Effect of Exogenous Interleukin-10 on Carbon Tetrachloride CCL_4-Induced Hepatic Fibrosis in Rats

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Abstract

Background: **Transforming growth factor β1 (TGF-β1) is one of the strongest profibrotic cytokines and TGF-β1/Smad signaling is the cardinal signal transduction pathway involved in fibrosis. Previous studies indicated that exogenous IL-10 down regulates collagen type I. It also exerts antifibrogenic effect by down regulating profibrogenic cytokines such as (TGF- β1). All these studies indicate that IL-10 might become a new therapeutic target.** ***Aim:*** **To investigate the potential therapeutic effect of exogenous interleukin-10 on reversing the well-established hepatic fibrosis of CCl_4 administration in experimental rats.** ***Materials and Methods:*** **Albino rats were divided into four groups (27 rats each): group A (control), group B1 (CCl_4-treated), group B2 (spontaneous recovery, SR) and group B3 (IL-10-treated). Rats' liver tissue was stained with i) hematoxylin and eosin (H & E) and, ii) Masson's trichrome stains for evaluation of the histological activity index (HAI). Serum TGF-β1 was determined by ELISA.** ***Results:*** **No inflammation was found in the control group, however, marked inflammation (grade 3) was observed in CCl_4-induced fibrosis group while moderate inflammation (grade 2) was observed in SR group. Meanwhile the administration of IL-10 in group D resulted in a marked decrease in the grading of inflammation (grade 1) compared to CCl_4-induced fibrosis group. No fibrosis was observed in the control group, however, a marked fibrosis reaching to cirrhosis (stages 3&4) was observed in CCl_4-induced fibrosis group. The degree of fibrosis showed a mild decrease (stage 3) in SR group. Meanwhile the administration of IL-10 in group D resulted in a marked decrease in the stage of fibrosis to (stage 1). Serum TGF-β1 concentration dramatically increased in CCl_4-induced fibrosis group compared to the control group. It also increased in SR group, but lesser than CCl_4 treated group while it was significantly reduced in the IL-10 treated group.** ***Conclusions:*** **Our results provide evidence towards the potential effect of IL-10 as anti-TGF-β1 that could lead to a reduction of hepatic fibrosis.**

Keywords: Liver fibrosis, IL-10, TGF-β1

Introduction

Fibrosis is a reversible wound healing process that occurs in almost all patients with chronic liver injury[(1, 2)]. It is the response to various insults, such as viral agents, alcohol, ischemia, medications and hepatotoxins[(3)]. Fibrosis and cirrhosis may ensue after any of the multiple types of liver injury. The major etiologies of cirrhosis include: chronic viral hepatitis (B or C), alcohol abuse, autoimmune hepatitis, hemochromatosis, Wilson's disease (copper overload), α-1-antitrypsin disease, recurrent injury to the bile ducts (primary biliary cirrhosis, and primary sclerosing cholangitis), and

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perhaps congenital lesions[2]. Broadly speaking, the causes of cirrhosis are multiple, and include many congenital, metabolic, inflammatory, and toxic liver diseases[3].

Regardless of the etiologic basis, with chronic injury and fibrosis, the clinical outcome is similar, liver architecture and metabolism is disrupted, eventually manifesting as cirrhosis and its complications[2]. Such as portal hypertension, ascites, encephalopathy, varices, synthetic dysfunction, and impaired metabolic capacity[1]. Thus fibrosis is deleterious both by its direct effects on cellular function and by its mechanical contribution to increased portal resistance[1,4].

According to Rockey[2], no matter what the cause of liver injury, increased production of extracellular matrix constituents is the key in all forms of hepatic fibrogenesis. The most prominent and abundant extracellular matrix types include interstitial collagens, types I, and III[5,6]. Quantitative and qualitative changes in many other matrix components have been described, including proteoglycans[7], and matrix glycoproteins, such as laminin[8], fibronectin[9], and tenascin[10]. Specific changes in matrix composition are similar in all forms of liver injury and hepatic fibrogenesis which suggests that the general mechanisms of fibrosis are similar[2].

