Therapeutic Effects and Molecular Mechanisms of Ginkgo Biloba Extract on Liver Fibrosis in Rats

Shi-Quan Liu,* Jie-Ping Yu,* Hong-Lei Chen,† He-Sheng Luo,* Shi-Ming Chen* and Hong-Gang Yu*

*Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan 430060, China
†Department of Pathology, Medical College of Wuhan University, Wuhan 430071, China

Abstract: Oxidative stress can be implicated as a cause of liver fibrosis. In this sense, Ginkgo Biloba Extract (EGB), an antioxidant, may be beneficial in restraining liver fibrosis. The aim of this study was to evaluate the effects of EGB on experimental liver fibrosis. Rat liver fibrosis was induced by intraperitoneal injection of carbon tetrachloride (CCl₄) twice a week for 8 weeks. Three groups of rats received EGB (0.25, 0.5 and 1.0 g/kg, respectively) by stomach everyday. CCl₄ administration induced liver fibrosis, which was inhibited by EGB in a dose-dependent manner. The histopathologic score of fibrosis, liver function and the levels of plasma hyaluronic acid (HA) and laminin (LN) were significantly improved in rats treated with CCl₄ + EGB, compared with those treated with CCl₄ only (p < 0.01 or p < 0.05). The activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were notably elevated, while malondialdehyde (MDA) content was significantly decreased in the rats treated with CCl₄ + EGB (p < 0.01 or p < 0.05). Inhibition of hepatic stellate cell (HSC) activation and nuclear factor kappaBp65 (NF-κBp65) expression was demonstrated in the livers of EGB-treated rats. The activation of NF-κB was significantly suppressed in EGB-treated rats determined by electrophoretic mobility shift assay (EMSA). Furthermore, EGB reduced expressions of transforming growth factor-β₁ (TGF-β₁) and collagen I mRNA. In conclusion, EGB is able to ameliorate liver injury and prevent rats from CCl₄-induced liver fibrosis by suppressing oxidative stress. This process may be related to inhibiting the induction of NF-κB on HSC activation and the expression of TGF-β₁.

Keywords: Liver Fibrosis; EGB; Oxidative Stress; NF-κB; TGF-β₁.

Correspondence to: Dr. Hong-Gang Yu, Department of Gastroenterology, Renmin Hospital of Wuhan University, Jiefang Road 238, Wuhan 430060, China. Tel: (+86) 27-6200-1478, E-mail: poempower@163.com
Introduction

Liver fibrosis is a common sequel to diverse liver injuries. Without effective treatments, reversible liver fibrosis at an early stage leads to irreversible cirrhosis. Accumulating evidences have indicated that oxidative stress plays a critical role in activation of hepatic stellate cell (HSC) (Friedman, 2000; Lee et al., 1995; Fernandez-Checa et al., 1998) and almost all the clinical and experimental conditions of chronic liver disease with different etiology, reactive oxygen species (ROS) injury and aldehydes from lipid peroxidation are main trigger factors of the development of liver fibrosis (Mahmood et al., 2004; Pietrangelo, 2003; Aboutwerat et al., 2003). Reducing oxidative stress by antioxidants, such as \( \alpha \)-tocopherol and butylated hydroxytoluene, blocked HSC activation and suppressed the expression of collagen genes in HSC in vitro (Lee et al., 1995), as well as preventing fibrosis in iron-overloaded rat liver in vivo (Pietrangelo et al., 1995). Experimental results suggested that reducing oxidative stress by antioxidants could be a potential and effective therapeutic strategy for treatment and prevention of liver fibrogenesis. However, the therapeutic efficacy of currently well-known antioxidants, such as superoxide dismutase and vitamin E, in treatment of human liver fibrosis is generally unimpressive.

Ginkgo Biloba Extract (EGB), a natural antioxidant, has recently attracted considerable attention for preventing oxidative stress-related diseases including cancers (Suzuki et al., 2004), cardiovascular diseases (Mahady, 2002), degenerative diseases (Kanowski and Hoerr, 2003) and central neural system disorders (Chandrasekaran et al., 2003). EGB is a well-known and inexpensive herb that has been used without side effects for centuries. It is an extract from green leaves of the Ginkgo biloba tree; the main pharmacological components of it are flavonoid glycosides and terpenoids. EGB has been showed to have hydroxyl scavenging property (Tian et al., 2003), lipid peroxidation restraining capacity (Yang et al., 2003; Eckert et al., 2003) and antioxidant enzyme-like activity (Naidu et al., 2002; Mechirova and Domorakova, 2002). Its potent antioxidant capability and long history without adverse health effects make it a possible substitute for therapeutic treatment for liver fibrosis.

