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World J Gastroenterol 2005;11(35):5444-5449
World Journal of Gastroenterology ISSN 1007-9327

• *BASIC RESEARCH* •

Hepatic fibrosis in biliary-obstructed rats is prevented by *Ginkgo biloba* treatment

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Received: 2005-01-15 Accepted: 2005-01-26

Abstract

AIM: To assess the antioxidant and antifibrotic effects of long-term *Ginkgo biloba* administration on liver fibrosis induced by biliary obstruction in rats.

METHODS: Liver fibrosis was induced in male Wistar albino rats by bile duct ligation and scission (BDL). *Ginkgo biloba* extract (EGb 761, 50 mg/kg·per d) or saline was administered for 28 d. At the end of the treatment period, all rats were killed. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) levels were determined to assess liver functions and tissue damage, respectively. Tumor necrosis factor-α (TNF-α) was also assayed in serum samples. Liver tissues were taken for determination of the hepatic malondialdehyde (MDA) and glutathione (GSH) levels, myeloperoxidase (MPO) activity and collagen content. Production of reactive oxidants was monitored by chemiluminescence (CL) assay. Serum AST, ALT, LDH, and TNF-α levels were elevated in the BDL group as compared to control group and were significantly decreased by EGb treatment.

RESULTS: Hepatic GSH level, depressed by BDL, was elevated back to control level in EGb-treated BDL group. Increase in tissue MDA level, MPO activity and collagen content due to BDL were also attenuated by EGb treatment. Furthermore, luminol and lucigenin CL values in BDL group increased dramatically compared to control and reduced by EGb treatment.

CONCLUSION: Our results suggest that *Ginkgo biloba* protects the liver from oxidative damage following BDL in rats. This effect possibly involves the inhibition of neutrophil infiltration and lipid peroxidation; thus, restoration of oxidant and antioxidant status in the tissue.

Key words: *Ginkgo biloba*; Bile duct ligation; Hepatic fibrosis; Oxidant

Şener G, Kabasakal L, Yüksel M, Gedik N, Alican İ. Hepatic fibrosis in biliary-obstructed rats is prevented by ginkgo biloba treatment. *World J Gastroenterol* 2005; 11(35): 5444-5449
http://www.wjgnet.com/1007-9327/11/5444.asp

INTRODUCTION

Hepatic fibrosis, which is usually initiated by hepatocyte damage, leads to recruitment of inflammatory cells and platelets, activation of Kupffer cells and subsequent release of cytokines and growth factors[1]. These factors probably link the inflammatory processes and oxygen free radicals, which are known to cause tissue fibrosis[2,3].

Accumulation of hydrophobic bile acids during cholestasis leads to generation of reactive oxygen free radicals in the liver[4,5]. Previous studies showed that hepatic mitochondria generate reactive oxygen species when isolated hepatocytes are exposed to hydrophobic bile acids[4,6]. Thus, mitochondrial free radical production may be an important mechanism of cholestatic liver injury and fibrosis[7-9]. Therapeutic attempts with anti-fibrotic drugs are still under investigation[10-12]. Disadvantages associated with anti-fibrotics are toxicity due to chronic administration and reduced therapeutic effect when used in clinical studies. Anti-fibrotics from natural sources may reduce the risk of toxicity and preserve therapeutic effectiveness in clinical usage[13-15].

An extract of the leaves of *Ginkgo biloba* L., a mixture mainly composed of flavonoid glycosides and terpenoides (ginkgolides and bilobalide), has been shown to exhibit a variety of pharmacological actions[16]. *Ginkgo biloba* extract has been reported to be a potent-free radical scavenger and an antioxidant. The leaf extract suppresses platelet aggregation induced by *tert*-butyl hydroperoxide and hydrogen peroxide through its antioxidant action[17]. Furthermore, the extract and its ingredients exhibit an antagonistic effect on platelet-activating factor[18], and inhibitory effect on the expression of inducible nitric oxide synthase and nitric oxide production[19]. They also provide protection in myocardial and brain ischemia/reperfusion injury[20,21].