Transforming growth factor β1 (TGF-β1) is one of the strongest profibrotic cytokines and TGF-β1/Smad signaling is the cardinal signal transduction pathway involved in fibrosis, which has been verified by several related studies[11]. The down regulation of TGF-β1 expression and modulation of TGF-β/Smad signaling may be effective in preventing liver fibrosis[12]. Interleukin-10 (IL-10), initially discovered in 1989, is a cytokine synthesis inhibitory factor for T lymphocytes[9]. It has anti-inflammatory and immunomodulatory effects and can regulate production of inflammatory cytokines, such as interleukin-1, interferon-gamma and interleukin-2 from T cells. It is produced by other cells of the immune system, including the liver cells. Within the liver, production of IL-10 has been documented within hepatocytes, sinusoidal cells, kup-ffer cells, stellate cells and liver-associated lymphocytes[13]. It was reported that endogenous IL-10 can decrease intrahepatic inflammatory response and fibrosis in several models of liver injury[14].

Previous studies indicate that exogenous IL-10 down regulates collagen type I in cultured hepatic stellate cells (HSCs) and hepatic fibrosis and up regulates metalloproteinase gene expression *in vitro*[15]. It also exerts antifibrogenic effect by down regulating profibrogenic cytokines such as (TGF- β1) and Tumor Necrosis Factor alpha (TNF-α). Previous research work strongly indicated that IL-10 might be a therapeutic target[16]. Therefore, we sought to scrutinize the possible effects of IL-10 on regression of liver fibrosis.

Materials and Methods

Animals

Adult male albino rats (n=108, weight=180-280g). All animals received food and water ad libitum and were housed in spacious wire mesh cages at room temperature.

Experimental design

The animals were divided into four main groups (27 rats in each group) and treated as described below. The method used is as that described by Huang et al[17]: *Group A:* The control group, which was injected subcutaneously with saline (2mL/kg) twice a week for 9 weeks. *Group B:* The experimental group which was injected subcutaneously with 0.2mL/100g of CCl_4 *(Algomhoria co., Egypt)* (dissolved 1:1 in sterile olive oil) twice a week for 9 weeks for induction of hepatic fibrosis. This group was subdivided into: *Group B1:* rats were put immedi-

ately to death after induction of fibrosis by CCl_4. *Group B2:* rats were left for spontaneous recovery (S.R) for 3 weeks after induction of fibrosis by CCl_4 and then put to death. *Group B3:* rats were treated with IL-10 (4µg/kg) subcutaneously 3 times/week for 3 weeks after induction of fibrosis by CCl_4 and then put to death.

Histological Technique

The albino rats were sacrificed and their livers were taken out. Midsections of the liver lobe a few mm thick were taken from each rat, and processed for observation by light microscopy. The process involved fixing the tissue specimen in 10% neutral buffered formalin solution, preparing the block in paraffin, cutting into 5-6 µm thick sections, and staining the sections H & E and Masson's trichrome.

Histological activity index (HAI) was evaluated using a numerical system as follows[18]: Inflammation was graded as Grade 1, focal collections of mononuclear inflammatory cells; Grade 2, diffuse infiltrates of mononuclear inflammatory cells; Grade 3, focal collections of polymorphonuclear cells in addition to mononuclear cell infiltrates; and Grade 4, diffuse infiltrates of polymorphonuclear cells in the parenchymal area or lobular area. The stage of liver fibrosis was graded with the METAVIR scale, which grades fibrosis on a five-point scale: Stage 0, no fibrosis; Stage 1, portal fibrosis without septa; Stage 2, portal fibrosis with a few septa; Stage 3, numerous septa without cirrhosis; and Stage 4, cirrhosis[19].

