Inhibitory effect of leflunomide on hepatic fibrosis induced by CCl_4 in rats[1]

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ABSTRACT

AIM: To study the effect of leflunomide on CCl_4-induced hepatic fibrosis in rats. **METHODS:** Hepatic fibrosis was induced by subcutaneous injection with 50 % CCl_4 in Sprague-Dawley rats. The amount of CCl_4 administered was 1 mg/kg. The alanine aminotransferase (ALT), aspartate aminotransferase (AST), nitric oxide (NO) levels in plasma and hydroxyproline (Hyp) contents in liver tissue were assayed by spectrophotometry. The hyaluronic acid (HA) and procollagen III (PC III) were assessed by radioimmunoassay. The transforming growth factor-β1 (TGF-β1) in serum was determined by ELISA. The nuclear factor-kappa B (NF-κB) in liver tissue was examined by immunohistochemistry. Liver samples collected after 12 weeks of CCl_4 treatment were stained with hematoxylin and eosin. **RESULTS:** Leflunomide (1, 3, and 9 mg/kg) significantly decreased indices of liver and spleen, the serum transaminase (AST, ALT) activities, HA and PC III levels, and Hyp contents in liver tissue in rats of hepatic fibrosis. Histopathological examination showed leflunomide had inhibitory effect on fibrogenesis and formation of pseudolobulus. Furthermore, leflunomide significantly inhibited NF-κB expression in liver tissue, and reduced elevated serum TGF-β1 and NO levels in rats of hepatic fibrosis. **CONCLUSION:** Leflunomide showed inhibitory action on hepatic fibrosis induced by CCl_4 in rats.

INTRODUCTION

A considerable body of evidence showed leflunomide, an isoxazole derivative, as a unique immunomodulatory agent, was capable of treating rheumatoid arthritis, allograft and xenograft rejection, systemic lupus erythematosus, and colon carcinoma[1-4]. Leflunomide was a prodrug that is rapidly converted in the cell to an active metabolite, A_{771726}[5,6]. The initial conversion involved the opening of isoxazole ring to produce A_{771726}, which constituted more than 95 % of the drug in the circulation. Our studies indicated that leflunomide had significantly therapeutic effects on the secondary inflammation response of adjuvant arthritis in rats. Recent evidence suggested the anti-inflammatory and immunoregulatory effects of leflunomide were related to its ability to suppress the activation of mediated nuclear factor-kappa B (NF-κB), a potent mediator of inflammation when stimulated by a wide variety of inflammatory stimuli, including tumor necrosis factor (TNF), lipopolysaccharide (LPS), phorbol ester, H_2O_2, and ceramide[7,8]. Treatment of a human T cell line (Jurkat) with leflunomide blocked TNF-mediated NF-κB activation in a dose- and time-dependent manner, with maximum inhibition at 5-10 μmol/L[8]. Jankovic *et al* reported that A_{771726} also had inhibitory effect on nitric oxide (NO) production and inducible nitric oxide

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synthase (iNOS) mRNA expression in Interferon-γ plus LPS-activated murine and rat primary fibroblast[9].

Hepatic fibrosis is a dynamic process caused by chronic liver injury due to various etiologies (viral, toxic, metabolic, autoimmune), eventually leading to cirrhosis. It is predominantly characterized by excessive accumulation of extracellular matrix caused by both an increased synthesis and decreased or unbalanced degradation of extracellular matrix. Current evidence indicates that the central mediator of hepatic fibrosis is the hepatic stellate cell (HSC)[10]. Upon activation, HSC changed their phenotype from retinoid-storing quiescent cell to extracellular matrix-producing myofibroblast. Activated HSC produced cytokines [transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), *etc*] and chemokines and attracted inflammatory cells (Kupffer cells, leukocytes, *etc*) to the site of injury. The expression of cytokine and chemokine receptors and adhesion molecules on HSC and the potential to stimulate the proliferation of allogenic inflammatory cells implied a bi-directional interaction between HSC and infiltrating inflammatory cells. HSC perpetuated their activation through autocrine and paracrine mechanisms that involved the secretion of cytokines and chemokines. Furthermore, several studies showed that HSC activation was associated with elevation of NF-κB activity[11-14]. Thus, inhibition of proinflammatory and profibrogenic factors and regulation of host immunity would be beneficial to prevent the progression of hepatic fibrosis.

