



Hepatoprotective activity of berberine is mediated by inhibition of TNF- α , COX-2, and iNOS expression in CCl₄-intoxicated mice

Robert Domitrović^{a,*}, Hrvoje Jakovac^b, Gordana Blagojević^b

^a Department of Chemistry and Biochemistry, School of Medicine, University of Rijeka, B. Branchetta 20, 51000 Rijeka, Croatia

^b Department of Physiology and Immunology, School of Medicine, University of Rijeka, 51000 Rijeka, Croatia

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ABSTRACT

This study investigated the protective effects of isoquinoline alkaloid berberine on the CCl₄-induced hepatotoxicity in mice. Berberine was administered as a single dose at 5 and 10 mg/kg intraperitoneally (i.p.), 1 h before CCl₄ (10%, v/v in olive oil, 2 ml/kg) injection and mice were euthanized 24 h later. The rise in serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) in CCl₄-intoxicated mice was markedly suppressed by berberine in a concentration-dependent manner. The decrease in hepatic activity of superoxide dismutase (Cu/Zn SOD) and an increase in lipid peroxidation were significantly prevented by berberine. Histopathological changes were reduced and the expression of tumor necrosis factor- α (TNF- α), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) was markedly attenuated by berberine 10 mg/mg. The results of this study indicate that berberine could be effective in protecting the liver from acute CCl₄-induced injury. The hepatoprotective mechanisms of berberine may be related to the free radical scavenging and attenuation of oxidative/nitrosative stress, as well as to the inhibition of inflammatory response in the liver.

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1. Introduction

The liver is a major organ responsible for the metabolism of drugs and toxic chemicals, and therefore is the primary target organ for nearly all toxic chemicals (Bissell et al., 2001). Natural antioxidants could prevent the deleterious effects of toxic agents by scavenging free radicals and other reactive oxygen species or by modulation of the inflammatory response (Grimble, 1994; Domitrović et al., 2009). TNF- α is the key mediator in many experimental liver injury models, including CCl₄-induced hepatitis (Schümann and Tiegs, 1999). An early rise of TNF- α levels induces proinflammatory genes, including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Akarasereenont et al., 1995; Ramadori and Ambrust, 1999). Persistent hepatic injury and inflammation may lead to the progressive liver damage, fibrosis, and finally cirrhosis. The inhibition of proinflammatory cytokines and enzymes may offer a new therapeutic strategy against inflammatory liver disease.

Berberine is an isoquinoline alkaloid of the protoberberine type, which could be found in the root, rhizome, and stem bark of many plant species traditionally used for treatment of hepatic disorders,

such as *Coptis chinensis* Franch., *Coptis japonica* Makino., *Berberis thunbergii* D.C., *Thalictrum lucidum* L., barberry (*Berberis vulgaris* L.), Oregon grape (*Berberis aquifolium* Pursh), and goldenseal (*Hydrastis canadensis* L.) (Imanshahidi and Hosseinzadeh, 2008). Extensive research within the past decade indicates that berberine possesses a wide range of pharmacological activities, including antioxidative (Rockova et al., 2004), anti-inflammatory (Küpeleli et al., 2002), and immunoregulative (Kim et al., 2003) activities. Several studies demonstrated the inhibitory effects of berberine on chemically induced cytotoxicity, lipid peroxidation, and oxidative stress in the liver (Hwang et al., 2002; Zhang et al., 2008), including CCl₄-induced liver damage (Janbaz and Gilani, 2000; Ye et al., 2009; Feng et al., 2010).

Silymarin is an antioxidant flavonoid complex derived from the herb milk thistle (*Silybium marianum* L.), traditionally used for the treatment of hepatobiliary disorders (Tamayo and Diamond, 2007). The major mechanisms of silymarin hepatoprotective activity that have been proposed include antioxidative, immunomodulating, anti-inflammatory, and antifibrotic activity (Luper, 1998). Although berberine does not share structural similarity with silymarin, both drugs seems to increase the cellular antioxidant defense machinery and modulate immune system function.

In the present study, we have investigated the protective effects of berberine against acute CCl₄-induced damage and a possible mechanism for its hepatoprotective activity. Silymarin has been used as the reference drug.

* Corresponding author. Tel.: +385 51 651 135; fax: +385 51 651 135.

E-mail address: robertd@medri.hr (R. Domitrović).

2. Materials and methods

2.1. Chemicals

Berberine was purchased from Polyphenols Laboratories AS (Sandnes, Norway). Carbon tetrachloride (CCl₄) and acetic acid were obtained from Kemika, (Zagreb, Croatia), olive oil, 1,1,3,3,-tetramethoxypropane (TMP), trichloroacetic acid (TCA), sodium dodecylsulphate (SDS), bovine superoxide dismutase (SOD), xanthine, xanthine oxidase, cytochrome c, ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Diagnostic kits for the determination of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from Herbos Diagnostics (Sisak, Croatia), and for alkaline phosphatase (ALP) from DiaSys Diagnostic Systems (Holzheim, Germany). HRP-conjugated anti-mouse IgG was from Sigma–Aldrich Chemie GmbH (Steinheim, Germany) and HRP-conjugated anti-rabbit IgG from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were of the highest grade commercially available.

2.2. Animals

Male Balb/c mice from our breeding colony, 2–3 month old, were randomly divided into 6 groups with 5 animals per group. Mice were fed a standard rodent diet (pellet, type 4RF21 GLP, Mucedola, Italy), and water *ad libitum*. The animals were maintained at 12 h light/dark cycle, at constant temperature (20 ± 1 °C) and humidity (50 ± 5%). All experimental procedures were performed in compliance with the appropriate laws and institutional guidelines, and were approved by the Ethical Committee of the Medical Faculty, University of Rijeka.

2.3. Experimental design

Berberine (5 or 10 mg/kg) and silymarin (100 mg/kg) dissolved in DMSO and diluted with saline (final DMSO solution 5%, v/v) were given intraperitoneally (i.p.) 1 h before CCl₄ dissolved in olive oil (10%, v/v, 2 ml/kg), at different sites of abdomen to avoid cross-reaction of chemicals. The control group and the group treated with berberine alone (10 mg/kg) received DMSO and berberine, respectively. The CCl₄ group received DMSO and CCl₄ 1 h later. Silymarin as the reference drug, in the therapeutic dosage of 100 mg/kg, has been used as a comparative control for hepatoprotective activity of berberine (Mansour et al., 2006). Berberine group alone was used for evaluating the potential hepatotoxicity of berberine.

Berberine was administered via i.p. route due to easier and more reliable dosage, considering the fact that previous studies shown efficiency of orally applied berberine, but also its low bioavailability as well as that it is not metabolized in the gut (Zuo et al., 2006). The doses were selected on the basis of our preliminary study (range of doses 1–10 mg/kg), by observing the extent of histological damage and the increase in serum enzymes, as well as of comparable doses in several previous reports (Kuo et al., 2004; Kulkarni and Dhir, 2007; Ho et al., 2009). Although the LD₅₀ for i.p. administration of berberine in mice is a relatively low, ≥50 mg/kg (Anis et al., 1999; Kheir et al., 2010), the doses used in this study (5 and 10 mg/kg) could be considered safe, since we did not observe mortality in any experimental group.