In the light of these findings, in this study, we investigated the anti-fibrotic and antioxidant effects of *Ginkgo biloba* extract on oxidative damage and liver fibrosis in bile duct-ligated rats.

MATERIALS AND METHODS

Animals

Male Wistar albino rats (200-250 g) were housed in a room at a mean constant temperature of 22± 2 ℃ with a 12-h light-dark cycle, and free access to standard pellet chow and water. The study was approved by the Marmara University School of Medicine Animal Care and Use Committee.

Induction of liver fibrosis

Rats were anesthetized (100 mg/kg ketamine and 0.75 mg/kg chlorpromazine; ip) and the common bile duct was exposed and ligated by double ligatures with suture silk. The first ligature was made below the junction of the hepatic ducts and the second ligature was made above the entrance of the pancreatic ducts. Finally, the common bile duct was resected between the double ligatures[15]. In sham-operated rats, abdominal incision was made without a ligation.

Experimental groups

Ginkgo biloba extract was administered to control (EGb group) and bile duct ligated (BDL+EGb group) rats for 28 d at a daily dose of 50 mg/kg, ip. The sham-operated control (C) and BDL groups received equal amounts of saline for 28 d.

After 28 d of treatment, the rats were killed and trunk blood was collected. Serum samples were used for the measurement of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactic dehydrogenase (LDH), bilirubin and tumor necrosis factor (TNF)-α levels. Liver samples were taken and stored at -70 ℃ for the measurement of malondialdehyde (MDA) and glutathione (GSH) levels, myeloperoxidase (MPO) activity and collagen contents. Formation of reactive oxygen species in liver samples was monitored by using chemiluminescence (CL) method.

Determination of serum AST, ALT, LDH, bilirubin, and TNF-α levels

Serum AST and ALT levels - as indicators of liver function - and LDH activity - as a marker of tissue injury - were assessed by using commercial kits (Roche Diagnostics, GmbH, D-68298, Mannheim, Germany) in Roche-Hitachi Modular Autoanalyzer (Roche Diagnostics, GmbH, D-68298, Mannheim, Germany)[22]. Serum total bilirubin level was assayed on Bayer Opera Autoanalyzer according to a method described previously[23]. Serum TNF-α level was evaluated by a radioimmunoassay-immunoradiometricassay method by using a commercial kit (Biosource Europe S.A., Nivelles, Belgium). The activity of radioactive assays was measured by gamma counter (LKB WALLAC 1270 RACK Gamma Counter, Canada).

Measurement of liver MDA and GSH levels

Tissue samples were homogenized with ice-cold trichloroacetic acid (1 g tissue+10 mL 10% TCA) in an Ultra Turrax tissue homogenizer. The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously[24]. Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of 1.56×10^5 mol/(L·cm) and results were expressed as nanomole MDA per gram tissue. GSH was determined by a spectrophotometric method, which was based on the use of Ellman's reagent[25]. GSH levels were calculated using an extinction coefficient of 13 600 mol/(L·cm) and the results were expressed in micromole per gram tissue.

Measurement of liver myeloperoxidase (MPO) activity

Tissue samples (0.2-0.3 g) were homogenized in 10 volumes of ice-cold potassium phosphate buffer (50 mmol/L K_2HPO_4, pH 6.0) containing hexadecyltrimethylammonium bromide (HETAB, 5 g/L). The homogenate was centrifuged at 30 000 *g* for 10 min at 4 ℃, and the supernatant was discarded. The pellet was then rehomogenized with an equivalent volume of 50 mmol/L K_2HPO_4 containing 5 g/L HETAB and 10 mmol/L EDTA (Sigma). MPO activity was assessed by measuring the H_2O_2-dependent oxidation of *o*-dianizidine·2HCl. One unit of enzyme activity was defined as the amount of the MPO present per gram of tissue weight that caused a change in absorbance of 1.0 per min at 460 nm and 37 ℃[26].

