future in controlling liver fibrosis and cirrhosis. These drugs have the advantages of being cheap, safe and easy to acquire, but most of them are limited in some animal experiments, rough clinical observation and lack of systemic study at molecular level.

The activation of hepatic stellate cells (HSC) "induced by some critical cytokines" is considered to be of great importance during the long period of liver fibrosis[14]. This activated HSC then becomes the main source of most cytokines and collagen proteins. Among the cytokines mediating factors, transforming growth factor beta 1 (TGFβ1) has been demonstrated in most research to be an essential pro-fibrogenesis factor[15-19]. In addition to that, TGFβ-Smad signaling pathway is the main pathway of TGF-β1[20-23], which transfers the stimulating signal from outside into the affected cells. The Smad proteins consists of a large family of transcription factors, which are also found in vertebrates, insects and nematodes. To date, Smads are the only TGF-β receptor substrates with a demonstrated ability to propagate signals. Briefly, two different transmembrane protein serine/threonine kinases, named as TGF-β receptor type Ⅰ and Ⅱ respectively, are brought together by the ligand, which acts as a receptor assembly factor[24]. Before this occurs, receptor Ⅰ is inactive because a wedge-shaped GS region is inserted into the kinase domain, dislocating the catalytic center. During the TGF-β signal transduction, receptor Ⅱ is activated firstly. TGF-β and its receptor then form a activated complex. In the ligand-induced complex, activated receptor Ⅱ phosphorylates the GS region of receptor type Ⅰ, resulting in the activation of the receptor Ⅰ kinase. The type Ⅰ receptors specifically recognize the Smad subgroup known as receptor-activated Smads (R-Smads), which are Smad 2 and Smad 3[25]. Then R-Smads are activated and forms a complex consisting of R-Smads and Smad 4, which belongs to Co-Smad.

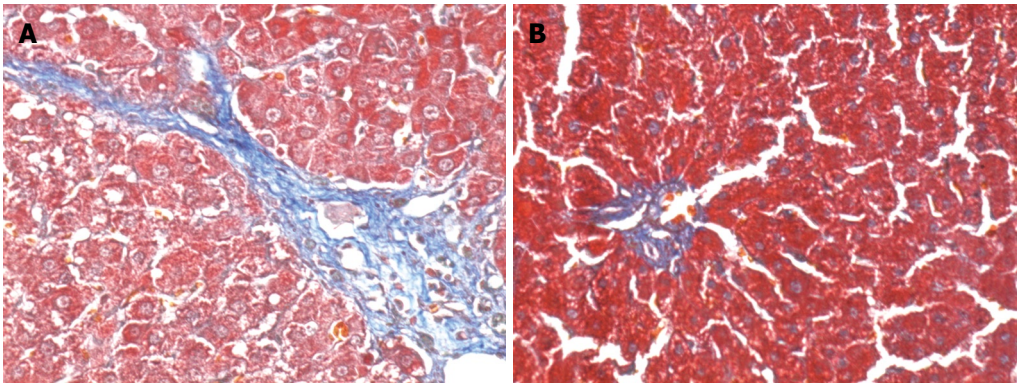

Figure 1 Subcutaneous injection of CCl_4 caused severe hepatic injury such as significant hepatic cell necrosis and excessive collagen deposition. With Masson staining, the collagen fiber was shown blue and hepatic cells were red. (**A**: Masson, × 200). Oxymarine treated livers showed less hepatic cell necrosis and less collagen deposition (**B**: Masson, × 200).