Although the underlying mechanisms remain undetermined, activated HSCs are thought to play a pivotal role in the development of liver fibrosis that takes place in chronic liver diseases. Data indicate that the activation of nuclear factor kappaB (NF-\( \kappa \)B) plays an important role in this process (Vasiliou et al., 2000; Lee et al., 2001). Furthermore, in the formation of liver fibrosis and cirrhosis, many cytokines produce marked effects through autocrine and paracrine (Alcolado et al., 1997; Olaso and Friedman, 1998). Molecular mechanisms involved in fibrogenesis reveal that transforming growth factor-\( \beta \) (TGF-\( \beta \)), especially TGF-\( \beta_1 \), plays a pivotal role (Okuno et al., 1997; Pinzani et al., 1998; Friedman et al., 2000). EGB may operate through these pathways. In the current study, therefore, we evaluate effects of EGB on liver fibrosis induced by carbon tetrachloride (CCL\(_4\)) and to detect its effects on oxidative stress, including NF-\( \kappa \)B expression and activation, HSC activation, mRNA expression of TGF-\( \beta_1 \) and collagen I, and to investigate the therapeutic potential and molecular mechanisms of EGB in treatment of liver fibrosis.
Materials and Methods

Establishment of Animal Model and EGB Treatment

Seventy male inbred Wistar rats (160 ± 20 g) were purchased from the Experimental Animal Center of Wuhan University, China. Animals were kept on standard rat cages with free access to tap water and received humane care in accordance with the animal care provisions. They were maintained in temperature and humidity controlled animal quarters under a 12-hour light-dark cycle. These rats were randomly assigned to normal group (N group, 10 rats), model group (M group, 15 rats), low dose EGB group (LD group, 15 rats), middle dose EGB group (MD group, 15 rats) and high dose EGB group (HD group, 15 rats). The liver fibrosis was induced by intraperitoneal injection CCl₄ (diluted 1:1 in liquid paraffin) 1.5 ml/kg twice a week for 8 weeks. Injection with saline instead of CCl₄ served as a negative control in N group. Rats of LD group, MD group and HD group were given 0.25, 0.5 and 1.0 g/kg EGB (Beaufour Ipsen Industry, France) by stomach everyday, additional to CCl₄ treatment, respectively. At the end of the 8th week, all rats were anesthetized with ether. Blood samples were taken by heart puncture and the plasma stored at −20ºC until to be detected. Then these rats were exsanguinated and the hepatic tissues, washed in situ with ice-cold isotonic saline, were harvested and stored at −70ºC.

Analysis of Hepatic Tissue Oxidative Stress Levels

Hepatic tissues were homogenized in 0.05 M ice-cold PBS and centrifuged at 4000 rpm for 10 minutes at 4ºC. The supernatant was taken for the assays of malondialdehyde (MDA) and antioxidant enzymes activities (all assay kits purchased from Nanjing Jiancheng Bioengineering Co. Ltd, China). MDA was assayed by the measurement of thiobarbituric acid-reactive substances (TBARS) levels at 532 nm. Results were expressed as nmol/mg protein. Superoxide dismutase (SOD) activity was measured through the inhibition of nitroblue tetrazolium (NBT) reduction by O₂⁻ generated by xanthine/xanthine oxidase system. One SOD activity unit was defined as the enzyme causing 50% inhibition in 1 ml reaction solution per mg tissue protein and the result expressed as U/mg protein. Glutathione peroxidase (GSH-Px) activity was tested by measuring the reduction of glutathione (GSH) per min on the base of its catalysis. GSH react with 5′-dithiobis-p-nitrobenzoic acid (DTNB), and produces yellow colored compounds, which were detected at 412 nm and represent the reduction of GSH. One unit of the enzyme activity was defined as a decrease of 1 µM GSH per minute for 1 mg tissue protein after the decrease of GSH of the non-enzymatic reaction is subtracted and the result expressed as U/mg protein. All above measurements were performed according to the kits’ protocols. The protein content was detected by the technique of Lowry et al. (1951), using bovine serum albumin as the standard.
Liver Function Examination and Radioimmunoassay of Hyaluronic Acid and Laminin

Blood samples were centrifuged at 3000 rpm for 10 minutes at 4°C and the plasma was harvested. Automative biochemical analyzer was applied to detect alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using commercial kits. Radioimmunoassay was exploited to detect plasma hyaluronic acid (HA) and laminin (LN), two important markers of liver fibrosis, according to the instruction of the assay kit (Shanghai Navy Medical Study Institute, China).