The mice were anaesthetized by inhalation of ethylether 24 h later, and blood was collected from retro-orbital sinus before mice were euthanized by cervical dislocation. Serum was separated for assessment of ALT, AST, and ALP activities. The abdomen was open and liver was removed, perfused with saline, blotted dry and divided into samples. Liver samples were used to assess the Cu/Zn SOD activity, thiobarbituric acid reactive species (TBARS) and protein content, and for immunoblotting. A portion of the livers was preserved in a buffered formalin solution to obtain histological sections.

2.4. Serum markers of liver damage

Serum levels of ALT, AST, and ALP as markers of hepatic function, were measured using commercial kits, according to manufacturer's instructions, in a microplate reader (Bio-Tek EL808 Ultra Microplate Reader (BioTek Instruments, Winooski, VT, USA).

2.5. Markers of oxidative stress

Livers were homogenized in 50 mM phosphate buffer saline (PBS), pH 7.4, and the homogenates were used for the determination of liver peroxidation. Alternatively, the homogenates were centrifuged at 12,000g for 15 min, 4 °C, and the supernatants were used for the measurement of Cu/Zn SOD activity. The protein content in liver samples was estimated by Bradford's method (Bradford, 1976).

The Cu/Zn SOD activity was measured indirectly by the decrease in cytochrome c reduction by superoxide radicals at 550 nm. The xanthine/xanthine oxidase system was used as the source of superoxide, as described by Flohé and Otting (1984). Briefly, 2.9 ml of reaction mixture (0.05 mM cytochrome c and 1 mM xanthine) was added to 50 µl of supernatant. The reaction was initiated by addition of 50 µl of 0.5 units of xanthine oxidase, and an increase in absorbance for 3 min was measured by a spectrophotometer (Cary 100, Varian, Mulgrave, Australia). One

unit of Cu/Zn SOD inhibits the rate of reduction of cytochrome c by 50% at pH 7.8 at 25 °C. The calibration curve was obtained with known concentrations of SOD.

The levels of lipid peroxidation were quantified by TBARS assay as previously described (Ohkawa et al., 1979), with minor modifications. In brief, 150 µl each of sample was mixed with 75 µl of 8.1% SDS. After 10 min of incubation at room temperature, 675 µl of 20% acetic acid solution (pH 3.5) was added and the mixture was centrifuged at 10,000g for 15 min. Equal volumes (800 µl) of supernatant and 0.8% aqueous solution of TCA were mixed and heated in a boiling water bath for 1 h. After cooling in cold water, the resulting chromogen was extracted with 1.6 ml of 15:1 1-butanol/pyridine solution and the organic phase was separated by centrifugation at 3000 rpm for 10 min. The amounts of TBARS were determined spectrophotometrically at 532 nm and were calculated as nmol malondialdehyde equivalent per mg of protein according to the standard curve prepared from TMP.

2.6. Histopathology

The liver tissues were placed in plastic cassettes and immersed in neutral buffered formalin for 24 h. The fixed tissues were processed routinely, embedded in paraffin, cut into 4 µm-thick sections and stained with hematoxylin and eosin (H&E). The extent of CCl₄-induced hepatic damage was evaluated by assessing the morphological changes in the liver sections.

2.7. Immunohistochemistry

Immunohistochemical studies were performed on paraffin embedded liver tissue sections of 4 µm thickness, previously deparaffinized and rehydrated using a standard technique. Mouse monoclonal anti-TNF-α diluted 1:50 (Abcam, Cambridge, UK), rabbit polyclonal anti-COX-2 diluted 1:50 (Lab Vision, Fremont, CA, USA), and rabbit polyclonal anti-iNOS diluted 1:50 (BD Transduction Laboratories, Lexington, KY, USA) were used as primary antibodies. Immunoreactions were visualized employing DAKO EnVision+System, Peroxidase/DAB kit according to the manufacturer's instructions (DAKO Corporation, Carpinteria, CA, USA), as described previously (Domitrović and Jakovac, 2010). Negative control sections were processed in an identical manner by substitution of primary antibodies with irrelevant immunoglobulins of matched isotype, used in the same conditions and dilutions as primary antibodies. Stained slides were analyzed by light microscopy (Olympus BX51, Tokyo, Japan). The Cell F v3.1 software, Olympus Soft Imaging Solutions (Münster, Germany) was used to quantify immunohistochemical staining. Captured images were subjected to intensity separation, which subsequently was inverted, resulting in gray scale images with different intensity range, depending on the strength of immunohistochemical signals. Regions of interest were set to measure the average gray level in specific areas that showed immunopositivity. Optical density of the corresponding negative control section was subtracted from that of the stained section.

2.8. Western blot

Liver samples were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 2 µg/ml of each aprotinin, leupeptin and pepstatin. After 10 min of centrifugation at 15,000 rpm, 4 °C, volumes equivalent to 50 µg of proteins were incubated with the sample buffer for 10 min at 96 °C and analyzed by 13% SDS-PAGE under non reducing conditions. After electrophoresis, polyacrylamide gels were blotted onto polyvinylidene fluoride membrane (Roche Diagnostics GmbH, Mannheim, Germany) at 60–70 V for 1 h. Membranes were washed in Tris–HCl-buffered saline (TBS; 50 mM, pH 7.5) and incubated in 1% blocking reagent (Roche Diagnostics GmbH, Mannheim, Germany) for 5 h, followed by over night incubation with anti-TNF-α (Abcam, Cambridge, UK), anti-COX-2 (Lab Vision, Fremont, CA, USA), anti-iNOS (BD Transduction Laboratories, Lexington, KY, USA), and anti-actin (Sigma–Aldrich Chemie GmbH, Germany) antibodies, three cycles of washing (TBS with 0.1% Tween 20; TBS-T buffer), and 60 min incubation with peroxidase-conjugated secondary reagent diluted in TBS buffer containing 0.5% blocking reagent. After washing three times with TBS-T buffer (pH 7.5), membranes were incubated with Super Signal West Dura extended duration substrate (Pierce Chemical Co., Rockford, IL, USA) for 5 min, and exposed to BioMax film (Kodak). Films were scanned (CanoScan LiDE 35, Cannon) and the intensity of the bands was assayed by computer image analysis software Cell F v 3.1, Olympus Soft Imaging Solutions (Münster, Germany).

2.9. Statistical analysis

Data were analyzed using StatSoft STATISTICA version 7.1 software. Differences between the groups were assessed by one-way ANOVA and Dunnett's post hoc test. Values in the text are means ± standard deviation (S.D.). Differences with *p* < 0.05 were considered to be statistically significant.