TGF-β1 assessment

TGF-β1 was assessed by Enzyme-linked immunesorbent assay (ELISA) as described before[20]. In brief, 100 µL of a serum sample was added to each well of the plate, followed by incubation for 2 h at 37°C. A Working Detector (100µL; Boster Biotechnology Co. Ltd) was loaded into each well, and the plate was incubated for an additional 1 h at room temperature (RT) before the addition of substrate solution (100µL; Boster Biotechnology Co. Ltd). The reaction was stopped by adding stop solution (1 drop; Boster Biotechnology Co. Ltd). The absorbance was read at 492 nm using a microplate reader. Calculation of the concentrations of TGF-β1 was performed in a log-log linear regression according to the instructions in the protocol.

Statistical Analysis

Student's t- test by using the mean and standard deviation and ANOVA were used to mean of TGF-β1 measurement and the histological activity index (HAI) was analyzed by using the Chi-square test. The SPSS 16.0 software *(SPSS Inc., Chicago, IL, USA)* was used for all statistical tests. *P value* < 0.05 was considered statistically significant.

Results

I. Histological and histopathological results

The effects of IL-10 on the protection of the liver from injury and fibrogenesis were initially evaluated by histological analyses. In the control group: the liver was covered by a thin connective tissue capsule. The structural unit of the liver was the hepatic lobule. They were separated from each other by hardly distinct connective tissue with central vein and radiating hepatic cords (Figure 1A). The portal triad was clearly surrounded by connective tissue, each enclosing a branch of the portal vein, a branch of hepatic artery and bile ductile (Figure 1B). Masson's trichrome (MT) stain demonstrated that the stroma of the liver had minimal connective tissue content. The fibrous septa between hepatic lobule were very fine and could not be easily demarcated (Figure 1D). Moreover, the connective

tissue fibers in the portal tract could be seen surrounding the portal venule, hepatic arteriole and the bile ductule and radiating for a short distance in between the hepatic lobules (Figure 1C).

In the CCl_4 treated group: the liver of 25 rats had severe pathological damages, such as marked steatosis, ballooning degeneration and marked inflammatory infiltrate (grade 3) and these changes were highly significant as compared to control group (Figure 2A). Masson's trichrome (MT) stain revealed outstanding thick bands of dark green fibrous tissue, sharply demarcated the cirrhotic nodules, bridging between the central vein and portal tract (stage 4 fibrosis) (Figure 2B). In the spontaneous recovery group: the liver of 21 rats showed severe pathological damages, such as moderate steatosis, ballooning degeneration and moderate inflammatory infiltrate (grade 2) (Figure 2C). Masson's trichrome (MT) stain revealed outstanding thick bands of dark green fibrous tissue bridging between the central vein and portal tract without formation of cirrhotic nodules, (stage 3 fibrosis) (Figure 2D). In the IL-10 treated group: The overall histological picture of the liver architecture 26 rats of this group of animals showed evident improvement, minimal steatosis, ballooning degeneration and mild inflammatory infiltrate (grade 1) (Figure 2E). Masson's trichrome (MT) stain revealed a relative decrease in the collagen fiber content of hepatic stroma was observed. In that respect, few thin strands of collagen fiber were seen surrounding the central veins and portal tracts extending between the degenerated hepatocytes. These changes were highly significant decreased as compared to CCl_4 treated and spontaneous recovery group (Figure 2F).

II. Results of TGF-β1 assessment

We examined the effect of IL-10 on the concentration of TGF-β1 in serum of the rat model. Our study showed that IL-10 decreases the levels of serum TGF-β1 in the rat model (Table 1). TGF-β1 in serum dramatically increased in the CCl4 group compared with the normal group (89.59±5.39 *vs.* 25.0±5.72 pg/ml P < 0.0001). The levels of TGF-β1 in serum were moderately increased in the spontaneous recovery group (55.26±6.07 pg/ml, *P*<0.0001 *vs.* control). The levels of TGF-β1 in serum were significantly reduced in the IL-10 group (30.89± 5.44 pg/ml, *P*< 0.0001 *vs.* CCl4 group). Although these were still higher than those of the normal group, these data indicated that IL-10 significantly reduced the levels of TGF-β1 nearly to its normal level (Figure 3).