Histopathological Examination

Hepatic tissues excised from each rat were fixed in 10% formalin and embedded in paraffin wax. Sections 3–4 µm thin from blocks were stained with hematoxylin-eozin (H&E). To quantify liver fibrosis, we used the Knodell index with some modification, scoring as the following: 0, absence of fibrosis; I, portal fibrous and/or fibrous portal expansion; II, septal fibrosis; III, bridging fibrosis (portal-portal or portal-central linkage); IV, cirrhosis. At least five fields containing a central vein of each specimen were analyzed, and the microscopic examination was performed in a blind way.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue blocks were serially sectioned at 4 µm. After de-waxing, sections were washed in 0.01M PBS. Endogenous peroxidase was quenched with 3.0% hydrogen peroxide in methanol for 10 minutes. Antigens retrieval were performed by microwave for 10 minutes and then they were blocked with normal goat serum at 37°C for 15 minutes. This was followed by incubation at 37°C with affinity purified mouse monoclonal NF-κBp65 (Santa Cruz Corporation, USA.) and α-smooth muscle actin specific IgG (α-SMA) (MBI Corporation, USA) at a dilution of 1:100 for 1 hour. Incubation with PBS instead of the primary antibody served as a negative control. Then, sections were washed three times with PBS for 2 minutes each and incubated with biotin-labeled anti-mouse IgG at 37°C for 15 minutes. After rinsing in PBS, the sections were stained by a streptavidin-peroxidase detection system. Finally, antibody binding was visualized using the diaminobenzidine as chromogen and counterstained with hematoxylin. Expressions of NF-κBp65 and α-SMA were semi-quantitated with an automatic image analyzer. The average value of positive cells’ absorbance (A) in ten randomly selected high power fields (400×) of each section was used to analyze their expressions.

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

After homogenization, total RNAs were extracted from fresh hepatic tissues, using TRIzol reagent (Invitrogen Life Technologies Co. Ltd., USA.). The first strand cDNA was synthesized from 1 µg of total RNA using oligo-dT primer and moloney murine leukemia virus reverse transcriptase (Promega, Southampton, UK) according to the instructions
from the manufacturer. Then PCR was performed by using the following primers: GAPDH (sense 5'-TCC CTC AAG ATT GTC AGC AA-3', antisense 5'-AGA TCC ACA ACG GAT ACA TT-3'); TGF-β1 (sense 5'-TGA GTG GCT GTC TTT TGA CG-3', antisense 5'-ACT TCC AAC CCA GGT CCT TC-3'); collagen I (sense 5'-TAC CGG GCC GAT GAT GC-3', antisense 5'-TCC TTG GGG TTT GGG CTG ATG TA-3'). The conditions of the PCR amplification were: 3 minutes at 94°C for one cycle, 30 seconds at 94°C, 45 seconds at 54°C and 60 seconds at 72°C for GAPDH; 3 minutes at 94°C for one cycle, 30 seconds at 94°C, 45 seconds at 55°C and 60 seconds at 72°C for TGF-β1; 3 min at 94°C for one cycle, 30 s at 94°C, 45 s at 57°C, and 60 s at 72°C for collagen I. The size of the PCR amplification products was 308 bp for GAPDH, 350 bp for TGF-β1, and 317 bp for collagen I. The PCR products were analyzed on 2% agarose gels and visualized by ethidium bromide staining. Quantity of expression levels was achieved after adjustment for the expression levels of the housekeeping gene GAPDH by densitometry (Bio-Rad, Hercules, CA, USA). A 100 bp DNA ladder (Gibco BRL, Paisley, UK) was used as a molecular weight marker on the gel.