Table 1
Relative liver weights and serum markers of liver damage.

	Liver weights (g/100 g b.w.)	AST (U/L)	ALT (U/L)	ALP (U/L)
Control	4.51 ± 0.07	52.3 ± 4.2	34.9 ± 3.1	54.2 ± 6.7
Berberine 10 mg/kg	4.56 ± 0.09	49.4 ± 3.1	32.9 ± 2.3	55.4 ± 4.9
CCl ₄	5.17 ± 0.48 ^a	3329 ± 355 ^a	7765 ± 875 ^a	358.4 ± 18.4 ^a
CCl ₄ + berberine 5 mg/kg	5.23 ± 0.28 ^a	3050 ± 392 ^a	7750 ± 686 ^a	249.1 ± 43.8 ^{a,b}
CCl ₄ + berberine 10 mg/kg	4.55 ± 0.24 ^b	261.5 ± 49.3 ^{a,b}	444.5 ± 86.3 ^{a,b}	105.2 ± 9.7 ^{a,b}
CCl ₄ + silymarin 100 mg/kg	5.12 ± 0.17 ^a	2849 ± 160 ^{a,b}	7012 ± 480 ^a	367.4 ± 37.0 ^a

Each value represents the mean ± S.D. for 5 mice.

^a $p < 0.05$ as compared with the control group.

^b $p < 0.05$ as compared with the CCl₄ group.

3. Results

3.1. Serum ALT, AST, and ALP activity

Serum ALT, AST, and ALP levels were highly elevated 24 h after CCl₄ treatment (Table 1). Treatment with berberine decreased aminotransferase and ALP levels in CCl₄-intoxicated mice in dose-dependent manner. Silymarin 100 mg/kg was less effective in attenuation of hepatocellular damage than berberine 10 mg/kg. The enzyme activity in the group treated with berberine alone was similar to controls. Liver weights increased in CCl₄-intoxicated mice, indicating development of the inflammatory hepatic oedema, which was prevented by berberine in the dose of 10 mg/kg.

3.2. Hepatic Cu/Zn SOD activity and TBARS content

CCl₄ administration significantly decreased Cu/Zn SOD activity in the livers, compared to the control group (Fig. 1). Berberine has ameliorated the loss of enzyme activity in a dose-dependent manner. Additionally, berberine 10 mg/kg was more effective in the attenuation of oxidative stress than silymarin 100 mg/kg. The Cu/Zn SOD activity in the group treated with berberine alone was similar to controls. Hepatic TBARS levels were markedly increased in the CCl₄ group and both berberine and silymarin decreased its values (Fig. 2). Mice treated with berberine alone had TBARS levels similar to controls.

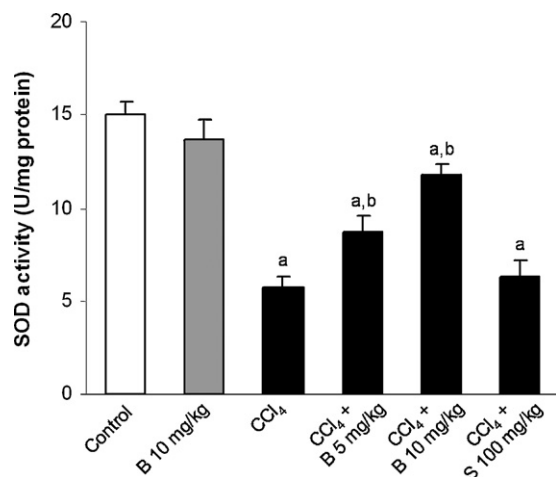


Fig. 1. The hepatic Cu/Zn SOD activity in experimental groups. Mice were treated with berberine (5 and 10 mg/kg) or silymarin 100 mg/kg, intraperitoneally (i.p.). One hour later, CCl₄ 2 ml/kg (10%, v/v in olive oil) was injected i.p., except the control group and the group receiving berberine alone, which received vehicle. Each value represents the mean ± S.D. for 5 mice. ^a $p < 0.05$ as compared with the control group. ^b $p < 0.05$ as compared with the CCl₄ group.

3.3. Histopathology

Liver sections from control mice (Fig. 3A) showed normal liver architecture, and berberine alone had no significant effect on liver histology (Fig. 3B). In the group treated with CCl₄, large areas of extensive, mainly pericentral necrosis with loss of hepatic architecture, vacuolar fatty change and mild inflammatory cell infiltration, comprised predominantly of mononuclear cells and macrophages, have been found (Fig. 3C). The low dose of berberine (5 mg/kg) did not prevent the toxic effect of CCl₄, and large necrotic areas were still present (Fig. 3D). However, the high dose of berberine (10 mg/kg) completely prevented liver necrosis, showing minimal hepatic damage (Fig. 3E). In contrast, silymarin in the dose of 100 mg/kg showed a weak hepatoprotective activity when compared to the high-dose berberine (Fig. 3F). Fig. 3G shows the extent of hepatocellular necrosis in the experimental groups.

3.4. TNF- α , COX-2, and iNOS expression

The effects of berberine on the TNF- α expression are shown in Fig. 4. The livers of control mice did not show substantial TNF- α immunopositivity (Fig. 4A). The TNF- α expression in the group receiving berberine alone was similar to controls (Fig. 4B). On the other hand, strong TNF- α expression was found in the CCl₄ group (Fig. 4C). TNF- α immunoreactivity was observed mainly in necrotic areas, showing predominantly pericentral staining. No apparent TNF- α expression was detected in nonparenchymal cells. TNF- α immunopositivity decreased in the livers of CCl₄-intoxicated mice

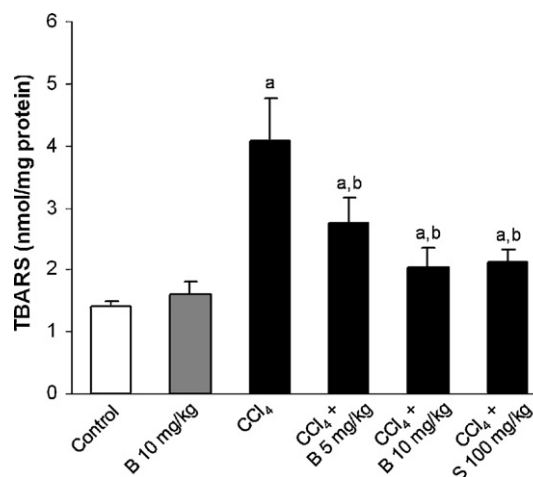


Fig. 2. The hepatic TBARS content in experimental groups. Mice were treated with berberine (5 and 10 mg/kg) or silymarin 100 mg/kg, intraperitoneally (i.p.). One hour later, CCl₄ 2 ml/kg (10%, v/v in olive oil) was injected i.p., except the control group and the group receiving berberine alone, which received vehicle. Each value represents the mean ± S.D. for 5 mice. ^a $p < 0.05$ as compared with the control group. ^b $p < 0.05$ as compared with the CCl₄ group.