Based on the immunological dysfunction in hepatic fibrosis and leflunomide's immunomodulatory feature with high effication and low toxicity, we assumed that leflunomide might have inhibitory effect on hepatic fibrosis. Our studies demonstrated that leflunomide had the therapeutic action on acute chemical and immunological liver injury[15-17]. The present work aimed at investigating the effect of leflunomide on hepatic fibrosis in rats and its mechanisms.

MATERIALS AND METHODS

Animal and materials Male Sprague-Dawley rats weighing 200-250 g were purchased from Animal Center of Anhui Medical University. Rats were allowed to take food and tap water *ad libitum*. Leflunomide was kindly donated by Cinkate Co, USA. Leflunomide was insoluble in water, therefore 1 % sodium carboxymethylcellulose was used as a carrier.

Induction of hepatic fibrosis[18] Male Sprague-Dawley rats were injected subcutaneously with 1 mL/kg of body weight sterile CCl_4 in a ratio of 1:1 with olive oil twice weekly for a total of 12 weeks. At the beginning of injection of CCl_4, the leflunomide was administered by intragastric injection at doses of 1, 3, 9 mg/kg daily for 12 weeks. The control group was administered with the same volume of 1 % sodium carboxymethylcellulose or colchicine (0.1 mg/kg). At 24 h after final injection of CCl_4, a laparotomy was performed and blood was drawn from the abdominal aorta under ether anesthesia, after which the animals were killed and the liver and spleen promptly were removed, and weighed. The serum was stored at -70 ºC after separation until assayed as described below. The tissues were also stored -70 ºC until required.

Indices of liver (liver wet weight/body weight) and spleen (spleen wet weight/body weight) were apparently increased in rats after subcutaneous injection of CCl_4 compared with normal animals (Tab 1). Serum ALT, AST, HA, PC III levels and Hyp contents in liver tissue were also significantly increased in model group (Tab 2, Fig 1). After subcutaneous injection of 1 mL/kg of body weight sterile CCl_4 in a ratio of 1:1 with olive oil twice weekly for a total of 12 weeks, there was obvious nodular fibrosis with delineated fibrous septae, which were continuous and extended throughout each section (Fig 2). The results indicated that hepatic fibrosis was successfully induced in rats.

Determination of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), NO, TGF-β1, hyaluronic acid (HA) and procollagen III (PC III) levels Plasma ALT and AST activities were determined using commercial kits produced by Institute of Shanghai Biological Products affiliated to the Ministry of Health. Serum NO was assayed using commercial kits produced by Beijing Biotinge-Tech Co LTD. Serum TGF-β1 level was determined using rat enzyme-linked immunosorbent assay (ELISA) kits obtained from Sigma Co. Serum HA and PC III levels were assessed using radioimmunoassay kits purchased from Beijing Jingmei Bio-Tech Co LTD.

Measurement of hydroxyproline (Hyp) contents and NF-κB expression in liver tissue The liver tissue was weighed, hydrolyzed in HCl, and analyzed for total Hyp content, according to the method of Bergman *et al*[19] with minor modifications. The NF-κB expression in liver tissue was analyzed using immunohistochemical kits purchased from Sigma Co.

Histological examination of liver specimens Formalin-fixed liver specimens were embedded in paraffin and stained with hematoxylin and eosin for conventional morphological evaluation.

Statistical analysis Results were expressed by mean±SD. Statistical significance of difference between groups was determined by ANOVA followed by *t*-test. $P<0.05$ was considered significant.

RESULTS

Effects of leflunomide on hepatic fibrosis induced by CCl_4 in rats Leflunomide (1, 3, and 9 mg/kg, ig) and colchicines (0.1 mg/kg, ig) significantly lowered the indices of liver and spleen (Tab 1). Leflunomide (1, 3, 9 mg/kg, ig) and colchicine (0.1 mg/kg, ig) significantly also decreased elevated serum ALT, AST, HA, PC III levels and Hyp contents in liver tissue (Fig 1, Tab 2).

Tab 1. Effect of leflunomide on indices of liver and spleen in hepatic fibrosis rats induced by CCl_4. *n*=8. Mean±SD. $^cP<0.01$ *vs* normal. $^eP<0.05$, $^fP<0.01$ *vs* model.