Isolation of Nuclear Proteins and Electrophoretic Mobility Shift Assay for NF-κB Activation

Nuclear protein extracts were harvested according to protocol described with some modification (Huang et al., 2000). In brief, fresh hepatic tissues were minced and homogenized in 400 µl of hypotonic lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin and 1 µg/ml leupeptin). Homogenized tissues were incubated on ice for 5 minutes, NP-40 was added to a final concentration of 5 g/L, and samples were vigorously mixed and centrifuged. The cytoplasmic proteins were removed and the pellet nuclei were resuspended in 50 µl buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin and 1 µg/ml leupeptin). After a 30-minute agitation at 4°C, the samples were centrifuged and supernatants, containing nuclear proteins, were transferred to a fresh vial. The protein concentrations of nuclear extracts were determined according to the method of Bradford. The nuclear extracts were stored at −70°C for later use.

Nuclear protein extracts of hepatic tissues of each group were analyzed by electrophoretic mobility shift assay (EMSA) for NF-κB nuclear translocation as described with some modification (Wong et al., 2003; Sosic et al., 2003). EMSA binding reaction mixture contained 8 µg protein of nuclear extracts, 2 µg of poly (deoxyinosinic-deoxycytidylic acid) (Sigma Co. USA), and [32P]-labeled double-stranded oligonucleotide containing the binding motif of NF-κB probe (4000 cpm) in binding buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 ml/L glycerol and 0.2 g/L albumin). The sequence of the double-stranded oligomer used for EMSA was 5'-AGT TGA GGG GAC TTT CCC AGG C-3'. The reaction was incubated at room temperature for 30 minutes before separation on a 50 g/L acrylamide gel, followed by autoradiography. For competition experiment and supershift assay, 100-fold of excess unlabeled double-stranded
oligonucleotide containing the binding motif of NF-κB probe and 2 µg NF-κBP65 mouse monoclonal antibody (Santa Cruz Biotechnology, USA) was incubated with the nuclear extracts for 15 minutes before the addition of the [32P]-labeled probe and then analyzed as described.

**Statistical Analysis**

Numerical data were presented as mean ± standard deviation. Statistical analyses were performed using one-way ANOVA with SPSS 10.0 software. The significance of the difference between the groups was assessed by Student's two-tailed t-test. Categorical data were assessed by Ridit analysis. p < 0.05 was considered significant.

**Results**

**Common Complexion**

The rats in N group lived healthily and all survived. Irritability, aggression, anorexia and reduced weight were present predominantly in the M group where three rats died. Generally, three EGB-treated rats were better of than those of the M group, while four, one and three rats were dead in LD, MD and HD groups, respectively.

**EGB Treatment Ameliorated Liver Function and Suppressed Fibril Deposition**

In the M group, not only the plasma levels of ALT and AST, but also the levels of HA and LN were obviously higher, when compared with the N group (p < 0.01), and these markers in EGB-treated groups (LD, MD and HD) were significantly lower than those of the M group (p < 0.05 or p < 0.01) (Table 1). Livers of M group were amaranthine, many granules were presented on their surface and the edge was blunt. The morphology of livers was also examined by light microscopy. The livers in the M group exhibited an obviously distorted tissue architecture, large fibrous septa and/or pseudolobules. These fields were populated by degenerate or necrotic hepatic cells (Fig. 1B). The liver damage varied from one area to another and ranged from moderate fibrosis to cirrhosis. The livers of EGB-treated groups showed a markedly less degenerate or necrotic hepatic cells than that of the M group, moreover, there were no obvious large fibrous septa or pseudolobules (Fig. 1C). The degree of liver fibrosis was significantly different between the N, M and EGB-treated groups (Table 2). These data confirmed that EGB could ameliorate liver function and restrain liver fibrogenesis.

**Analysis of Oxidative Stress of Hepatic Tissues**

As shown in Table 3, compared with the N group, the activities of SOD and GSH-Px were notably decreased, while the content of MDA was obviously increased in the M group
Table 1. Liver Function and the Plasma Levels of HA and LN in Each Group