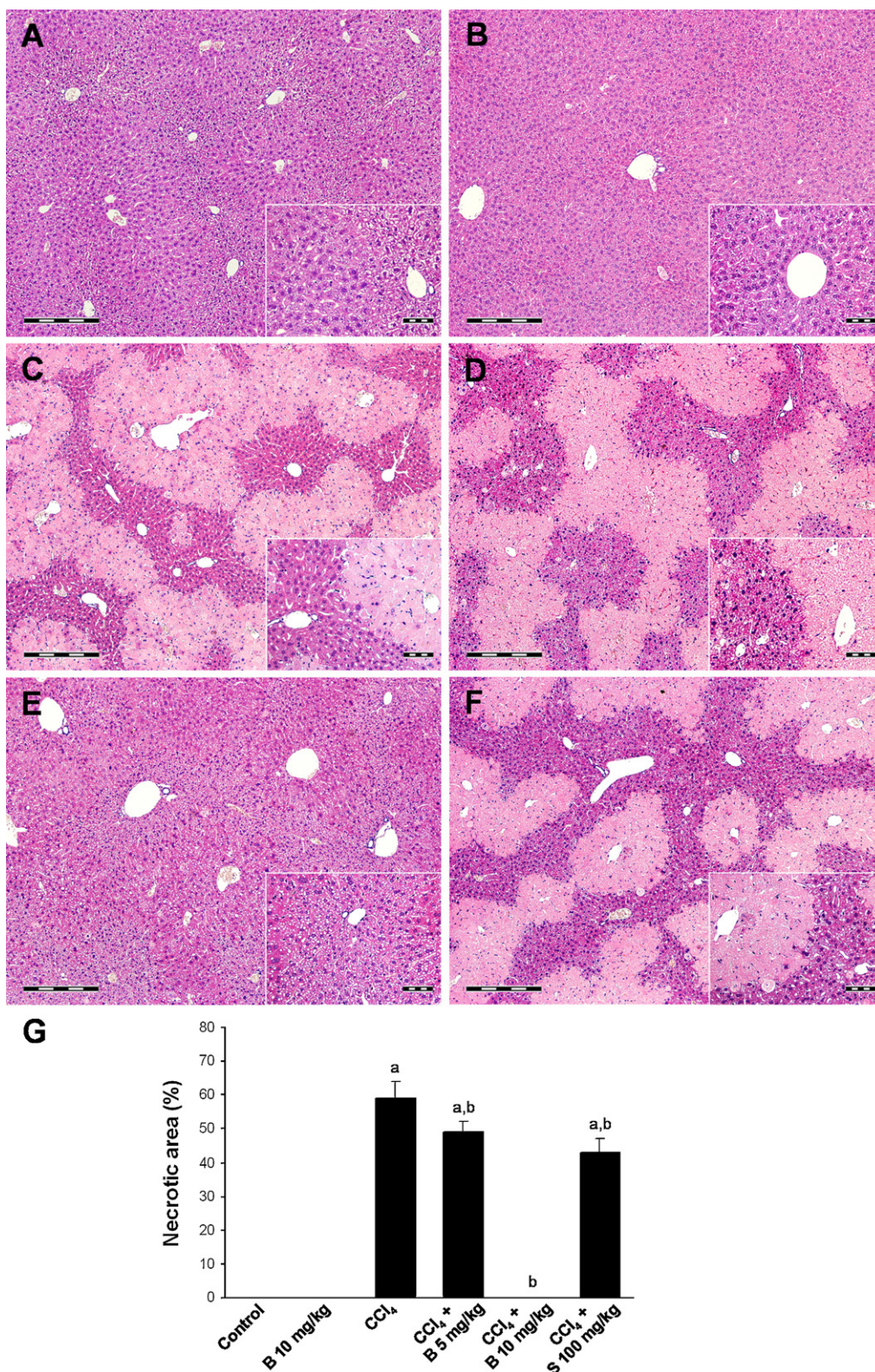


Fig. 3. The effect of berberine and silymarin on histological changes in the liver of CCl₄-treated mice. (A) The control group showing normal lobular architecture and cell structure. (B) The livers of mice receiving berberine 10 mg/kg alone were histologically similar to controls. (C) The CCl₄ group showing severe hepatic lesions, degenerated and necrotic hepatocytes, and microvesicular steatosis. (D) Berberine 5 mg/kg did not have a noticeable effect on hepatic damage, while (E) berberine 10 mg/kg completely reduced necrotic lesions. (F) Silymarin 100 mg/kg did not substantially ameliorate hepatic lesions. Scale bar = 500 μm, inset scale bar = 50 μm. H&E stain. Representative results from five similarly treated mice. (G) The percentage of necrotic area in the livers (mean ± S.D., N=5). ^a*p* < 0.05 as compared with the control group. ^b*p* < 0.05 as compared with the CCl₄ group.