Groups	Dose/ mg·kg^{-1}	Index of liver/%	Index of spleen/%
Normal		4.0±0.5	0.54±0.08
Model		6.4±0.5^c	0.72±0.08^c
Leflunomide	1	6.3±0.5	0.71±0.06
	3	5.7±0.4^f	0.68±0.05
	9	5.3±0.3^f	0.62±0.06^e
Colchicine	0.1	4.3±0.4^f	0.60±0.09^f

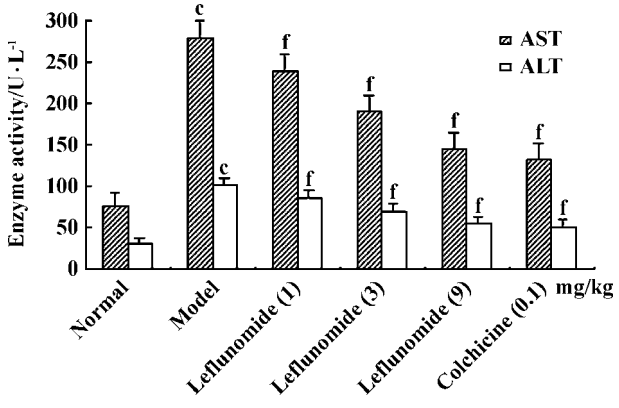

Fig 1. Effect of leflunomide on serum AST, ALT activities in rats of hepatic fibrosis induced by CCl_4. *n*=8. Mean±SD. $^cP<0.01$ *vs* Normal. $^fP<0.01$ *vs* Model.

Tab 2. Effect of leflunomide on serum HA, PC III level and Hyp contents of liver tissue in hepatic fibrosis rats induced by CCl_4. *n*=8. Mean±SD. $^cP<0.01$ *vs* normal. $^eP<0.05$, $^fP<0.01$ *vs* model.

Groups	Dose/ mg·kg^{-1}	HA/ng·L^{-1}	PC III/ng·L^{-1}	Hyp/μg·mg^{-1} liver tissue
Normal		72.7±15.2	58.8±14.2	2.3±0.6
Model		575.3±58.6^c	350.1±32.5^c	9.3±0.8^c
Leflunomide	1	474.3±58.5^f	263.1±32.5^f	8.2±0.7^e
	3	287.2±58.4^f	197.1±29.1^f	7.1±0.7^f
	9	153.0±49.9^f	111.4±16.0^f	6.1±0.6^f
Colchicine	0.1	135.6±43.8^f	94.0 ±11.4^f	5.7±0.7^f

Effect of leflunomide on hepatic histological examination Administration of leflunomide (9 mg/kg, ig) and colchicines (0.1 mg/kg, ig) significantly inhibited fibrogenesis and formation of pseudolobulus in liver (Fig 2).

Effect of leflunomide on NF-κB expression in liver tissue Immunohistochemical analysis of liver tissue sections revealed that NF-κB expression, as brown or yellow granulae in cytoplasm and nuleus, was apparently increased in response to 12-week subcutaneous injection of CCl_4. Leflunomide (9 mg/kg, ig) and colchicines (0.1 mg/kg, ig) significantly reduced NF-κB expression (Fig 3).

Effect of leflunomide on serum NO, TGF-β1 CCl_4 (sc) caused a significant rise in serum concentration of NO and TGF-β1 to 171.9±25.9 μmol/L and 895.2 ±110.4 μg/L compared with normal group ($P<0.01$), respectively. Intragastric administration of leflunomide (1, 3, and 9 mg/kg) and colchicines (0.1 mg/kg) sig-

Tab 3. Effect of leflunomide on serum TGF-β1 and NO level in hepatic fibrosis rats induced by CCl_4. *n*=8. Mean±SD. $^cP<0.01$ *vs* normal. $^fP<0.01$ *vs* model.

Groups	Dose/mg·kg^{-1}	TGF-β1/ μg·L^{-1}	Inhibition/%	NO/μmol·L^{-1}	Inhibition/%
Normal		87.0±21.3	-	67.3±17.0	-
Model		895.2±110.4^c	0	171.9±25.9^c	0
Leflunomide	1	738.5±60.3^f	17.5	151.3±22.8	12.0
	3	561.0±60.5^f	37.3	128.5±19.3^f	26.8
	9	296.4±36.1^f	66.9	94.5±13.1^f	45.0
Colchicine	0.1	240.3±62.8^f	73.2	93.8±10.3^f	45.4