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>HA (µg/L)</th>
<th>LN (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>10</td>
<td>40.3 ± 8.5</td>
<td>109.3 ± 35.6</td>
<td>50.3 ± 18.7</td>
<td>28.9 ± 7.8</td>
</tr>
<tr>
<td>M</td>
<td>12</td>
<td>265.1 ± 43.2</td>
<td>230.8 ± 41.5</td>
<td>160.2 ± 38.7</td>
<td>70.3 ± 20.1</td>
</tr>
<tr>
<td>LD</td>
<td>11</td>
<td>172.6 ± 24.5</td>
<td>195.4 ± 15.6</td>
<td>130.9 ± 17.0</td>
<td>44.3 ± 16.2</td>
</tr>
<tr>
<td>MD</td>
<td>14</td>
<td>83.5 ± 39.3</td>
<td>141.5 ± 38.6</td>
<td>78.2 ± 11.3</td>
<td>39.3 ± 13.4</td>
</tr>
<tr>
<td>HD</td>
<td>12</td>
<td>91.6 ± 15.8</td>
<td>123.9 ± 35.7</td>
<td>80.3 ± 10.2</td>
<td>35.5 ± 15.6</td>
</tr>
</tbody>
</table>

*p < 0.01 versus the N group; †p < 0.05, ‡p < 0.01 versus the M group.

Figure 1. H&E staining of liver tissues. The liver tissues of (A) the N group (200×), (B) the M group (200×) and (C) the EGB-treated groups (200×).

Table 2. Histological Grading of Hepatic Fibrosis in Each Group

<table>
<thead>
<tr>
<th>Degrees of Liver Fibrosis</th>
<th>N</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>10</td>
<td>9</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M*</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>LD†</td>
<td>11</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>MD†</td>
<td>14</td>
<td>0</td>
<td>5</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>HD†</td>
<td>12</td>
<td>0</td>
<td>5</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

*p < 0.01 versus the N group; †p < 0.01 versus the M group.

Table 3. SOD, GSH-Px and MDA Levels of Liver Tissues in Each Group

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>SOD (U/mg)</th>
<th>GSH-Px (U/mg)</th>
<th>MDA (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>10</td>
<td>23.6 ± 2.9</td>
<td>54.3 ± 6.3</td>
<td>1.93 ± 0.52</td>
</tr>
<tr>
<td>M</td>
<td>12</td>
<td>14.3 ± 3.2†</td>
<td>42.1 ± 3.9†</td>
<td>2.96 ± 0.21†</td>
</tr>
<tr>
<td>LD</td>
<td>11</td>
<td>18.5 ± 4.8†</td>
<td>48.2 ± 8.1†</td>
<td>2.34 ± 0.29†</td>
</tr>
<tr>
<td>MD</td>
<td>14</td>
<td>20.9 ± 3.7†</td>
<td>50.1 ± 6.8†</td>
<td>2.19 ± 0.45†</td>
</tr>
<tr>
<td>HD</td>
<td>12</td>
<td>25.3 ± 4.7†</td>
<td>51.3 ± 5.4†</td>
<td>2.01 ± 0.17†</td>
</tr>
</tbody>
</table>

*p < 0.01 versus the N group; †p < 0.05, ‡p < 0.01 versus the M group.
(p < 0.01). EGB (LD, MD and HD) evidently elevated the activities of SOD and GSH-Px, at the same time, reducing the content of MDA (p < 0.05 or p < 0.01). Furthermore, the effects were exerted in a dose-dependent manner (Fig. 2).

**EGB Reduced Expressions of NF-κBP_65 and α-SMA in Hepatic Tissue of CCl_4-Treated Rats**

There was a very weak expression of NF-κBP_65 in the N group (Fig. 3A1), while the expression of NF-κBP_65 in the M group was strong, the positive expression mainly located in cells near fibrous septa (Fig. 3A2). Positive expression of α-SMA was mostly in the blood vessel wall of the N group (Fig. 3B1); while in the M group, it was not only in the blood vessel wall, but also located in fibrous septa, areas full of inflammatory cells and HSC near fibrous septa (Fig. 3B2). Compared with the M group, expressions of NF-κBP_65 and α-SMA in EGB groups were weakened significantly in a dose-dependent manner (p < 0.05 or p < 0.01) (Figs. 3A3, 3B3 and 4).

**EGB Suppressed NF-κB Activation**

We confirmed the NF-κB-DNA binding activity by EMSA. The binding specificity of the observed band shift complexes was demonstrated by competition experiment and super shift experiment. Our results showed that the activation of NF-κB in the N group was weak; it was elevated significantly in the M group. EGB treatment suppressed the activation in a dose-dependent manner to some degree (Fig. 5).
EFFECTS OF GINKGO BILOBA EXTRACT ON LIVER FIBROSIS

Figure 3. Immunohistochemical staining for NF-κBp65 and α-SMA. Expression of (A1) NF-κBp65 in the N group (brown staining, 400×), (A2) NF-κBp65 in the M group (brown staining, 400×), (A3) NF-κBp65 in the EGB-treated groups (brown staining, 400×), (B1) α-SMA in the N group (brown staining, 400×), (B2) α-SMA in the M group (brown staining, 400×) and (B3) α-SMA in the EGB-treated groups (brown staining, 400×).