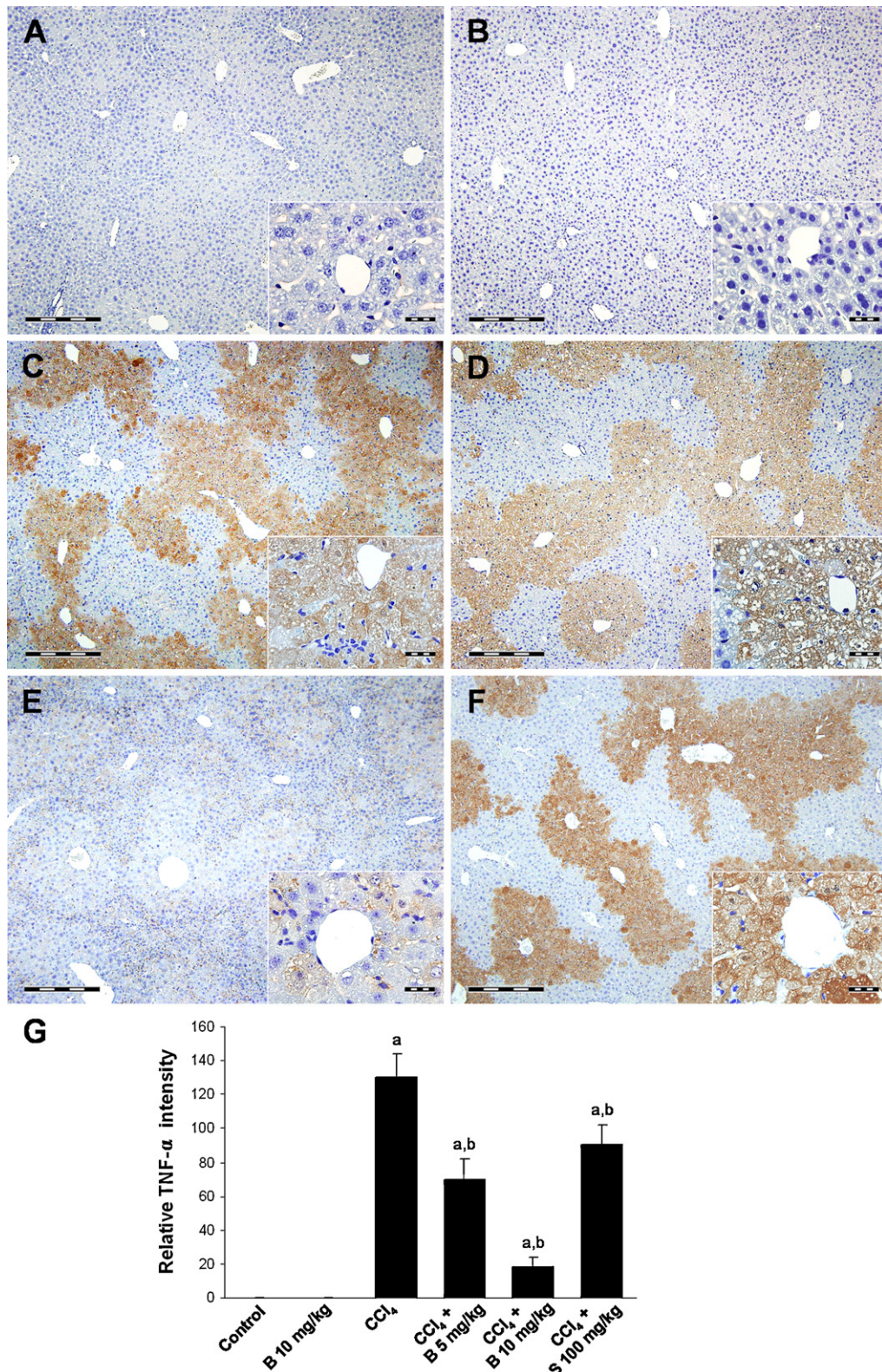


Fig. 4. The effect of berberine and silymarin on the expression and specific hepatic distribution of TNF- α . (A) In control livers, TNF- α expression was negligible. (B) The livers of mice receiving berberine 10 mg/kg alone were similar to controls. (C) Strong TNF- α immunopositivity in CCl₄-intoxicated mice. (D) Moderate TNF- α immunopositivity in mice treated with the low dose of berberine (5 mg/kg). (E) The high dose of berberine (10 mg/kg) markedly reduced TNF- α immunopositivity, compared to the low dose. (F) Strong TNF- α immunostaining in mice treated with silymarin 100 mg/kg. Scale bar = 500 μ m, inset scale bar = 50 μ m. Immunohistochemical stain for TNF- α . Representative results from five similarly treated mice. (G) The quantification of TNF- α staining (mean \pm S.D., $N = 5$). ^a $p < 0.05$ as compared with the control group. ^b $p < 0.05$ as compared with the CCl₄ group.

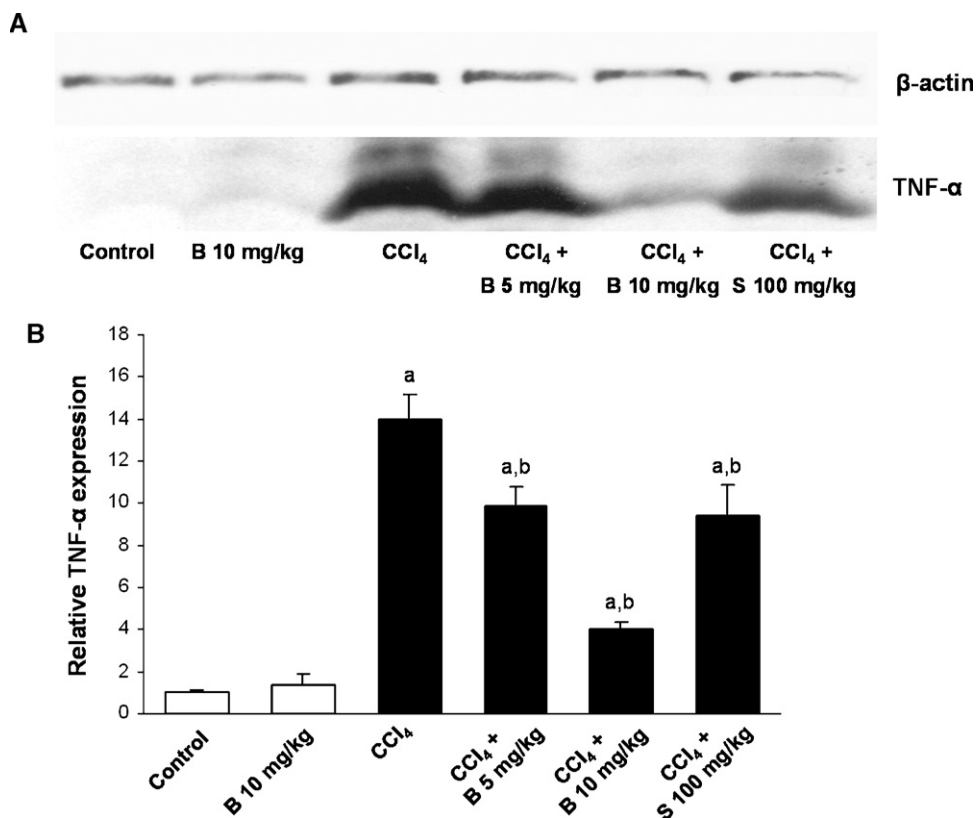


Fig. 5. Western blot analysis of hepatic TNF- α expression. (A) Western blot showing the effect of berberine and silymarin on TNF- α levels in acute liver injury. (B) The relative expression of TNF- α in relation to β -actin. Berberine alone did not significantly affect on TNF- α expression when compared to controls. TNF- α levels in the CCl₄ group were increased when compared to controls. Berberine 5 mg/kg slightly decreased TNF- α immunoreactivity, whereas berberine 10 mg/kg markedly attenuated TNF- α expression. Silymarin 100 mg/kg partially reduced TNF- α expression, comparable with the low dose of berberine. Each value represents the mean \pm S.D. for 5 mice. ^a $p < 0.05$ as compared with the control group. ^b $p < 0.05$ as compared with the CCl₄ group.

treated with the low dose of berberine (5 mg/kg) (Fig. 4D), and the high dose of berberine (10 mg/kg) further substantially reduced TNF- α expression (Fig. 4E). In contrast, silymarin in the dose of 100 mg/kg was not effective in reducing TNF- α expression (Fig. 4F). The quantification of TNF- α staining showed a 2- and 7-fold reduction of TNF- α expression by berberine 5 and 10 mg/kg, respectively, when compared to the CCl₄-group (Fig. 4G). The results obtained by immunohistochemistry were confirmed by immunoblotting analysis (Fig. 5). The TNF- α specific bands at 17 kDa were detected in all groups, with the highest intensity in the CCl₄ group (Fig. 5A). The high dose of berberine significantly reduced TNF- α protein level, whereas the effect of silymarin was less pronounced (Fig. 5B).