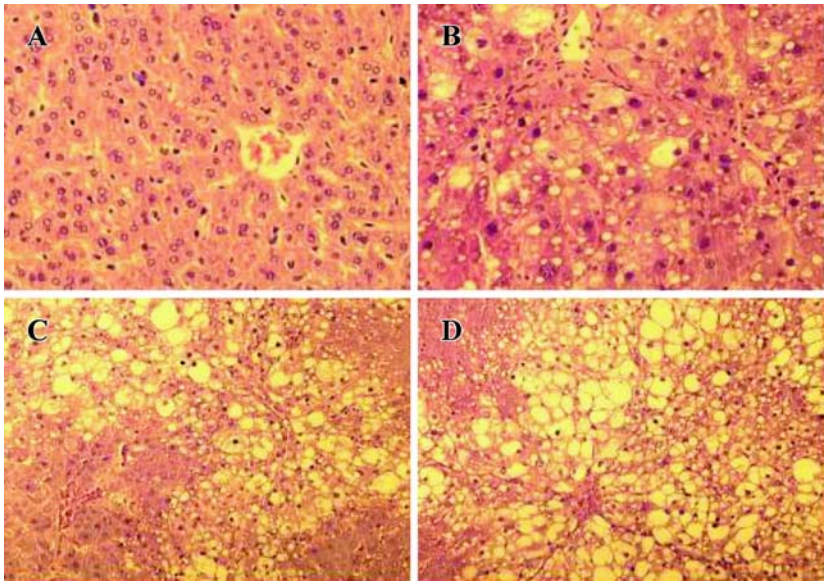

Fig 2. The photomicrographs of liver section taken from rats. A: Control (saline injection only). B: CCl_4 (1 mL/kg) injection only. C: CCl_4+leflunomide (9 mg/kg). D: CCl_4+colchicine (0.1 mg/kg). HE stain, ×200.

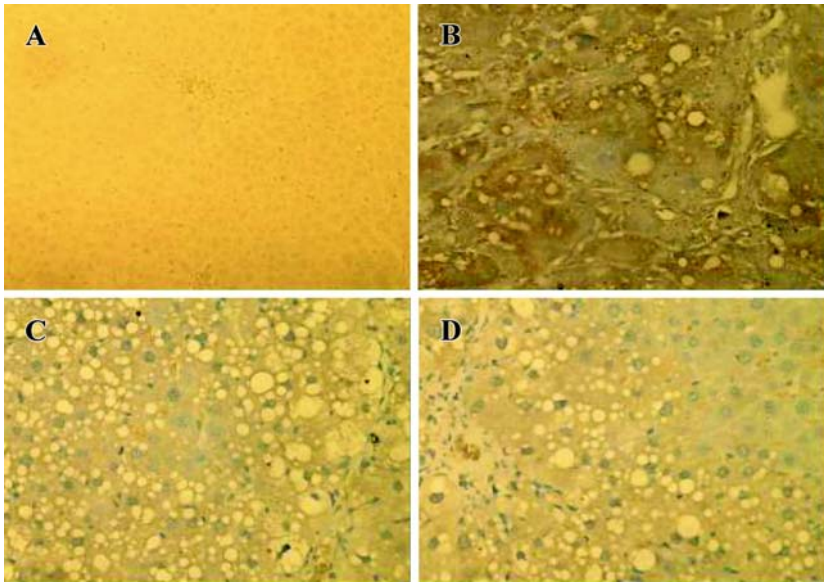

Fig 3. Expression of NF-κB in liver section taken from rats. A: Control (saline injection only). B: CCl_4 (1 mL/kg) injection only. C: CCl_4+leflunomide (9 mg/kg). D: CCl_4+colchicine (0.1 mg/kg). Immunohistochemical stain, ×200.

nificantly lowered elevated serum NO and TGF-β1 levels (Tab 3).

DISCUSSION

CCl_4 is one of the most widely used hepatic toxins for experimental induction of hepatic fibrosis and cirrhosis in laboratory animals. CCl_4-induced fibrosis or cirrhosis in experimental animals resembled human cirrhosis in some aspects of morphology and pathophysiology. For example, in both cases regeneration of hepatocytes occurs after necrosis, and fibrotic infiltration is almost irreversible in the advanced stage of cirrhosis. Thus, CCl_4-induced hepatic fibrosis has also been used to assess the efficacy of anti-fibrotic reagents and to verify correlation between pathophysiologic feature of the liver and serum marker of fibrosis[20].