Measurement of liver collagen content

Tissue samples were cut with a razor blade and immediately fixed in 40 g/L formaldehyde in 0.1 mol/L phosphate buffer (pH 7.2) in paraffin and sections of 15-μm thick were obtained. Collagen content was measured according to a method described by Lopez de Leon and Rojkind[27]. The method is based on selective binding of the dyes Sirius red and fast green FCF to collagen and noncollagenous components, respectively. Both dyes were eluted readily and simultaneously by using 0.1 N NaOH-methanol (1∶1, v/v). Finally, the absorbances at 540 and 605 nm were used to determine the amount of collagen and protein, respectively.

Chemiluminescence (CL) assay

Luminescence of the hepatic tissue samples was recorded at room temperature using Mini Lumat LB 9 506 luminometer (EG&G Berthold, Germany) in the presence of luminol or lucigenin, 0.2 mmol/L each. All counts were obtained at 15-s intervals for 5 min and the results were expressed as area under the curve of relative light unit (rlu) for 5 min/mg tissue[28].

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). All data were expressed as mean±SE. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Values of $P<0.05$ were regarded as significant.

RESULTS

As shown in Table 1, serum bilirubin, AST, ALT, and LDH levels were significantly higher in BDL rats compared to controls ($P<0.001$) and EGb administration to BDL group reduced these values ($P<0.001$). In untreated BDL group, serum TNF-α level showed a significant increase compared to that of the sham-operated control group ($P<0.001$). Similarly, BDL-induced rise in serum TNF-α level was significantly reduced by EGb treatment ($P<0.001$, Figure 1).

Table 1 Serum total bilirubin, AST, ALT, LDH and TNF-α levels in control (C), EGb, BDL and BDL+EGb groups

Groups	C (n = 8)	EGb (n = 8)	BDL (n = 8)	BDL+EGb (n = 8)
Total bilirubin (mg/dL)	0.36±0.3	0.35±0.6	10.1±0.6[d]	1.68±0.3[b,f]
AST (mg/dL)	148±16.6	150±12.4	300±18[d]	155±12[f]
ALT (mg/dL)	68±7.3	77±6.1	135±9.7[d]	67±5.7[f]
LDH (U/L)	1 672±240	1 499±219	5 082±225[d]	1 763±226[f]

[b]P<0.01, [d]P<0.001 *vs* control group. [f]P<0.001, *vs* untreated BDL group.

The liver MDA was found to be significantly higher in the BDL group (51.0±3.3 nmol/g; P<0.01) compared to that of the sham-operated control group (32.2±3.1 nmol/g). Treatment with EGb reversed the MDA level (33.8±2.6 nmol/g; P<0.01) back to control level (Figure 2).

The hepatic GSH level showed a marked reduction in the BDL group (0.88±0.07 μmol/g; P<0.001) compared to that of the sham-operated control group (2.1±0.15 μmol/g). Similarly, treatment with EGb reversed this effect (1.8±0.12 μmol/g; P<0.01) (Figure 3).

Liver MPO activity, which was observed to be higher in the BDL group (21.8±2.0 U/g; P<0.01) compared to sham-operated control group (13.8±1.3 U/g) was also attenuated by EGb treatment (13.3±0.7 U/g; P<0.01) (Figure 4).

Hepatic collagen content of the untreated BDL rats (21.3±2.1 μg/mg protein; P<0.01) was found to be significantly higher than that of the control group (12.8±1.4 μg/mg protein). EGb treatment significantly reduced the increase in hepatic collagen content (13.2±1.2 μg/mg protein; P<0.05) to the levels that were close to control values (Figure 5).

Luminol and lucigenin CL values of the liver samples of the BDL group (18.7±1.1 and 38.3±3.2 rlu/mg, respectively) were found to be significantly higher than those of the control group (11.1±1.0 and 14.8±1.1 rlu/mg; P<0.01 and P<0.001, respectively) and EGb treatment reversed these values (12.5±2.0 and 16.1±2.6 rlu/mg; P<0.05 and P<0.001, respectively) (Figures 6 and 7).