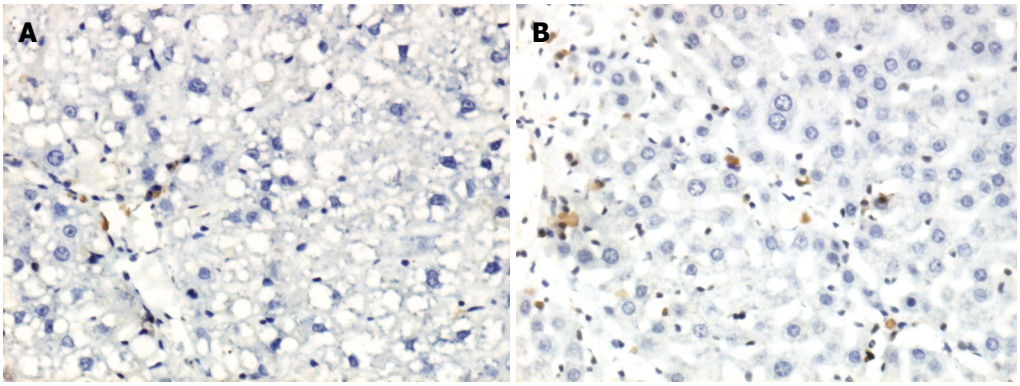

Figure 2 Expression of Smad 7 protein in liver tissue of model rats was increased when detected with immunohistochemistry (**A**: IH, × 200) when compared to the control group. However, the expression of Smad 7 protein in the livers of the Oxymatrine-treated group was even more significantly increased (**B**: IH, × 200). The positive rate of Smad 7 protein expression was 1.9% and 4.3% in the model group and the treated group, respectively (data obtained from statistical software, $P < 0.05$).

The Smads-complex then accumulates in the nucleus. This procedure leads to the formation of the functional transcriptional complexes. Both the R-Smads and the Co-Smads in this complex may participate in DNA-binding and recruitment of transcriptional cofactors[26,27]. CBP (Creb binding protein) is the main downstream molecule and the general transcriptional co-activator. After transfering into the nucleus, the transcriptional complex bind to the certain domain of the target gene and cause the gene expression such as collagen production. The excess collagen production would lead to collagen deposition in liver tissue and hepatic fibrosis or cirrhosis at last. In this pathway, there are two inhibitant Smads (I-Smads), named as Smad 6 and Smad 7, which could combine to the Smads-complex in cytoplasm. Smad 6 and Smad 7 could prevent the Smads-complex to transfer into the nucleus, thus prevent the stimulating signal being transferred from outside into cell nucleus. Since the TGFβ-Smad signaling pathway is very important in the formation of hepatic fibrosis, inhibiting the transduction of it may inhibit hepatic fibrosis. As it was shown in some research, inhibiting the TGFβ-Smad signaling pathway or modulating the gene expression of certain Smads could interfere with hepatic fibrosis effectively[28].

Oxymatrine, which is the main component of Sophora flavescentis Ait, has been used clinically in preventing chronic liver disease[29,30]. Many studies have shown that it has the effect of protecting hepatocytes, inhibiting the inflammation in liver and reducing the deposition of collagen protein. The present study aimed at exploring the potential mechanisms of Oxymatrine in the prevention of CCl_4-induced hepatic fibrosis in rats.

In this study, chronic administration of CCl_4 caused liver fibrosis and cirrhosis in experimental rats, which is indicated by the histopathological and molecular biological changes in liver tissues. The serum concentration of TGFβ1 in the model group was significantly increased (3.59 ± 1.23 μg/L *vs* 1.34 ± 0.25 μg/L in the control group, $P < 0.01$), along with the significant collagen deposition. The collagen area was 290.86 ± 89.37 μm^2, significantly higher than that of the control group (56.12 ± 21.45 μm^2, $P < 0.05$). Under the optical microscope, the liver fibrosis/cirrhosis was verified by the classical liver structure: damage of liver lobular, hepatic cell necrosis and excessive collagen deposition. Some of the samples have even shown pseudo-lobular formation, which was a pathological symbol of liver cirrhosis. However, with the administration of Oxymatrine, the serum concentration of TGFβ1 was significantly decreased to 1.82 ± 0.61 μg/L ($P < 0.05$ *vs* model group). The HE and Masson stained histopathological slices showed mild necrosis and less collagen deposition. The semi-quantitative fibrosis staging scores were also decreased obviously ($P < 0.01$ *vs* model group). Since the main machanism of CCl_4-induced liver fibrosis was toxicosis, there was slight inflammation in the livers of both groups. In the normal control group, the expression of Smad 3, Smad 7 and CBP were very low and could hardly be detected. Along with the formation of liver fibrosis, CCl_4 injection also caused an increase in Smad 7, Smad 3 and CBP gene expression, and the expression of Smad 3 and CBP was increased more significantly than Smad 7. We could show that the expression of Smads in liver fibrosis was unbalanced compared to normal liver. The expression of Smad 7 protein and Smad 7 mRNA