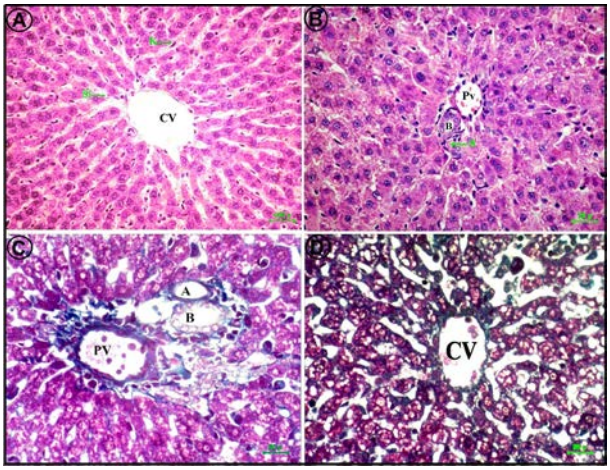

Figure 1: (A) liver of control rat showing a hepatic lobule with central vein (H&E stain x400). (B) portal triad enclosing a branch of the portal vein, a branch of hepatic artery and bile ductile (H&E stain x400). (C) connective tissue fibers in the portal tract surround the portal venule, hepatic arteriole and the bile ductule. (MT stain x400). (D) stroma of the liver with minimal connective tissue content (MT stain x400).

Discussion

Hepatic fibrosis is a progressive pathological process involving multi-cellular and molecular events that ultimately lead to deposition of excess matrix proteins in the extracellular space[21]. Advanced fibrosis and cirrhosis are generally considered to be irreversible conditions even after removal of

the injurious agent[22]. In the present work, we used chemically induced fibrosis using hepatotoxic agent. Carbon tetrachloride (CCl_4) is the most commonly used liver-damaging agent to induce liver fibrosis. The administration of other several toxic compounds is used for induction of fibrosis, including dimethylnitrosamine, galactosamine, thioacetamide, and ethanol[23].

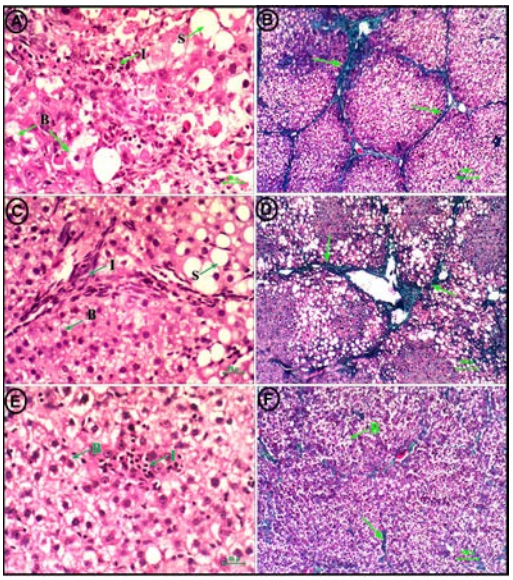

Figure 2: (A & B): histology of the rat liver treated with CCL4 for 9 weeks (H&E stain x400 & MT stain x100). (C & D): histology of the rat liver after being left for spontaneous recovery for 3 weeks (H&E stain x400 & MT stain x100). (E & F): histology of the rat liver treated with IL-10 for 3 weeks (H&E stain x400 & MT stain x100).