DISCUSSION

The present study demonstrates that EGb treatment improved BDL-induced impairment in liver functions and decreased BDL-induced elevations in serum LDH activity and TNF-α levels. Furthermore, increase in hepatic lipid peroxidation, MPO activity, oxidant production and collagen content, and

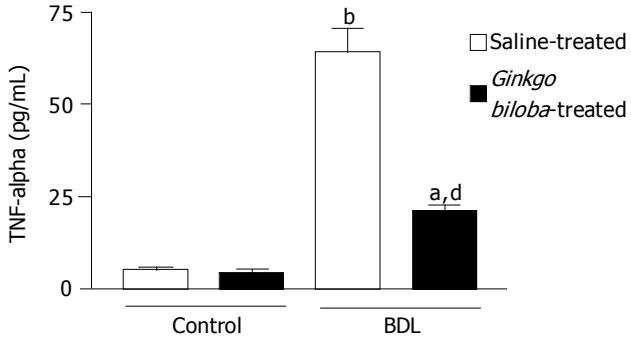

Figure 1 Serum TNF-α levels of the control, *Ginkgo-biloba*-treated, saline-treated BDL and *Ginkgo-biloba*-treated BDL groups. [a]P<0.05, [b]P<0.001 *vs* control; [d]P<0.001 *vs* saline-treated BDL group.

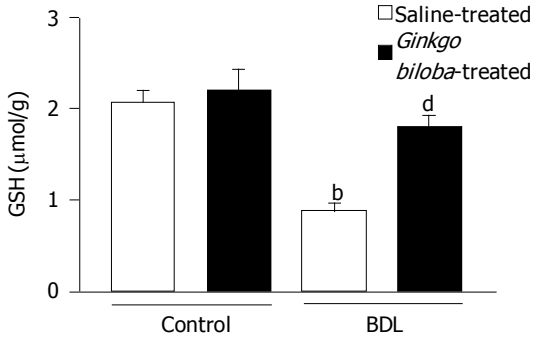

Figure 3 Liver GSH levels of the control, *Ginkgo-biloba*-treated, saline-treated-BDL, and *Ginkgo-biloba*-treated BDL groups. [b]P<0.001 *vs* control; [d]P<0.001 *vs* saline-treated BDL group.

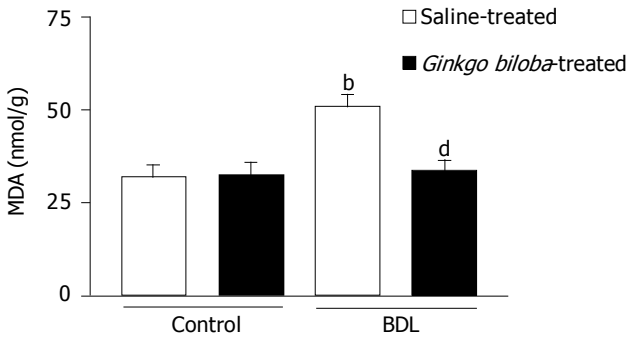

Figure 2 Liver MDA levels of the control, *Ginkgo-biloba*-treated, saline-treated-BDL, and *Ginkgo-biloba*-treated BDL groups. [b]P<0.01 *vs* control; [d]P<0.01 *vs* saline-treated BDL group.

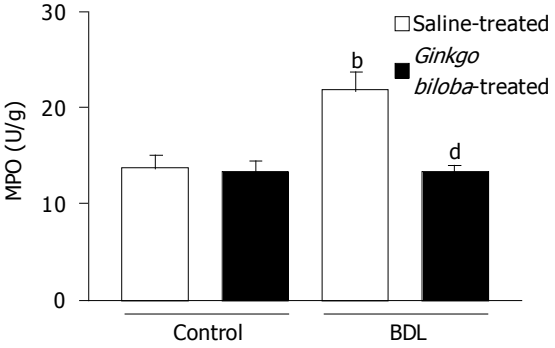

Figure 4 Liver MPO activities of the control, *Ginkgo-biloba*-treated, saline-treated BDL, and *Ginkgo-biloba*-treated BDL groups. [b]P<0.01 *vs* control; [d]P<0.01 *vs* saline-treated BDL group.

