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• BASIC RESEARCH •

Ginkgo biloba extract reverses CCl_4–induced liver fibrosis in rats

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Abstract

AIM: To study the reversing effect of Ginkgo biloba extract (GbE) on established liver fibrosis in rats.

METHODS: Following confirmation of CCl_4-induced liver fibrosis, GbE or saline was administrated to the rats for 4 weeks. The remaining rats received neither CCl_4 nor GbE as normal control. The four groups were compared in terms of serum enzymes, tissue damage, expression of αSMA and tissue inhibitor-1 of metalloproteinase (TIMP-1) and metalloproteinase-1 (MMP-1).

RESULTS: Compared with saline-treated group, liver fibrosis rats treated with GbE had decreased serum total bilirubin ($P<0.01$) and aminotransferase levels ($P<0.01$) and increased levels of serum albumin ($P<0.01$). Microscopic studies revealed that the livers of rats receiving GbE showed allieviation in fibrosis ($P<0.05$) as well as expression of αSMA ($P<0.01$). The liver collagen and reticulum contents were lower in rats treated with GbE than saline-treated group ($P<0.01$). RT-PCR revealed that the level of TIMP-1 decreased while the level of MMP-1 increased in GbE group.

CONCLUSION: Administration of GbE improved CCl_4–induced liver fibrosis. It is possibly attributed to its effect of inhibiting the expression of TIMP-1 and promoting the apoptosis of hepatic stellate cells.

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INTRODUCTION

Hepatic fibrosis represents the response of the liver to diverse chronic insults such as parasitic disease, chronic viral infection (hepatitis B and C), immunologic attack (autoimmune hepatitis), hereditary metal overload, toxic damage, *etc*. Because of the worldwide prevalence of these insults, liver fibrosis is common and is associated with significant morbidity and mortality[1-3]. Ginkgo biloba extract (GbE) is an extract from green leaves of the Ginkgo biloba tree. GbE has been shown to have an SOD-like activity and a hydroxyl radical scavening activity[4-10]. We have demonstrated that GbE concomitant administration to rats subjected to CCl4-induced liver fibrosis resulted in a reliable hepatoprotection against liver damage, as well as a curtailing process in the progression to liver fibrosis[11]. Therefore, the aim of this study was to further evaluate the beneficial action of GbE in reversing a well-established liver fibrosis after 8 wk of administration.

MATERIALS AND METHODS

Animals and treatment

Twenty-four 2-month-old male inbred Wistar rats were purchased from the Experimental Animal Center of Wuhan University of Medical Science. Six normal rats were treated with neither CCl_4 nor GbE (group N). GbE was provided by Wuhan Wushi Pharmaceutical Company, China. (No 21003). GbE contains two groups of major components: flavonoid (>24%) and terpenoids (>6%). The GbE and double-distilled water were mixed to 0.1 mg/mL suspension and subjected to full vibration. Carbon tetrachloride (CCl_4) and liquid paraffin were purchased from Sigma Corporation, USA. CCl_4 was injected intraperitoneally at 0.15 mL per rat (diluted 1:1 in liquid paraffin) twice weekly for 8 wk to produce liver fibrosis. After completing the CCl_4 treatment, 3 d after withdrawal of the hepatotoxin, six rats were anaesthetized with ether (group C). One blood sample was taken and the plasma stored until analysis. After this, the animals were exsanguinated and the liver was quickly washed *in situ* with ice-cold isotonic saline, removed, weighed, and divided into two portions, one for histological study (immunohistochemical staining, HE, Gorden-Sweet and Masson staining), the other was immediately frozen in liquid nitrogen. Following establishment of CCl_4-induced liver fibrosis, GbE (200 mg/kg per day given orally daily with gavage) or saline was administrated for 4 wk (group E and group Z, respectively). Three days after the last GbE administration, animals (groups N, E and Z) were anaesthetized with ether and kept at a constant temperature of 37.0±0.5℃. One blood sample was taken, centrifugated (3 000 rpm for 10 min), and the plasma stored until analysis. After this, the animals were exsanguinated and the liver was quickly washed *in situ* with ice-cold isotonic saline, removed, weighed, and divided into two portions, one was for histological study, the other immediately frozen in liquid nitrogen. Serum levels of TBIL, albumin and the activities of ALT and AST were determined by routine laboratory methods.