Figure 4. Average absorbance (A) value of NF-κBp65 and α-SMA immunohistochemical staining sections in each group. The average value of positive cells' A in ten randomly selected high power fields (400×) of each section was used to analyze for their expressions. *p < 0.01 versus the N group; #p < 0.05, ##p < 0.01 versus the M group.
Expressions of TGF-β1 and Collagen I mRNA of Hepatic Tissues in Each Group

We examined mean expression levels of TGF-β1 and collagen I mRNA in each group using a semi-quantitative RT-PCR assay. The RT-PCR was controlled by equalization of input RNA for each sample and comparable amplification efficiencies were validated by the uniformity of control GAPDH RT-PCR product yields. The RT-PCR results showed that all tissues constitutively expressed TGF-β1 and collagen I mRNA, but the expression levels varied between these groups. The expressions of TGF-β1 and collagen I mRNA of the N group were weak, but they were elevated in the M group. EGB treatment decreased the expressions of TGF-β1 and collagen I mRNA in a dose-dependent manner to some degree (Fig. 6).
Discussion

Chronic liver injury leads to a progressive wound healing response that eventually results in liver fibrosis characterized by both quantity and quality alteration of hepatic extracellular matrix (ECM). The pivotal cellular event underlying this response is HSC activation towards a myofibroblast-like phenotype. Upon stimulation, quiescent HSCs become activated and trans-differentiate into myofibroblast-like cells characterized by several key phenotypic changes. This includes an increase in proliferation, accumulation of ECM, including collagen I, expression of α-SMA and loss of stored vitamin A droplets (Friedman, 2000). Evidence indicates that oxidative stresses play critical roles in activation of HSC during liver fibrogenesis (Zamara et al., 2004; Nieto et al., 2002). Oxidative stresses have been detected in almost all the clinical and experimental conditions of chronic liver diseases with different etiology and fibrosis progression rats often in association with decreased antioxidant defenses (Poli, 2000). MDA is a degradative by-product of lipid peroxidation. The level of MDA is often used as an indication of oxidative damage. In this study, the level of MDA in the M group increased significantly, as compared with that of the N group, which may result from a strong oxidative stress and enhanced ROS formation. Moreover, the activities of GSH-Px and SOD, two important catalases, were both down-regulated in the M group. The total antioxidant capacity of hepatic cells might be insufficient to scavenge the ROS generated in the M group hepatic tissues. The lower activities of SOD and GSH-Px in the M group could be a consequence of depleting effect due to excess ROS generation. All these observations agree with the view that the oxidative stresses likely contribute to onset and progression of liver fibrosis (Parola and Robino, 2001).

EGB, a leaf extract of *Ginkgo biloba* which plays a role in scavenging the free radicals (Ozkur et al., 2002; Pehlivan et al., 2002), is thought to be beneficial in fighting against free radical injuries. EGB can specially protect mitochondrial ATP synthesis against anoxia/reoxygenation injury by scavenging the superoxide anion generated by mitochondria (Du et al., 1999; DeFeudis and Drieu, 2000). In this study, the MDA content was decreased while the activities of GSH-Px and SOD were elevated in the EGB-treated groups compared with those in the M group. These effects of EGB were also associated with a striking improvement of liver function, which indicates EGB has the capacity of protecting hepatic cells. These results confirm that EGB is able to inhibit lipid peroxidation in hepatic tissues and protect the membrane proteins by preventing the polymerization induced by lipid peroxidation. The plasma levels of HA and LN, two important markers of liver fibrosis, were obviously lower in the EGB-treated groups and histopathologic examinations showed fibrosis degree was significantly lower when treated with EGB. These indicate that EGB is able to ameliorate chronic liver injury and prevent liver fibrosis by restraining oxidative stress.