The histological activity index (HAI) of rats in the present study, was assessed for both the degree of inflammation and the stage of fibrosis. Regarding, grading of inflammation: It was observed that there was no inflammation in the control group. In contrast, there was marked inflammation (grade 3) in CCl_4-induced fibrosis group and moderate inflammation (grade 2) in the spontaneous recovery group. Meanwhile the administration of IL-10 in group D resulted in marked decreasing of the grading of inflammation (grade 1) compared to CCl_4-induced fibrosis group. These inflammatory changes are thought to be due to the action of CCl_4. Mochizuki, et al[24] reported that CCl_4 is metabolized in the liver by cytochrome P450 into the free radical CCl_3.

Table 1: Effect of IL-10 on TGF-β1 concentration in serum of the rat model

Group	TGF-ß1 mean ± SD
Group A (n=27)	25.0 ± 5.72
Group B1 (n=27)	89.59 ± 5.39 [b,c,d]
Group B2 (n=27)	55.26 ± 6.07 [b,e]
Group B3 (n=27)	30.89 ± 5.44 [a]

ANOVA test: a- P<0.01 compared to control group. b- P<0.000001 compared to control group. c- P<0.00001 compared to IL-10 treated group. d- P<0.0001 compared to S.R. group. e- P<0.0001 compared to IL-10 treated group.

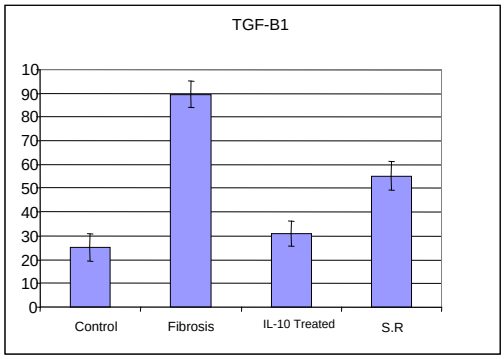

Figure 3: TGF-β1 in the different groups

The free radical attacks hepatocytes leads to lipid peroxidation and membrane damage, which results in a reversible acute centrilobular liver necrosis, which promotes inflammatory responses in the liver. In group D, the inflammatory changes were ameliorated under the effect of IL-10. Huang, et al[17] reported that the degree of hepatocyte necrosis and degeneration was decreased markedly, and there were a few inflammatory cells infiltrate around central lobular veins after treatment with IL-10 in fibrosed liver. Nelson, et al[25] had treated 24 patients with chronic hepatitis C with IL-10, they found that IL-10 normalized serum ALT levels, decreased hepatic inflammation, reduced liver fibrosis and was well tolerated in patients. This agrees with the

results of the present work, not only in ameliorating the inflammatory response, but also in correction of the levels of liver enzymes.

Regarding, staging of fibrosis: It was observed that there was no fibrosis in the control group. In contrast, there was a marked fibrosis reaching to cirrhosis (stages 3 &4) in CCl_4-induced fibrosis group. The degree of fibrosis showed mild decreased (stage 3) in the spontaneous recovery group. Meanwhile the administration of IL-10 in group D resulted in a marked decrease in the stage of fibrosis to (stage 1).

A similar study was carried out by Huang, et al[17]. They showed that advanced fibrosis or cirrhosis was established after 9 weeks administration of CCL_4, most of the fibrotic septa were resolved, and only small fibrotic fragments could be found after 3 weeks of IL-10 treatment. The therapeutic effect of IL-10 on hepatic fibrosis is not only related to removal of deposited collagen, and expression levels of MMP-2, and TIMP-1, but also related to the degree of inflammation. The results indicated that exogenous IL-10 had therapeutic effect on advanced fibrosis. Zhang, et al[16] reported that the exogenous IL-10 could alleviate liver fibrosis induced by CCl_4 in rats. IL-10 down regulates collagen type I while up regulates metalloproteinase gene expression. It also has antifibrogenic properties by down regulating profibrogenic cytokines, like TGF-β1 and TNF-α. In our research, serum TGF-β1 concentration was measured by ELISA. The concentration was dramatically increased in CCl_4-induced fibrosis group compared to the control group. The level of TGF-β1 in serum was increased in the spontaneous recovery group, but less than the levels of CCl_4-induced fibrosis group and was significantly reduced in the IL-10 treated group. This indicates that IL-10 had anti TGF-β1 effect leading to decreasing of fibrosis. Many studies have detected the presence of TGF-β1 in the fibrotic tissues of animal models or human samples of those suffering from fibrosis or cirrhosis[26]. Partial inhibition of the accumulation of ECM using either anti-TGF-β1 serum or a TGF-β1-binding protein has been reported in fibrosis models[20].