Animals were kept on standard rat chow with free access to tap water and received humane care in accordance with the animal care provisions, maintained in temperature- and humidity-controlled animal quarters under a 12 h light-dark cycle. The rats were weighed daily.

Histopathological examination

Liver tissue sections were fixed in 4 g/L formaldehyde saline and processed in paraffin wax. Sections from blocks were stained with hematoxylin-eosin (HE), reticulum (Gordon-Sweet staining) and Massaon's Trihrome. Qualitative and quantitative histological analyses were performed blindly under a light microscope and computer image analysis system. The image intensity level was kept the same throughout the study. To quantify hepatic fibrosis, we used the Knodell index, scoring as the following: 0, absence of fibrosis; 1, portal fibrous; 2, fibrous portal expansion; 3, bridging fibrosis (portal-portal or portal-central linkage); 4, cirrhosis. For each sample the

collagenous deposits at centrilobular field of the hepatic acinus, and at surrounding terminal hepatic veins were deserved at 100× magnification. In order to avoid possible bias due to the sampling of the individual fields, for every specimen, we analyzed at least 5 fields each containing a centrilobular vein. The microscopic examinations were performed in a blind fashion. Actin, smooth muscle Ab-1 was from NeoMarkers and immunohistochemical streptavidin/ peroxidase (SP) kit from Zhongshan Corporation. Immunohistochemistry of αSMA was performed using an indirect SP technique. At least 5 fields each containing a centrilobular vein were observed and the areas of positive hepatocytes were quantitated at 400×.

RT-PCR

Total RNA was extracted using Trizol (Biostar Biologic Technology Co. Ltd. USA.) according to the manufacturer's directions. Then total RNA was reverse by transcribed into cDNA. PCR was performed using the following primer pairs: β-actin: sense 5' -ATC ATG TTT GAG ACC TTC AAC ACC-3', antisense 5' -CAT GGT GGT GCC GCC AGA CAG-3'; TIMP-1[12]: sense 5' -ACA GCT TTC TGC AAC TCG-3', antisense 5' -CTA TAG GTC TTT ACG AAG GCC-3'. MMP-1[12]: sense 5' -AGC TTG GCC ACT CGC TCG GTC TG-3', antisense 5' -GTC TCG GGA TGC ATG CTC GTA TGC-3'. The amplified products were electrophoresed on a 12 g/L agarose gel containing 0.5 μg/mL ethidium bromide, and visualised under UV light.

RESULTS

Body, liver and spleen weight

Irritability, aggression, and weight loss were present predominantly in group C rats. Liver and body weight (LW and BW) of rats are presented in Table 1. No changes in body weight were observed in the rats of group Z and group E regardless of the treatment. Animals in group C showed an evident hepato- and splenomegaly. GbE (group E) blocked the hepatosplenomegaly more significantly than saline (group Z).