The low level of COX-2 immunoreactivity in normal livers was restricted to nonparenchymal cells, morphologically corresponding to the Kupffer cells (Fig. 6A). Berberine alone had no substantial effect on COX-2 expression, compared to controls (Fig. 5B). CCl₄ injection dramatically increased COX-2 immunopositivity in necrotic areas of the livers (Fig. 6C), which was attenuated by the low dose of berberine (Fig. 6D). The high dose of berberine markedly reduced COX-2 expression when compared to the low dose (Fig. 6E). COX-2 immunoreactivity was strongly expressed in the cytoplasm and sporadically in the nuclei of individual hepatocytes. Silymarin in the dose of 100 mg/kg also reduced COX-2 immunoreactivity, however, this effect was less pronounced (Fig. 6F). The quantification of COX-2 staining showed a 2- and 4-fold reduction of COX-2 expression by berberine 5 and 10 mg/kg, respectively, when compared to the CCl₄-group (Fig. 4G). Western blot analysis did not show detectable COX-2 immunopositivity.

Weak iNOS immunopositivity in the livers of control mice was restricted to nonparenchymal cells in sinusoidal walls (Fig. 7A). In the group treated with berberine alone, iNOS expression was similar to controls (Fig. 7B). CCl₄ intoxication strikingly increased iNOS immunoreactivity in necrotic areas (Fig. 7C), which was slightly ameliorated by berberine 5 mg/kg (Fig. 7D). Berberine in the dose of 10 mg/kg markedly reduced cytoplasmic iNOS immunoreactivity when compared to the low dose (Fig. 7E), whereas silymarin has not been effective in the attenuation of iNOS expression (Fig. 7F). The quantification of iNOS staining showed a minor decrease (21%) in iNOS expression by berberine 5 mg/kg and a 3-fold reduction by berberine 10 mg/kg, when compared to the CCl₄-group (Fig. 7G). Western blot analysis did not show detectable iNOS immunoreactivity.

4. Discussion

CCl₄-induced hepatic injury is an experimental model widely used for hepatoprotective drug screening. The goal of this study was to determine a possible mechanism for the hepatoprotective activity of berberine in CCl₄-intoxicated mice. Our results suggest that berberine can prevent acute hepatotoxicity induced by CCl₄, by attenuation of oxidative and nitrosative stress as well as the inflammatory response in the liver. The decrease in serum aminotransferases and ALP activity by the high-dose berberine in CCl₄-intoxicated mice indicates that berberine preserves the structural integrity of hepatocellular membrane, which was supported by the histological findings. Furthermore, berberine seems to possess considerably stronger activity against CCl₄-induced liver

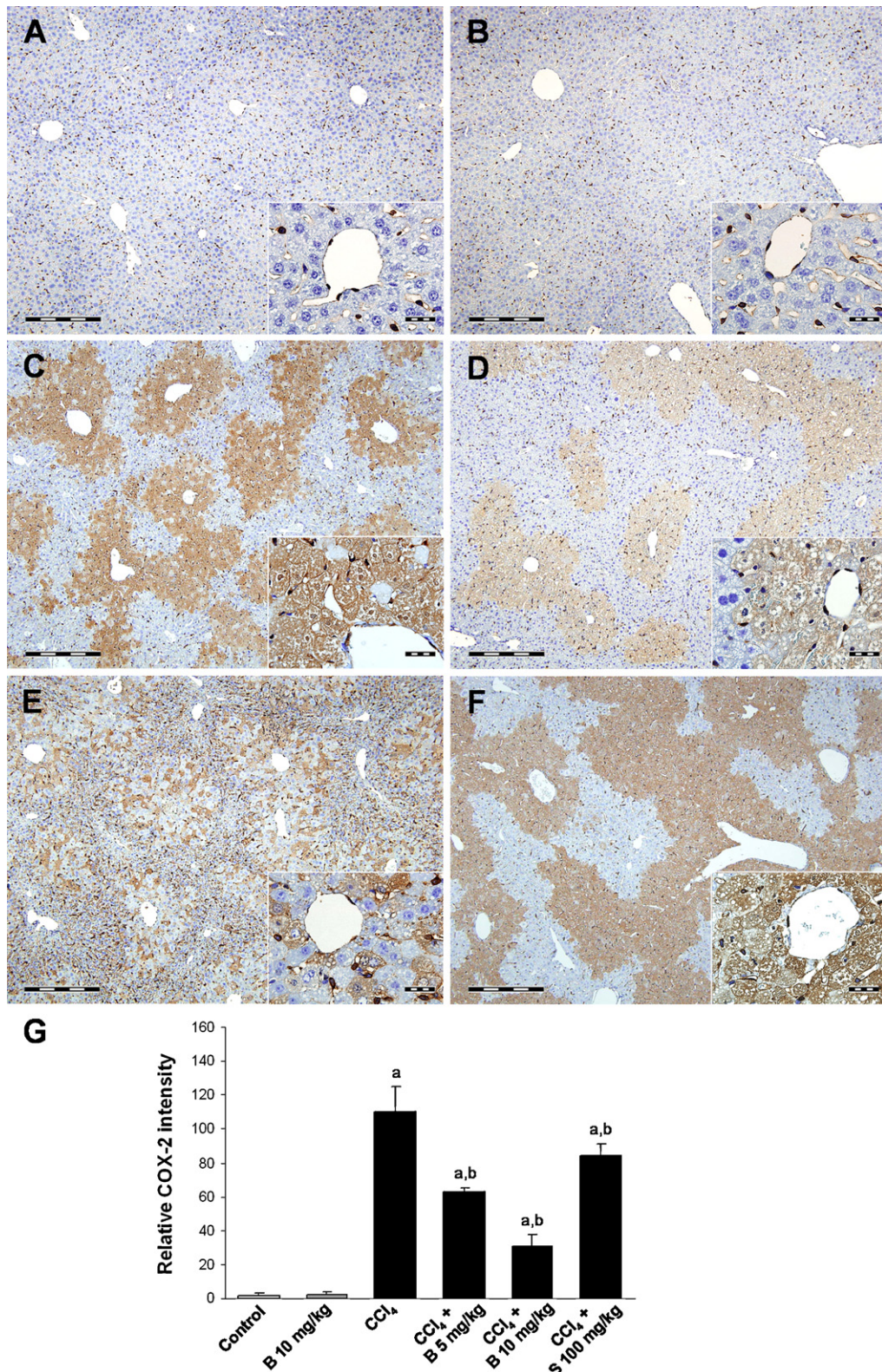


Fig. 6. The effect of berberine and silymarin on the expression and specific hepatic distribution of COX-2. (A) COX-2 immunopositive macrophages in the control group. (B) Berberine alone showed COX-2 expression pattern similar to controls. (C) Strong COX-2 immunopositivity in necrotic areas of CCl₄-intoxicated mice. (D) The low-dose berberine (5 mg/kg) slightly decreased COX-2 immunoreactivity, compared to the CCl₄ group, while (E) COX-2 immunopositivity was markedly reduced in mice treated with berberine 10 mg/kg. (F) Moderate COX-2 immunopositivity in mice treated with silymarin 100 mg/kg. Scale bar = 500 μm, inset scale bar = 50 μm. Immunohistochemical stain for COX-2. Representative results from five similarly treated mice. (G) The quantification of COX-2 staining (mean ± S.D., N = 5). ^a*p* < 0.05 as compared with the control group. ^b*p* < 0.05 as compared with the CCl₄ group.