Shi, et al[27] reported that, with the development of hepatic fibrosis, TGF- β 1 increased in hepatic fibrosis rats and decreased after treatment with IL-10. The results of ELISA proved that IL-10 exerted its inhibitory effects on the expression of TGF-β 1 not only in the liver, but also in serum by decreasing the level 44% lower than fibrosed group, suggesting that IL-10 can inhibit the expression of TGF-β1. The above findings agree with our results that IL-10 had anti TGF-β1 effect leading to decreasing of fibrosis by decreasing its expression 35.5% lower than CCl_4-induced fibrosis group.

Conclusion

The present data provide evidence that IL-10 is active as an antifibrogenic drug able to reduce the biological effects of TGF-β1 in ongoing fibrogenesis.

References

1. Friedman SL. Hepatic Fibrosis. In: Schiff ER, Sorrell MF, Maddrey WC, Eds. Sciff's Diseases of the Liver, Ninth Edition, Vol. 1. Philadelphia: Lippincott Williams &Wilkins 2003, p 409-427.
2. Rockey DC. Hepatic Fibrosis, Stellate Cells, and Portal Hypertension. Clin Liver Dis 2006; 10 (3): 459-479.
3. Prosser CC, Yen RD, WU J. Molecular therapy for hepatic injury and fibrosis: Where are we? World J Gastroenterol 2006; 12 (4): 509-515.
4. Boyer TD, Wright TL, Manns MP, Eds. Zakim and Boyer's Hepatology. A textbook of liver disease. 5th edition; Philadelphia: Saunders, 2006, Vol 1, 333-346.