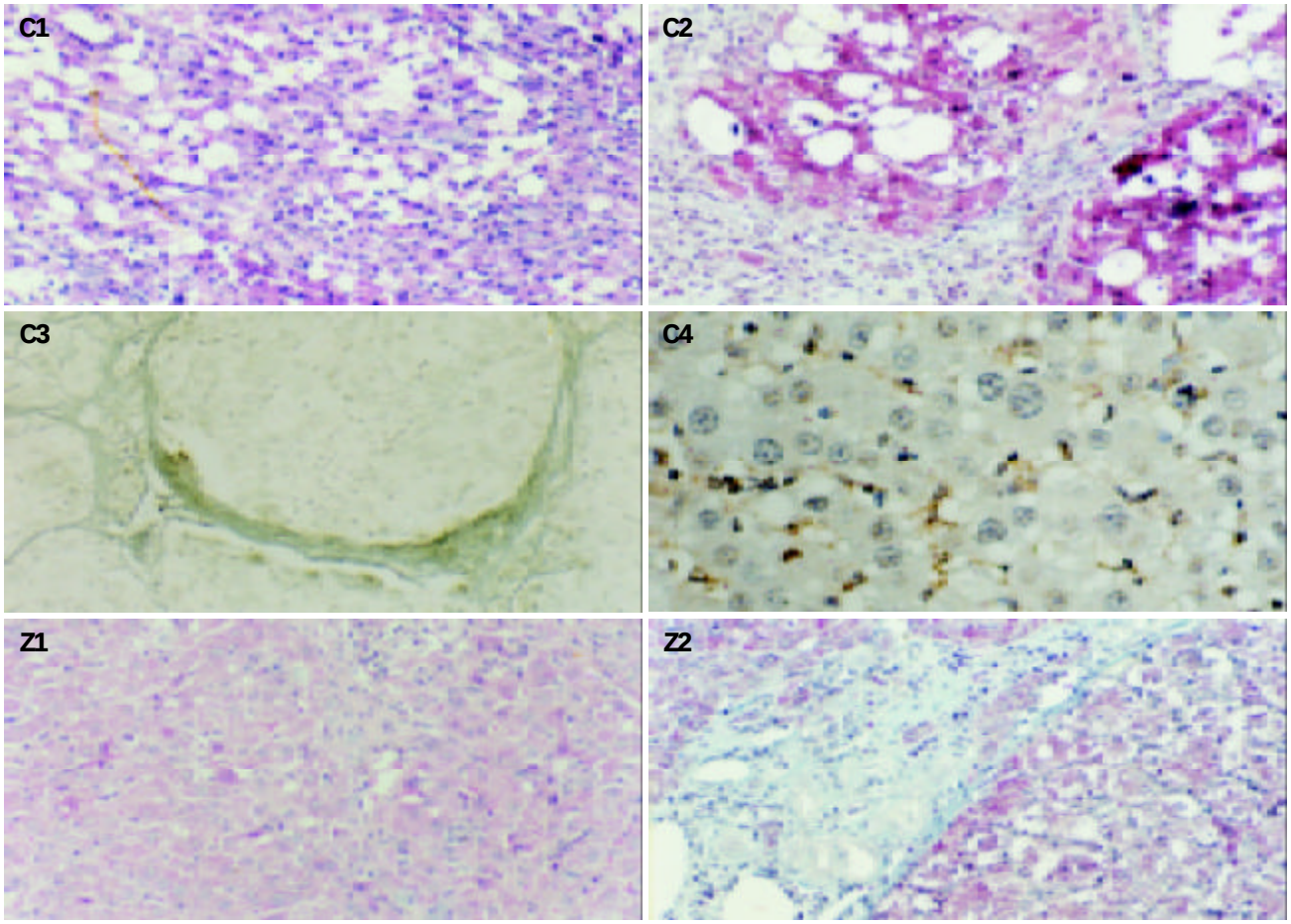
Table 1 Rat liver weight (LW), body weight (BW) and spleen weight (SW)

	BW (g)	LW (g)	LW / BW(%)	SW(g)	SW / BW (%)
C	297.0 ± 39.6^{d}	14.6 ± 3.0	4.9 ± 0.7^{c}	1.5 ± 0.2	0.5 ± 0.06^{d}
E	343.3 ± 25.7^{b}	9.6 ± 4.2^{bc}	2.8 ± 0.4^{bc}	1.0 ± 0.1^{bd}	0.3 ± 0.03^{bd}
Z	351.1 ± 21.6^{b}	13.2 ± 3.3	3.7 ± 0.4^{a}	1.4 ± 0.1	0.4 ± 0.04^{b}
N	358.3 ± 72.2^{a}	11.4 ± 0.5^{ac}	3.2 ± 0.1^{ad}	0.7 ± 0.1^{bd}	0.2 ± 0.02^{bd}

$^{a}P<0.05$, $^{b}P<0.01$ *vs* C group; $^{c}P<0.05$, $^{d}P<0.01$ *vs* Z group.

Histopathology

The morphology of the rat livers was assessed by light microscopy and is presented in Table 2 and Figure 1. αSMA positive staining of immunohistochemistry was localized in the cytoplasm and membrane. Chronic administration of CCl_4 for 8 weeks induced liver fibrosis. The liver exhibited a marked increase in ECM content and displayed bundles of collagen surrounding the lobules, which resulted in large fibrous septa and distorted tissue architecture. These septa were populated by αSMA-positive cells. The liver damage varied from one area to another, and ranged from moderate fibrosis to cirrhosis. The degree of liver fibrosis was classified according to five stages in the development of fibrosis and the difference between group Z and E, group E and C was statistically significant (group Z *vs* group E, $q=6.00$, $P<0.01$; group E *vs* group C, $q=9.46$, $P<0.01$; group Z *vs* group N, $q=6.74$, $P<0.01$; group N *vs* group C, $q=50.19$, $P<0.01$; but group Z *vs* group C, $q=2.29$, $P>0.05$). In group Z, liver collagenous and reticulum proteins as well as expression of αSMA decreased. Microscopic studies revealed that the livers of rats receiving GbE showed decreases in fibrosis and the expression of αSMA was only surrounding blood vessels.