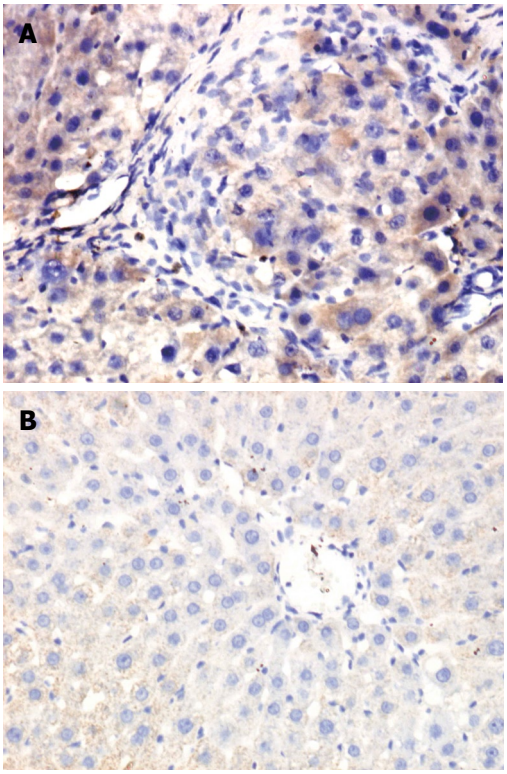

Figure 3 In the control group, the expression of CBP mRNA was at a very low level, the *A* (optical densit) value of it was nearly 0. After CCl_4 injection, the expression of CBP mRNA in the liver of fibrotic rats was significantly enhanced (**A**: ISH, × 200). However, in the livers of the Oxymatrine-treated rats, the expression of CBP was also increased compared to the control group (**B**: ISH, × 200), but it was significantly reduced compared to the model group. The *A* value of CBP mRNA in model group and treated group were 0.235 ± 0.025 and 0.065 ± 0.049, respectively ($P < 0.05$).

was increased in the Oxymatrine group compared to the model group. The percentage of Smad 7 protein was 0.019 ± 0.002 in the model group, while in Oxymatrine it was 0.043 ± 0.009 ($P < 0.05$ *vs* model group). The *A* value of Smad 7 mRNA was 0.074 ± 0.012 in the model group and 0.175 ± 0.065 in Oxymatrine group. The gene expression of Smad 3 mRNA and CBP mRNA were significantly increased in the model group and the O.D. value of them were 0.167 ± 0.092 and 0.235 ± 0.025, respectively. After Oxymatrine treatment, both Smad 3 and CBP mRNA were inhibited significantly (0.034 ± 0.090 and 0.065 ± 0.049, both $P < 0.05$) when detected with semi-quantitative evaluation after *in situ* hybridization. However, even with Oxymatrine treatment, Smad 3, Smad 7 and CBP expression remained still significantly increased compared to control group. The detection with *in situ* hybridization and immunohistochemistry could clearly show the change of molecular expression in liver tissue slice. The pathohistological damage of experimental liver could be revealed in the same visual field when the samples were observed under an optical microscope. However, the detection with *in situ* hybridization and immunohistochemistry could not gain an accurate quantity of the molecular expression.

In conclusion, the traditional Chinese medicine Oxymatrine shows significant anti-fibrotic effects in CCl_4-induced liver fibrosis in rats. It can inhibit the expression of Smad 3 and CBP, and promotes the expression of Smad7. Further studies are needed to explore the exact molecular mechanisms of Oxymatrine in anti-fibrosis.

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