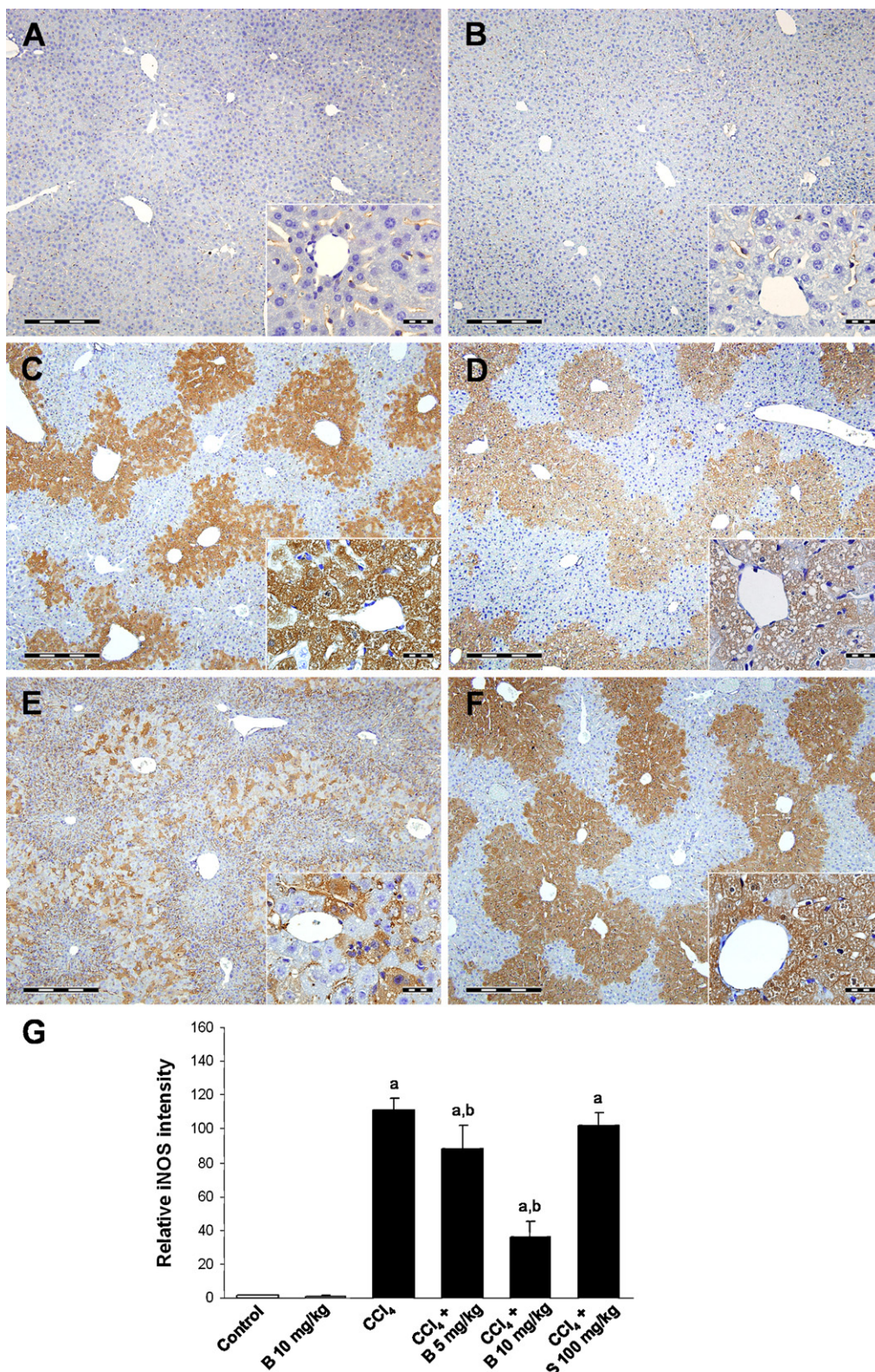


Fig. 7. The effect of berberine and silymarin on the expression and specific hepatic distribution of iNOS. (A) iNOS immunopositivity in sinusoidal lining cells of control mice. (B) iNOS expression in mice receiving berberine alone was similar to controls. (C) Strong iNOS immunopositivity in necrotic areas in CCl₄-intoxicated mice. (D) iNOS immunoreactivity decreased in mice treated with the low dose of berberine (5 mg/kg), compared to the CCl₄ group, whereas (E) the high dose of berberine (10 mg/kg) markedly attenuated iNOS expression. (F) Strong iNOS immunopositivity in the silymarin (100 mg/kg)-treated group. Scale bar = 500 μ m, inset scale bar = 50 μ m. Immunohistochemical stain for iNOS. Representative results from five similarly treated mice. (G) The quantification of iNOS staining (mean \pm S.D., $N = 5$). ^a $p < 0.05$ as compared with the control group. ^b $p < 0.05$ as compared with the CCl₄ group.

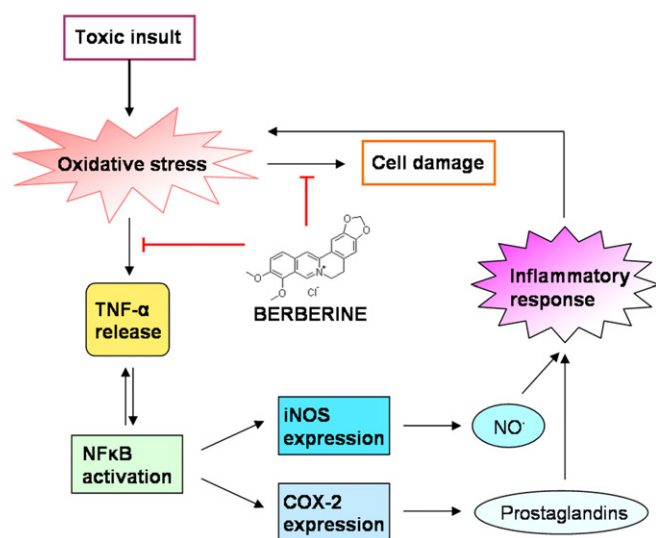


Fig. 8. Scheme showing the proposed mechanisms of hepatoprotective activity of berberine. Toxic exposure could induce oxidative stress in the liver due to its metabolism into highly reactive free radicals. Oxidative stress triggers the TNF- α release from Kupffer cells and injured hepatocytes, which further activates NF κ B, allowing its nuclear translocation. Consequently, NF κ B stimulates the expression of iNOS and COX-2 at the level of transcription, translation, and the enzyme level. The final products of iNOS and COX-2, NO \cdot and prostaglandins, respectively, contribute to nitrosative stress and, on the other hand, initiate the cascade of inflammatory response in injured liver. Inflammation, in turn, is associated with the release of highly reactive oxygen and nitrogen species from inflammatory cells, further exacerbating oxidative and nitrosative stress. Berberine prevents oxidative damage, as indicated by the decrease in lipid peroxidation, and improves the antioxidant status. Furthermore, berberine suppresses the inflammatory response by downregulating the proinflammatory cascade initiated by TNF- α , and attenuates nitrosative stress by the iNOS inhibition.