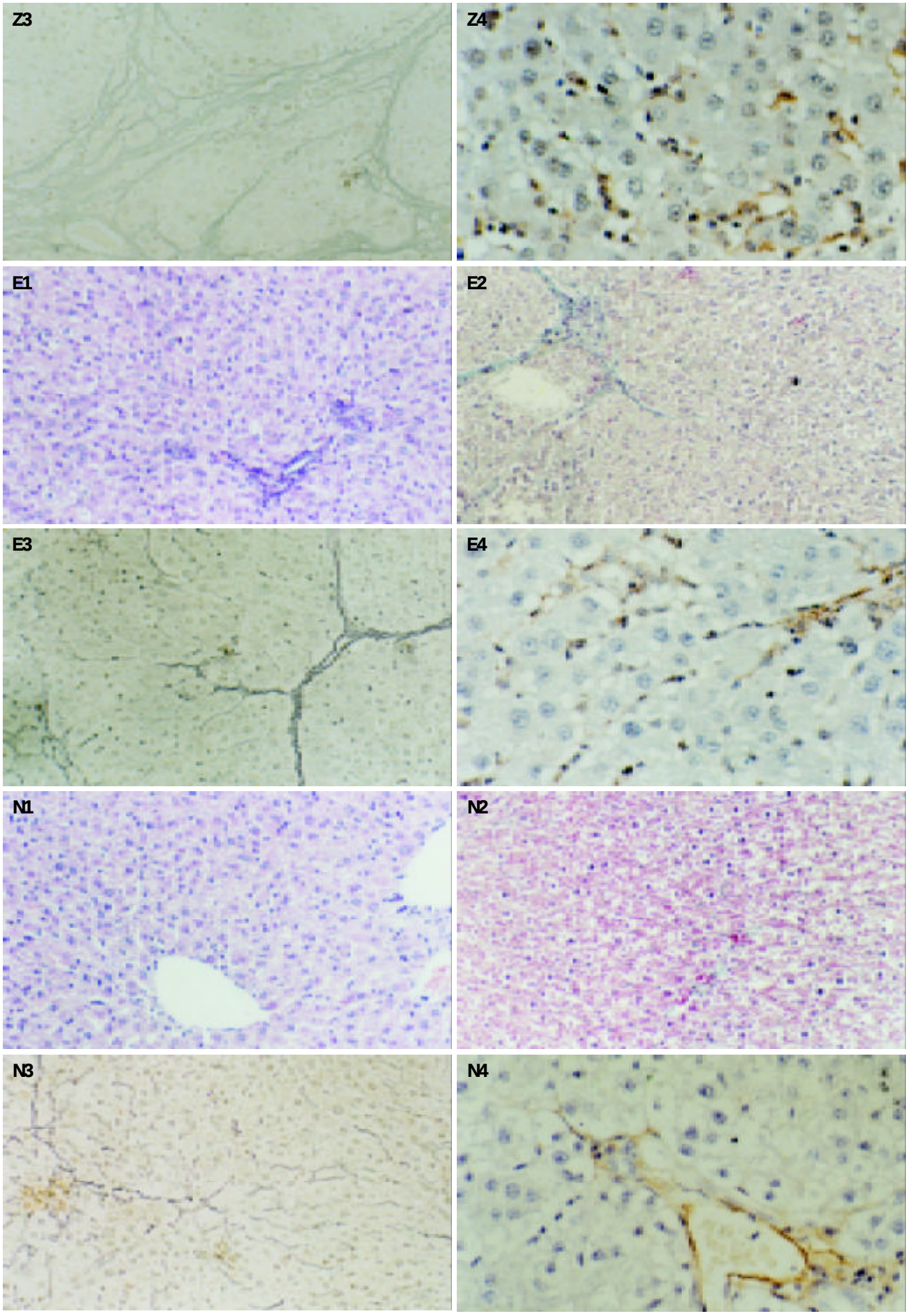


Figure 1 Histology of liver of normal rats (N) and rats treated with CCl_4 for 8 weeks (C) and then treated with saline (Z) or GbE (E) for 4 weeks. The samples were stained with HE (1), Masson (2), Reticulum staining (3) and immunohistochemistry of αSMA (4). (1)-(3) 100×, (4) 200×.