In this article, hepatic fibrosis was successfully induced by subcutaneous injection with sterile CCl_4 1 mL/kg body weight in a ratio of 1:1 with olive oil twice weekly for a total of 12 weeks. On this basis, leflunomide (1, 3, and 9 mg/kg, ig) significantly reduced elevated indices of liver and spleen, serum transaminase,

HA, PC III levels, and Hyp contents in liver tissue. Histological examination showed leflunomide evidently alleviated the progression of hepatic fibrosis. Therefore, intragastric administration of leflunomide had apparently inhibitory effect on hepatic fibrosis induced by CCl_4 in rats.

NF-κB is a ubiquitous transcription factor that plays a critical role in hepatic fibrosis. Once activated, NF-κB dimers were transported to the nucleus where they stimulated the transcription of genes that carried NF-κB DNA binding motifs, including the genes encoding TGF-β and NO[21]. Several studies showed that HSC activation was associated with elevation of NF-κB activity[11-14]. Activated HSC predominantly expressed the classic p65:p50 complex. Lang reported that NF-κB was a regulator of HSC activation and proliferation, and protected HSC against tumor necrosis factor α (TNF-α)–induced apoptosis[13]. This important observation suggested that NF-κB functioned to promote the persistence of activated HSC and a chronic wound healing response. In this investigation, leflunomide apparently inhibited NF-κB expression in liver tissue of hepatic fibrosis, which agreed with the results of Manna's report that treatment of a human T cell line (Jurkat) with leflunomide significantly blocked a wide variety of inflammatory stimuli-mediated NF-κB activation in a dose- and time-dependent manner[7,8]. As noted above, NF-κB was a regulator of HSC activation and proliferation, and protected HSC against TNF-α induced apoptosis. It deserved further investigation on about whether inhibitory effect of leflunomide on hepatic fibrosis was related with inactivating of NF-κB and promoting apoptosis of HSC, thus preventing the progression of hepatic fibrosis.

TGF-β is one of the most powerful and widely distributed profibrogenic mediators in the body. In the progress of hepatic fibrosis, TGF-β1 is produced by a number of non-parenchymal cells, such as activated HSC, Kupffer cells, and endothelial cells[22] . TGF-β1 not only caused an increase in the production of type I, III, and IV collagen but also in fibronectin and proteoglycan. TGF-β1 also altered the balance of matrix turnover against matrix degradation and in favor of matrix accumulation, by inhibiting production of interstitial collagenase, stromelysin and promoting the production of the potent collagenase inhibitor, TIMP-1 and plasminogen activator inhibitor-1. A further significant effect of TGF-β1 was the stimulation of a positive autocrine loop. Therefore, blocking TGF-β1 synthesis or activation appeared an attractive prospect for therapy of fibrosis. According to our investigation, the inhibitory effects of leflunomide on hepatic fibrosis might well be related with it function of decreasing the generation of TGF-β1.

Nadler *et al* showed that the synthesis of NO was regulated by many proinflammatory factors including TNF-α, IL-1, and NF-κB[23]. LPS could also induce HSC to express iNOS and synthesis a large amount of NO[24]. In the progress of hepatic fibrosis induced by CCl_4, the level of NO in serum was positive correlated with degree of hepatic fibrosis[25]. In this paper, leflunomide significantly reduced elevated serum NO level in the model of hepatic fibrosis induced by CCl_4. The results indicated the inhibitory effect of leflunomide on hepatic fibrosis was associated with its ability to decrease serum NO level.

All these results showed that the leflunomide was beneficial to hepatic fibrosis induced by CCl_4. Although the mechanism was not completely clear, the present study revealed that the inhibitory effect of leflunomide on hepatic fibrosis was associated with its ability to modulate NF-κB expression, TGF-β1, and NO production.

REFERENCES

1 Sanders S, Harisdangkul V. Leflunomide for the treatment of rheumatoid arthritis and autoimmunity. Am J Med Sci 2002; 323: 190-3.

2 Williams JW, Mital D, Chong A, Kottayil A, Millis M, Longstreth J, *et al*. Experience with leflunomide in solid organ transplantation. Transplantation 2002; 73: 358-66.

3 Kessel A, Toubi E. Leflunomide in systemic lupus erythematosus. Harefuah 2002; 141: 355-7, 409.

4 Mall JW, Myers JA, Xu X, Saclarides TJ, Philipp AW, Pollmann C. Leflunomide reduces the angiogenesis score and tumor growth of subcutaneously implanted colon carcinoma cells in the mouse model. Chirurg 2002; 73: 716-20.