damage than silymarin, widely recognised as a potent hepatoprotective agent. Based on our results and previous studies, we proposed a mechanism of berberine-mediated hepatoprotection, summarized in Fig. 8.

Increased production of free radicals and oxidative stress have been implicated in the pathogenesis of acute hepatic disorders, including CCl₄-intoxication (Slater, 1984). The metabolism of CCl₄ by cytochrome P450 leads to the formation of free radicals, inducing lipid peroxidation and impairs antioxidant status, as shown here, which was successfully prevented by berberine (Fig. 8). Our results suggest that the antioxidant properties may be responsible for berberine hepatoprotective effect, also shown in previous studies (Ye et al., 2009; Feng et al., 2010). On the other hand, the results of this study, for the first time, show antinitrosative and anti-inflammatory effect of berberine in CCl₄-induced liver injury.

TNF- α is a pleiotropic cytokine associated with a variety of physiological and pathological conditions (Beyaert and Fiers, 1998). The TNF- α expression rise in acute liver diseases, as well as following exposure to hepatotoxic chemicals (Luster et al., 1999; Simeonova et al., 2001), contributing to the hepatotoxicity induced by CCl₄ (Czaja et al., 1995; Morio et al., 2001). TNF- α seems to be responsible for regulating products that stimulate inflammation and fibrosis, but not direct hepatocyte necrosis in CCl₄-induced hepatotoxicity (Simeonova et al., 2001). The results of current research coincide with studies on several cell lines showing that, independently from the kind of inflammatory stimulus, berberine effectively suppresses the expression of proinflammatory cytokines, including TNF- α , subsequently inhibiting downstream mediators of inflammation, such as iNOS and COX-2 (Fig. 8) (González-Amaro et al., 1994; Hsiang et al., 2005; Jeong et al., 2009). In this study, the modulation of TNF- α expression by berber-

ine suggests its important role in the attenuation of CCl₄-induced inflammatory cascade in the liver.

In addition to other functions, TNF- α induces iNOS and stimulates production of nitric oxide (NO \cdot), contributing to nitrosative stress. NO \cdot may react with superoxide (O₂ \cdot ⁻) in the mitochondria to produce peroxynitrite (ONOO⁻), both of which are important mediators of cell dysfunction. Overexpression of iNOS has been seen in many acute and chronic diseases (Nussler and Billiar, 1993; Hierholzer et al., 1998), but the role of NO \cdot in tissue damage is still controversial. Although a hepatoprotective function of iNOS has been observed in different types of liver injury, including chronic CCl₄-intoxication (Muriel, 1998; Hu et al., 2010), hepatotoxic effects of iNOS were reported (Thiemermann et al., 1995; Isobe et al., 1999; Park et al., 2008). It seems that iNOS-derived NO \cdot could regulate proinflammatory genes expression, thereby contributing to inflammatory liver injury (Sass et al., 2001). In various experimental models it has been demonstrated that berberine suppresses the activation of NF κ B and downstream proinflammatory mediators, including iNOS (Fig. 8) (Kim et al., 2007; Jiang et al., 2010). In this study, increased iNOS expression in the livers of CCl₄-intoxicated mice indicates enhanced production of NO \cdot and nitrosative stress, as a response to liver injury. Our results suggest that iNOS mediates acute CCl₄-induced liver injury and its inhibition by berberine exerts beneficial effects in the prevention of acute hepatic damage.

COX-2 is an inducible form of the prostaglandin synthase enzymes, which catalyse the committed step in the prostaglandin production pathway (Dubois et al., 1998). COX-2 expression is increased in inflammatory conditions as a result of induction by several different stimuli, including proinflammatory cytokines TNF- α , IL-1 β , and EGF (Fig. 8) (Akarasereenont et al., 1995). The inhibition of COX-2 has been shown to exert the hepatoprotective effect in CCl₄-induced liver damage (Vadiraja et al., 1998). In this study, the decrease in COX-2 expression by berberine indicates suppression of prostaglandin synthesis and amelioration of the inflammatory response in CCl₄-intoxicated mice, as reported previously (Kuo et al., 2004). Additionally, the positive correlation between COX-2 and iNOS expression in the livers, observed in this study, is in agreement with previous findings that NO \cdot is capable to modulate COX-2 expression, and there probably a cross-talk between the iNOS and COX-2 systems exists (Swierkosz et al., 1995). In fact, both the iNOS and COX-2 genes share NF κ B binding sites in their promoter regions (D'acquisto et al., 2000). COX-2 is also involved in the regulation of nuclear functions, such as the cell cycle progression, proliferation, and apoptosis (Hla et al., 1993; Dubois et al., 1998). The COX-2 localisation in the nuclei of hepatocytes in group treated with the high-dose berberine, suggests the involvement of COX-2 in other cellular events, besides the inflammatory response mediated by prostaglandins.

Several studies demonstrated hepatoprotective activity of berberine, both preventive and curative, in CCl₄-intoxicated rats, with discrepancies which could be attributed to the differences in the dosage of both berberine and CCl₄ and the route of their administration (Janbaz and Gilani, 2000; Feng et al., 2010). In this study, liver protection was achieved by relatively low i.p. doses of berberine, as observed by other researchers (Hwang et al., 2002), but oral application, due to a poor bioavailability (Sheng et al., 1993), requires much higher doses (Zhang et al., 2008; Ye et al., 2009; Feng et al., 2010). Nevertheless, the same metabolites could be found in the rat urine after either oral or i.p. administration, suggesting that the metabolites of berberine are formed after absorption rather than in the gastrointestinal tract (Qiu et al., 2008). Furthermore, it seems that these metabolites are the active components of berberine (Tsai and Tsai, 2004; Zuo et al., 2006).

In conclusion, berberine shows dose-dependent protective effects against CCl₄-induced liver damage by inhibiting oxida-

tive/nitrosative stress and the inflammatory response. Berberine seems to be more potent hepatoprotective drug than silymarin, and its use in maintaining a healthy liver and preventing toxic liver damage deserves consideration and further examination.

Conflicts of interest

The authors have declared no conflicts of interest.

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