5. Rojkind M, Martinez-Palomo A. Increase in type I and type III collagens in human alcoholic cirrhosis. Proc Natl Acad Sci USA, 1976;73:539-43.1998.
6. Schuppan D. Structure of the extracellular matrix in normal and fibrotic liver: collagens and glycoproteins. Semin Liver Dis, 73 (2)539- 543.
7. Gallai M, Kovalszky I, Knittel T, Neubauer K, Armbrust T, Ramadori G. Expression of extracellular matrix proteoglycans, perlecan and decorin in carbon-tetra-chloride-injured rat liver and in isolated liver cells. Am J Pathol 1996; 148 (5); 1463-1471.
8. Jezequel AM, Ballardini G, Mancini R, Paolucci F, Bianchi FB, Orlandi F. Modulation of extracellular matrix components during dimethylnitrosamine-induced cirrhosis. J Hepatol 1992; 11 (2): 206-214.
9. Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med 1989; 170 (6): 2081-2095.
10. Van Eyken P, Geerts A, De Bleser P, Lazou JM, Vrijsen R, Sciot R, Wisse E, Desmet VJ. Localization and cellular source of the extracellular matrix protein, tenascin in normal and fibrotic rat liver. Hepatology 1992; 15 (5):909-916.
11. Decologne N, Kolb M, Margetts PJ, Menetrier F, Artur Y, Garrido C, Gauldie J, Camus P, Bonniaud P. TGF-beta1 induces progressive pleural scarring and subpleural fibrosis. J Immunol 2007; 179 (9): 6043-6051.
12. Yang Y, Yang S, Chen M, Zhang X, Zou Y, Zhang X. Compound Astragalus and Salvia miltiorrhiza Extract exerts anti-fibrosis by mediating TGF- beta/Smad in myofibroblasts. J Ethnopharmacol 2008; 118 (2): 264-270.
13. Le Moine O, Louis H, Sermon F, Goldman M, Deviere J. Interleukin-10 and liver diseases. Acta Gastroentrol Belg 1999; 62 (1): 1-8.
14. Louis H, Van Laethem JL, Wu W, Quertinmont E, Degraef C, Van den Berg K, Demols A, Goldman M, Le Moine O, Geerts A, Deviere J. Interleukin-10 controls neutronphilic infiltration, hepatocyte proliferation, and liver fibrosis induced by carbon tetrachloride in mice. Hepatology 1998; 28 (6):1607–1615.
15. Zheng WD, Zhang LJ, Shi MN, Chen ZX, Chen YX, Huang YH, Wang XZ. Expression of matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-1 in hepatic stellate cells during rat hepatic fibrosis and its intervention by IL-10. World J Gastroenterol 2005;11 (12):1753-1758.
16. Zhang LJ, Yu JP, Li D, Huang YH, Chen ZX, Wang XZ. Effects of cytokines on carbon tetrachloride-induced hepatic fibrogenesis in rats. World J Gastroentrol 2006; 10 (1): 77-81.
17. Huang YH, Shi MN, Zheng WD, Zhang LJ, Chen ZX, Wang XZ. Therapeutic effect of interleukin-10 on CCl4-induced hepatic fibrosis in rats. World J Gastroentrol 2006; 12 (9):1386-1391.
18. Knodell RG, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, Kiernan TW, Wollman J. Formulation and application of a numerical scoring system for assessing histological activity in a symptomatic chronic active hepatitis. J Hepatol 2003; 38 (4): 382-386.
19. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology 1996;24 (2):289–293.
20. Dong MX, Jia Y, Zhang YB, Li CC, Geng YT, Zhou L, Li XY, Liu JC, Niu YC. Emodin protects rat liver from CCl_4-induced fibrogenesis via inhibition of hepatic stellate cells activation. World J Gastroenterol 2009; 15 (38):4753-4762.
21. Murphy FR, Issa R, Zhou X, Ratnarajah S, Nagase H, Arthur MJ, Benyon C, Iredale JP. Inhibition of apoptosis of activated hepatic stellate cells by tissue inhibitor of metalloproteinase-1 is mediated via effects on matrix metalloproteinase inhibition: implications for reversibility of liver fibrosis. J Biol Chem 2002; 277 (13):11069-11076.
22. Luo YJ, Yu JP, Shi ZH, Wang L. Ginkgo biloba extract reverses CCl4- induced liver fibrosis in rats. World J Gastroenterol 2004; 10 (7):1037-1042.

23. French SW. Intragastric ethanol infusion model for cellular and molecular studies of alcoholic liver disease. J Biomed Sci 2001; 8 (1):20-27.
24. Mochizuki M, Shimizu S, Urasoko Y, Umeshita K, Kamata T, Kitazawa T, Nakamura D, Nishihata Y, Ohishi T, Edamoto H. Carbon tetrachloride-induced hepatotoxicity in pregnant and lactating rats. J Toxicol Sci 2009; 34 (2):175-181.
25. Nelson DR, Lauwers GY, Lau JY, Davis GL. : Interleukin 10 treatment reduces fibrosis in patients with chronic hepatitis C: a pilot trial of interferon nonresponders. Gastroenterology 2000; 118 (4):655-660.
26. Nakamuta M, Morizono S, Tsuruta S, Kohjima M, Kotoh K, Enjoji M. Remote delivery and expression of soluble type II TGF-beta receptor in muscle prevents hepatic fibrosis in rats. Int J Mol Med 2005; 16 (1):59-64.
27. Shi M, Huang YH, Zheng WD, Zhang LJ, Chen ZX, Wang XZ. Relationship between transforming growth factor beta1 and antifibrotic effect of interleukin-10 World J Gastroenterol 2006; 12 (15):2357-2362.