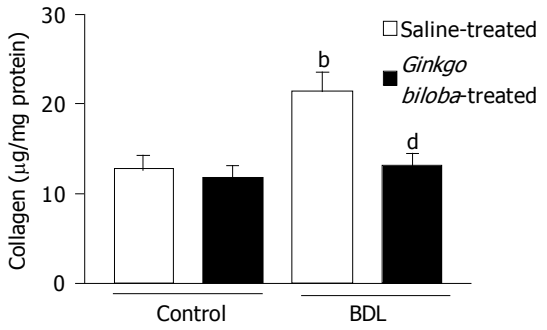

Figure 5 Liver collagen levels of the control, *Ginkgo-biloba*-treated, saline-treated-BDL, and *Ginkgo-biloba*-treated BDL groups. $^{b}P<0.01$ *vs* control; $^{d}P<0.01$ *vs* saline-treated BDL group.

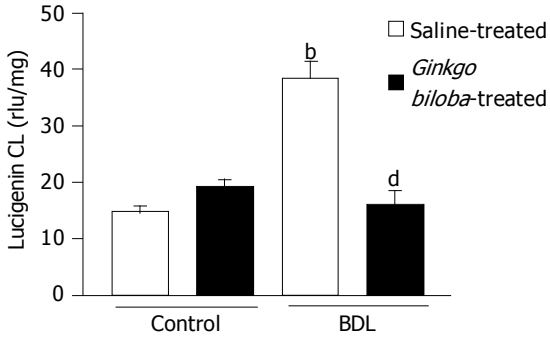

Figure 7 Liver lucigenin CL values of the control, *Ginkgo-biloba*-treated, saline-treated BDL, and *Ginkgo-biloba*-treated BDL groups. $^{b}P<0.001$ *vs* control; $^{d}P<0.001$ *vs* saline-treated BDL group.

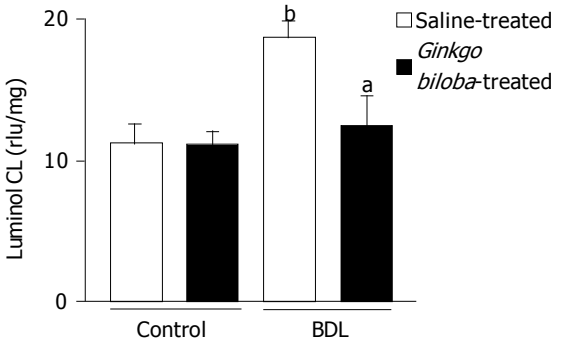

Figure 6 Liver luminol CL values of the control, *Ginkgo-biloba*-treated, saline-treated BDL, and *Ginkgo-biloba*-treated BDL groups. $^{a}P<0.05$ *vs* saline-treated BDL group; $^{b}P<0.01$ *vs* control.

decrease in GSH levels following BDL were reversed by EGb treatment.

Hepatic fibrosis, characterized by an increased production and deposition of extracellular matrix component accompanies most chronic liver disorders and its presence is a major factor contributing to hepatic failure[29,30]. Although the mechanism of liver fibrosis is not fully understood, activated hepatic stellate cells play an important role in connective tissue synthesis and deposition during fibrogenesis[12]. On the other hand, oxidative stress caused by accumulation of hydrophobic bile acids also have a critical role in fibrogenesis[3,31].

Bile duct ligation (BDL) induces a type of liver fibrosis, which etiologically and pathogenitically resembles the biliary fibrosis in the human beings. Injury to hepatocytes results in the generation of lipid peroxides, which may have a direct stimulatory effect on matrix production by activated stellate cells[8,32]. Liu *et al.*, demonstrated excessive production of superoxide radicals and hydroxyl radicals in blood and liver in rats with obstructive jaundice induced by common BDL[33]. In the present study, BDL caused significant increases in the hepatic MDA level -end product of lipid peroxidation - and EGb treatment prevented the increase in MDA, probably in part by scavenging the very reactive hydroxyl and peroxyl radicals. Moreover, attenuation of the increase in hepatic luminol- and lucigenin-enhanced CL levels by EGb treatment in BDL animals also supports the scavenging ability of the agent.