However, as a therapeutic agent, the mechanisms of EGB in preventing liver fibrosis still needs to be investigated. It has been reported that hepatocytes, which are undergoing oxidative stress, release ROS that stimulate HSC proliferation and transformation into α-SMA-positive myofibroblast-like cells (Svegliati Baroni et al., 1998). The key to this process may be due to the activation of NF-κB, a redox-sensitive transcription factor that
transactivate promoters of many inflammations, infection and stress genes, including cytokines (Lee et al., 2001; Paik et al., 2003; Schwabe et al., 2001). It was reported that bile duct ligation increased 4-hydroxynonenal, a product of lipid peroxidation, activated NF-κB and increased synthesis of TNF-α and TGF-β, these effects were also blunted significantly by Ad-Mn-SOD (Zhong et al., 2002). Much research has been devoted to identification of upstream signaling for activation of NF-κB, but the precise mechanism by which oxidant stress participates in this signaling is yet to be determined. Clues to this key question may be attained through studies on the mechanisms of sustained and/or accentuated NF-κB activation in chronic liver diseases. In this experiment, the activity of NF-κB and expression of NF-κBp65 and α-SMA were significantly increased in the M group, synchronously. Activated NF-κB and NF-κB induced genes expressions only in activated but not in quiescent HSCs (Hellerbrand et al., 1998; Takahra et al., 2004). Mechanisms involved in this process are not elucidated completely. In vivo study showed that HSC activation was associated with the expressions of C-myb and NF-κB, which bind to the specific regulating sequence of α-SMA gene (Vasiliou et al., 2000; Buck et al., 2000). Therefore, NF-κB and C-myb may play an essential role in the activation of HSC. On the other hand, NF-κB has the capacity of restraining apoptosis in many type cells, including HSC (Saile et al., 2001; Lang et al., 2000). This may be another mechanism of liver fibrosis. Furthermore, a series of studies have outlined a close relationship between oxidative stress, redox sensitive transcription factor NF-κB and activation of HSCs (Chen et al., 2002; Mann and Smart, 2002; Xu et al., 2003). As showed in this study, in contrast with the M group, the activity of NF-κB and the expression of NF-κBp65, α-SMA and collagen I were reduced in the EGB-treated groups consistently. Oxidative stress may play an essential role through the induction of NF-κB on HSC activation (Lee et al., 2001) and this process can be inhibited by EGB.

TGF-β is a major fibrogenic cytokine, regulating the production, degradation and accumulation of ECM proteins in liver fibrogenesis. This cytokine induces its own expression in activated HSCs, thereby creating a self-perpetuating cycle of events, referred to as an autocrine loop. TGF-β gene expression correlates with the extent of liver fibrosis (Shek and Benyon, 2004), and an increased production of ROS such as H₂O₂ in fibrotic livers is associated with the up-regulation of TGF-β (Garcia-Trevijano et al., 1999). Data also demonstrate a direct connection between TGF-β-mediated accumulation of H₂O₂ and the up-regulation of collagen I in HSCs (Garcia-Trevijano et al., 1999). Secreted as a latent precursor, TGF-β is activated at sites of injury. Active TGF-β binds to specific, high-affinity receptors present on most cells, initiating a signaling cascade that results in biological effects. The findings of the present study suggest that treatment with EGB leads to a significant decrease of ROS generation, MDA production and TGF-β1 mRNA expression, causing the potent inhibition of activation of NF-κB and HSC with a significant decrease in both α-SMA expression and collagen I production, compared with the M group. TGF-β may act as surviving factors for activated rat HSC through up-regulating the anti-apoptotic factors NF-κB (Saile et al., 2001). EGB may prevent rats from liver fibrosis through suppressing this pathway.
In summary, EGB has a strong antioxidative capacity. This antioxidant is able to ameliorate liver injury and prevent \( \text{CCl}_4 \)-induced liver fibrosis by suppressing oxidative stress. HSC may be activated via the activation of NF-\( \kappa \)B, which is induced by oxidative stress and may be related to TGF-\( \beta_1 \) — this process can be inhibited by EGB. This may be the main mechanism behind EGB protection against liver fibrosis. However, as a therapeutic agent, the effect of EGB on restraining liver fibrosis still needs to be investigated. Further studies are necessary to investigate the upstream and downstream pathways of NF-\( \kappa \)B and TGF-\( \beta \) in order to elucidate the underlying molecular mechanisms. Of course, other mechanisms may also be involved in this process.

Acknowledgments

This work was supported by grants from Youth Chenguang Project of Science and Technology of Wuhan City, No. 20025001023.

References


EFFECTS OF GINKGO BILOBA EXTRACT ON LIVER FIBROSIS