Table 2 Rat liver histopathology

	Liver fibrosis					Collagen (%)	Reticulum (%)	αSMA(+) (%)
	0	I	II	III	IV			
C	0	0	0	3	3	11.4±1.2[d]	12.4±0.9[d]	4.8±2.1[c]
E	5	1	0	0	0	4.6±0.9[bd]	4.0±1.1[bd]	2.6±0.4[bc]
Z	0	1	3	2	0	9.1±1.0[b]	10.4±0.9[b]	3.5±1.6[a]
N	6	0	0	0	0	0.1±0.1[bd]	1.0±0.3[bd]	1.0±0.1[bd]

[a]$P<0.05$, [b]$P<0.01$ *vs* group C; [c]$P<0.05$, [d]$P<0.01$ *vs* group Z.

Liver function

Table 3 shows the serum parameters in the four groups of rats. Serum ALT, AST and TBIL concentrations in group C were all 1.3-fold more than that in group Z, and there was a more significant decrease in GbE group. GbE produced improvement both in markers of hepatocellular damage (AST and ALT) and in parameters that indicate synthetic activity (albumin). All the differences were statistically significant compared with group Z.

Table 3 Liver function parameters

	ALT (U/L)	AST (U/L)	Albumin (g/L)	TBIL (mg/L)
C	188.8±52.8	267.8±45.4[c]	22.2±1.7[d]	9.3±1.1[d]
E	80.8±15.2[bd]	108.1±23.4[bd]	35.8±3.2[bd]	5.6±0.9[bd]
Z	143.1±35.0	211.6±53.1[a]	28.7±1.9[b]	7.0±0.8[b]
N	43.4±7.7[bd]	101.3±22.2[bd]	36.3±1.3[bd]	5.3±0.8[bd]

[a]$P<0.05$, [b]$P<0.01$ *vs* group C; [c]$P<0.05$, [d]$P<0.01$ *vs* group Z.

Expression of TIMP-1 and MMP-1

Remarkable TIMP-1 mRNA expression was observed in group C and TIMP-1 mRNA expression decreased in group Z. In groups E and N, RT-PCR analysis revealed very slight TIMP-1 mRNA expression (Figure 2). In groups C, Z and N, the expressions of MMP-1 were all slight while the expression in group E was remarkable.

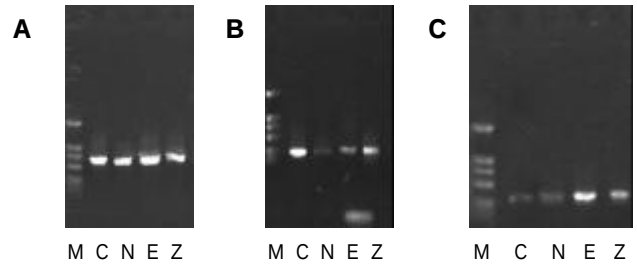


Figure 2 RT-PCR. M, 2 000 bp DNA marker, A. β-actin (556 bp), B. TIMP-1 (218 bp), C. MMP-1 (365 bp).

DISCUSSION

Incidence of liver fibrosis is growing as a result of the widespread occurrence of chronic hepatitis (predominantly type C). Cameron and Krunaratne first reported the reversibility of hepatic fibrosis after removal of the toxic agent CCl_4 in the CCl_4-induced liver fibrosis model. Since then, fibrolysis after the removal of the causative agents has been observed in experimental models of fibrosis of various types[13-20]. The reversibility of hepatic fibrosis has also been observed in alcoholic liver disease by clinicians. In this study, 4 wk after CCl_4 withdrawal, significant decreases in liver and spleen size as well as liver fibrosis state were recorded in the liver fibrosis rats treated with saline. But liver fibrosis is a complex process, and current therapies targeting at arresting or reversing liver fibrosis are largely ineffective and some has unacceptable side effects in long-term therapy[21-29]. For example, glucocorticosteroids, which have been shown to inhibit collagen synthesis in culture and in animal models, rarely suppressed fibrogenesis or prevented progression to liver fibrosis[30]. In addition, long-term administration of glucocorticoids may cause serious adverse side effects, which that prevent their use as a general treatment of liver fibrosis. D-penicillamine, which blocks intra- and interchain crosslinking in the newly formed collagen molecules, was found to be ineffective on preventing the progression of hepatic fibrosis and was associated with a high incidence of serious side effects. Medicinally useful plants have made a significant contribution to current medical practice and traditional Chinese herbs are well known for their cheap prices and negligible side effects[31-41]. GbE is a well-known and inexpensive herb that has been used to improve blood circulation without ill effects for centuries in traditional Chinese medicine. GbE contains two groups of major components: flavonoid glycosides and terpenoids. Furthermore, it has been recently reported that GbE has the property of inactivating oxo-ferryl radical species, which are more efficient oxidative agents than classical hydroxyl radicals[3-8]. Li *et al*.[41] demonstrated that procollagen II peptide, laminin, SOD and MDA were significantly decreased after GbE treatment in patients with chronic hepatitis B. In our previous study, the biochemical and histological protocol demonstrated that GbE[12], administrated at a safe dosage with minimal side effects, effectively prevented both the biochemical and histological changes associated with liver fibrosis in CCl_4-injured rats.