5 Lucient J, Dias VC, LeGatt DF, Yatscoff RW. Blood distribution and single-dose pharmacokinetics of leflunomide. Ther Drug Monit 1995; 17: 454-9.

6 Li J, Yao HW, Jin Y, Zhang YF, Li CY, Xu SY. Pharmacokinetics of leflunomide in Chinese healthy volunteers. Acta Pharmacol Sin 2002; 23: 551-5.

7 Manna SK, Aggarwal BB. Immunosuppressive leflunomide metabolite (A_{771726}) blocks TNF-dependent nuclear factor-κB activation and gene expression. J Immunol 1999; 162: 2095-102.

8 Manna SK, Mukhopadhyay A, Aggarwal BB. Leflunomide suppresses TNF-induced cellular responses: effects on NF-kappa B, activator protein-1, c-Jun *N*-terminal protein kinase, and apoptosis. J Immunol 2000; 165: 5962-9.

9 Jankovic V, Samardzic T, Stosic-Grujicic S, Popadic D, Trajkovic V. Cell-specific inhibition of inducible nitric oxide synthase activation by leflunomide. Cell Immunol 2000; 199: 73-80.
10 Beljaars L, Meijer DK, Poelstra K. Targeting hepatic stellate cells for cell-specific treatment of liver fibrosis. Front Biosci 2002; 7: e214-22.
11 Wang JY, Guo JS, Li H, Liu SL, Zern MA. Inhibitory effect of glycyrrhizin on NF-kappaB binding activity in CCl_4- plus ethanol-induced liver cirrhosis in rats. Liver 1998; 18: 180-5.
12 Hellerbrand C, Jobin C, Iimuro Y, Licato L, Sartor RB, Brenner DA. Inhibition of NFkappaB in activated rat hepatic stellate cells by proteasome inhibitors and an IkappaB super-repressor. Hepatology 1998; 27: 1285-95.
13 Lang A, Schoonhoven R, Tuvia S, Brenner DA, Rippe RA. Nuclear factor kappaB in proliferation, activation, and apoptosis in rat hepatic stellate cells. J Hepatol 2000; 33: 49-58.
14 Wright MC, Issa R, Smart DE, Trim N, Murray GI, Primrose JN, *et al*. Gliotoxin stimulates the apoptosis of human and rat hepatic stellate cells and enhances the resolution of liver fibrosis in rats. Gastroenterology 2001; 121: 685-98.
15 Yao HW, Li J, Jin Y, Zhang YF, Li CY, Xu SY. Effect of leflunomide on immunological injury in mice. World J Gastroenterol 2003; 9: 320-3.
16 Yao HW, Jin Y, Li J, Zhang YF, Li CY, Xu SY. Effect of leflunomide on immunological injury. Acta Pharm Sin 2002; 36: 727-30.
17 Yao HW, Jin Y, Li J, Zhang YF, Li CY, Xu SY. Effect of leflunomide on the acute chemical injury. Anhui Yiyao 2002; 5: 7-9.
18 Zhang JP, Zhang M, Zhou JP, Liu FT, Zhou B, Xie WF, *et al*. Antifibrotic effects of matrine on *in vitro* and *in vivo* models of liver fibrosis in rats. Acta Pharmacol Sin 2001; 22: 183-6.
19 Bergman T, Loxley R. Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. Anal Chem 1963; 35: 1961-5.
20 WU J, Norton PA. Animal models of liver fibrosis. Scand J Gastroenterol 1996; 31: 1137-43.
21 Ghost S, May MJ, Kopp EB. NF-κB and Rel proteins: evolutionary conserved mediators of immune responses. Annu Rev Immunol 1998; 16: 225-60.
22 Wu J, Zern MA. Hepatic stellate cells: a target for the treatment of liver fibrosis. J Gastroenterol 2000; 35: 665-72.
23 Nadler EP, Dickinson EC, Beerstolz D, Alber SM, Watkins SC, Pratt DW, *et al*. Scavenging nitric oxide reduces hepatocellular injury after endotoxin challenge. Am J Physiol Gastrointest Liver Physiol 2001; 281: G173-G181.
24 Rockey DC, Chung JJ. Inducible nitric oxide synthase in rat hepatic lipocytes and the effect of nitric oxide on lipocyte contractility. J Clin Invest 1995; 95: 1199-206.
25 Fang JZ, Gao JS, Wu H. Implication of dynamic changes of nitric oxide in rats of experimental liver fibrosis. Immunol J 1997; 13: 26-8