An extract of the leaves of *Ginkgo biloba L.*, a mixture mainly composed of flavonoid glycosides and terpenoides (ginkgolides and bilobalide), has been shown to exhibit a variety of pharmacological actions. The leaf extract acts as a scavenger of reactive oxygen species, that is, superoxide radical, peroxyl radical and nitric oxide[34-36]. It has been reported that the extract suppresses platelet aggregation induced by *tert*-butyl hydroperoxide and hydrogen peroxide through its antioxidant action[17]. Moreover, the extract and its ingredients (especially ginkgolide C) exhibit a potent antagonistic effect on platelet-activating factor and inhibitory effects on the expression of inducible nitric oxide synthase as well as nitric oxide production[18,19,37,38]. It decreased ethanol- or cold stress-induced gastric injury, protected against cerulein-induced acute pancreatitis and hepatic damage evoked by CCl_4 in the rat[39-42]. It has also been demonstrated that EGb has protective effects on aging liver. Thus, these effects suggest that EGb is beneficial in various cardiovascular, cerebrovascular, and neurological disorders in which oxidants are involved.

GSH, a key antioxidant, is an important constituent of intracellular protective mechanisms against various noxious stimuli, including oxidative stress. On the other hand, reduced GSH, which constitutes the main component of endogenous non-protein sulfhydryl pool, is known to be a major low molecular weight scavenger of free radicals in the cytoplasm[43]. Because of their exposed sulfhydryl groups, non-protein sulfhydryls bind a variety of electrophilic radicals and metabolites that may be damaging to the cells[44]. Huang *et al.*, studied the mitochondrial functions in bile duct-ligated rats and suggested that biochemical and molecular changes are related to oxidative stress in the liver[45]. In accordance with the previous reports, our results also support the notion that depletion of tissue GSH, as observed in the BDL-induced hepatic injury, is one of the major factors that permit lipid peroxidation and subsequent tissue damage. Since administration of *Ginkgo biloba* extract prevented the hepatic GSH depletion, it appears that the protective effect of the extract involves the maintenance of antioxidant capacity in protecting the hepatic tissue against oxidative stress.

Excessive production of hydroxyl radicals in blood and liver has previously been demonstrated by Liu *et al.*, in rats with obstructive jaundice induced by common BDL[33]. It has been demonstrated by various investigators that circulating

proinflammatory cytokines, such as TNF-α, interleukin-1-β (IL-1β) and IL-6, which trigger hepatic injury, were increased, at least in part, by a free radical-mediated apoptotic mechanism. Therefore, it seems reasonable to propose that antioxidants or free radical scavengers counteract the oxidant stress produced by cholestasis. In our study, increased TNF-α levels of BDL rats tended to decrease following the treatment by EGb, also supports the notion that EGb ameliorates oxidative liver injury caused by BDL through its antioxidant effect.

Observations suggest that reactive oxygen metabolites (ROM) play a role in the recruitment of neutrophils into damaged tissue, but activated neutrophils are also a potential source of ROM[46,47]. Although it is not certain whether neutrophil accumulation and activation are the causes or the results of injury, increasing evidence suggests that mesangial cells and neutrophils release chemotactic substances (e.g., IL-8), which further promote neutrophil migration and activation[48]. Several methods have been used to define the role of neutrophils in the tissue injury. MPO, which is an essential enzyme for normal neutrophil function, is released as a response to various stimulatory substances[49]. In the present study, increased hepatic MPO activity due to BDL, which indicates that tissue injury involves the contribution of neutrophil infiltration, was effectively reversed by EGb treatment. Moreover, BDL-induced increase in fibrotic activity, as assessed by hepatic collagen content, was also reduced by EGb treatment. These findings suggest that EGb has an additional protective effect on inflammation-induced production and deposition of extracellular matrix components, which result in hepatic fibrosis.

In conclusion, the findings of our present study demonstrate that *Ginkgo biloba* extract, with its potent free radical scavenging and antioxidant properties, seems to be a highly promising agent in protecting hepatic tissue against oxidative damage and in preventing hepatic fibrosis and dysfunction due to obstructive jaundice.

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