The CCl_4-treated rat is frequently used as an experimental model to study hepatic fibrosis. CCl_4 treatment generates free radicals that trigger a cascade of events resulting in hepatic fibrosis. In this study, when treated with CCl_4 twice weekly for 8 wk, the liver exhibited a marked increase in ECM content and displayed bundles of collagen surrounding the lobules leading to large fibrous septa and distorted tissue architecture. These septa were populated by αSMA-positive cells. All of these are characteristics of advanced fibrosis. In liver fibrosis rats, there was also evident liver dysfunction, as reflected by significantly decreased serum albumin and increased bilirubin contents. In addition, serum levels of ALT and AST were elevated.

When these animals received GbE, hepatomegaly was absent. A primary consideration in the assessment of the efficacy of a potential therapeutic agent for hepatic fibrosis is its effect on liver histology. Those livers from disease control (group C) had a high degree of fibrosis. Group Z had some improvement in histological scores compared to group C. GbE administration to liver fibrosis rats apparently accelerated the reversion of liver fibrosis and lowered the high levels of serum ALT and AST activity, indicating that GbE was also effective on reversing liver cirrhosis.

Hepatic fibrosis, regardless of the cause, is characterized by an increase in extracellular matrix (ECM) constituents. There is now overwhelming evidence suggesting that the hepatic stellate cells (HSC), lying in the space of Disse beneath the endothelial cell layer, are the principal cells involved in hepatic fibrogenesis. Thus, to prevent or reverse liver fibrosis depends greatly on controlling of HSC[42-46]. These cells are usually quiescent, with a low proliferation rate. On activation, probably because of hepatocyte injury, they differentiate into myofibroblast-like cells, with a high proliferative capacity. It has been shown that activated HSCs constitute the source of various collagenases that are necessary for the ECM remodeling. In group C, large fibrous septa were populated by αSMA-positive cells. GbE given orally promoted the apoptosis of most of the HSC and only traces of αSMA-positive cells were detected. It suggests that GbE could enhance the apoptosis of HSC.

Matrix degradation occurs predominantly as a consequence of the action of a family of enzymes called matrix metalloproteinases (MMPs), and the expression of these enzymes are in turn inhibited by a family of TIMPs[47-53]. To explore the way in which this herb results in a significant reduction in fibrosis, we investigated the effect of GbE treatment on the expression of genes known to have a role in hepatic fibrosis such as TIMP-1 and MMP-1 by reverse transcription-polymerase chain reaction (RT-PCR). In group Z, there was a rapid and significant decrease in the expression level of TIMP-1. We also systematically evaluated the mechanism of action of GbE at the molecular level by analyzing TIMP-1 transcript expression. GbE treatment was associated with an increased collagenolytic activity and a prompt normalization of liver levels of TIMP-1 and also caused a more marked reduction in the expression level of TIMP-1 transcript than group Z while increased the level of MMP-1. A lower expression of TIMP-1 indicated decreased hepatic fibrogenesis and might be an effect correlated with enhanced apoptosis in activated myofibroblast-like stellate cells. The expression levels of TIMP-1 in groups Z and E were lower than that in group C.

In summary, our results indicate that treatment with GbE after the establishment of CCl4-induced hepatic fibrosis significantly reduces and even reverses the fibrosis in rats. This effect is related to an increased removal of deposited collagen, enhanced collagenolytic activity due to decreased TIMP-1 levels and enhanced apoptosis of HSC.

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