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Boschniakia rossica prevents the carbon tetrachloride-induced hepatotoxicity in rat

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ABSTRACT

The present study was undertaken to investigate the hepatoprotective effect of *Boschniakia rossica* extract (BRE), rich in phenylpropanoid glycoside and iridoid glucoside, on CCl_4-induced liver damage. Male Wistar rats were randomly divided into six groups of ten each. While the first group was maintained as normal control, groups II–VI were administered 0.5 ml/kg CCl_4 (model), 100 mg/kg BRE plus CCl_4, 200 mg/kg BRE plus CCl_4, 50 mg/kg silymarin plus CCl_4 and 200 mg/kg BRE, respectively. CCl_4 challenge not only elevated the serum marker enzyme activities and reduced albumin (ALB) level but also increased liver oxidative stress, as evidenced by elevated lipid hydroperoxide (LOOH) and malondialdehyde (MDA) concentrations, combined with suppressed potential of hepatic antioxidative defense system including superoxide dismutase (SOD), glutathione peroxidase (GPx) activities and reduced glutathione (GSH) content. Furthermore, serum tumor necrosis factor-α (TNF-α), hepatic nitrite level, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein contents were elevated while cytochrome P450 2E1 (CYP2E1) expression and function were inhibited. Preadministration of BRE not only reversed the significant changes in serum toxicity markers, hepatic oxidative stress, xenobiotic metabolizing enzymes and proinflammatory mediators induced by CCl_4 but also restored liver CYP2E1 level and function. Interestingly, the protein expression of heme oxygenase-1 (HO-1) was further elevated by BRE treatment, which was markedly increased after CCl_4 challenge. These results demonstrate that BRE exhibits protective effect on CCl_4-induced acute hepatic injury *via*, at least in part, reduced oxidative stress, suppressed inflammatory responses and induced HO-1 protein expression combined with improved CYP2E1 level and function in liver.

Introduction

Carbon tetrachloride (CCl_4) is a well-known hepatotoxin that is widely used to induce toxic liver injury in a range of laboratory animals. CCl_4-induced hepatotoxicity is believed to involve two phases. The initial phase involves the metabolism of CCl_4 by cytochrome P450 to the trichloromethyl radicals ($CCl_3\cdot$ and/or $CCl_3OO\cdot$), which lead to membrane lipid peroxidation and finally to cell necrosis (Basu, 2003; Manibusan et al., 2007). The second phase of CCl_4-induced hepatotoxicity involves the activation of Kupffer cells, which is accompanied by the production of proinflammatory mediators (Planagumà et al., 2005). Several microarray studies have been reported describing gene expression changes caused by acute CCl_4 toxicity (Harries et al., 2001), although the significance of these changes has not been fully understood.

Herbs have attracted a great deal of interest as physiologically functional foods and as a source for the development of drugs. Herbal medicines derived from plant extracts are increasingly being utilized to treat a wide variety of clinical diseases, with relatively little knowledge on their modes of action. So far, there has been considerable interest in the role of complementary and alternative medicines for the treatment of liver diseases (Seeff et al., 2001).

Boschniakia rossica Fedtsch. *et* Flerov (*Orobanchaceae*), a parasitic plant growing on the root of plants of the genus *Alnus* (Betulaceae), is widely used in Chinese traditional medicine as a substitute for *Cistanchis Herba*, a famous staminal tonic agent. It is called an "anti senility herb" in China and is valued for its ability to tonify kidney and strengthen *Yang*. Although the crude extracts of *B. rossica* have been reported to display a variety of pharmacological activities (Yin et al., 2000; Tusda et al., 1994a, 1994b; Shen and Yin, 1999), the tonic principle has not been well elucidated. *B. rossica*, similar to other *Orobanchaceae* plants, contains phenylpropanoid glycoside and iridoid glucoside substances (Lin et al., 2004; Lee et al., 2004) that exhibit a variety of pharmacological activities including anti-inflammatory (Diaz

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et al., 2004), anti-lipid peroxidative and free-radical scavenging activities (Gao et al., 1999). The major constituents of *B. rossica* are the phenylpropanoid glycoside rossicaside B and the iridoid glucosides boschnaloside and 8-epideoxyloganic acid (Konishi and Shoji, 1981; Yin et al., 1999; Lin and Chen, 2004). Based on the data from our laboratory and others, we suggested that *B. rossica* extract (BRE), rich in phenylpropanoid glycoside and the iridoid glucoside, may be effective in protecting the liver against acute CCl_4 toxicity. So far, however, there has been little research reported on the *in vivo* hepatoprotective mechanism of BRE.

To test our hypothesis, a classic CCl_4-induced liver injury model was chosen to study the liver protective effect of BRE in rats. The effect of BRE on liver injury was compared to that of silymarin in this study. Silymarin, a polyphenolic flavonoid isolated from milk thistle (*Silybum marianum*), is used clinically in Europe and Asia for the treatment of liver diseases. Various studies indicate that silymarin exhibits strong antioxidative activity (Müzes et al., 1991; Dehmlow et al., 1996), induces superoxide dismutase (SOD) (Müzes et al., 1991) and increases cellular glutathione (GSH) content (Valenzuela et al., 1989). By inhibiting lipid peroxidation, silymarin protects against hepatic toxicity induced by a wide variety of agents (Letteron et al., 1990; Boigk et al., 1997). In this study, for evaluation of the hepatoprotective mechanisms of BRE, serum tumor necrosis factor-α (TNF-α), liver oxidative stress and antioxidative defense system, hepatic proinflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), heme oxygenase-1 (HO-1) as well as cytochrome P450 2E1 (CYP2E1) protein expression were determined.

Materials and methods

Preparation of the test substances

The whole plant of *B. rossica* was collected at foot of Mt. Changbai, China, and identified and authenticated by Dr. Zongzhu Yin of Yanbian University, Yanji, China. A voucher specimen was deposited in the Herbarium of Institute of Applied Ecology, Chinese Academy of Science. BRE was extracted by a procedure previously described with a slight modification (Konishi and Shoji, 1981; Yin et al., 1999; Lin and Chen, 2004). Briefly, the dried whole plants (10 kg) of *B. rossica* were chopped and extracted with 90% EtOH, and evaporated *in vacuo* to give 1.86 kg of a dark brown powder, which was successively extracted with n-hexane, $CHCl_3$, EtOAc and *n*-BuOH. The *n*-BuOH extract was concentrated and lyophilized to give BRE (yield 12.9%), which was mostly consisted of iridoid glucosides boschnaloside (30.1%) and 8-epideoxyloganic acid (16.6%), as well as phenylpropanoid glycosides rossicaside B (32.2%) according to the HPLC analysis, and is referred to hereafter as BRE.

Animals

Male Wistar rats weighing 160–180 g were obtained from the animal house section of Yanbian University Health Science Center, China. Animals were housed in plastic cages and maintained at 23 ± 1 °C under a natural light–dark cycle in a well-ventilated room. They were fed with standard pellet food and tap water *ad libitum*. The food and water intake was assessed daily for the animals during the treatment. The composition of the control diet was as follows (g/kg): casein 250, corn starch 300, sucrose 250, cellulose powder 50, corn oil 100, mineral mixture 35, vitamin mixture 10, methionine 3 and choline bitartrate 2. The composition of the mineral mixture was according to AIN-93G MX and that of the vitamin mixture to AIN-93G VX (Reeves et al., 1993). The experimental procedures were in accordance with internationally accepted guidelines for animal use and care (EEC Directive of 1986; 86/09/EEC; National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*, revised 1988) with the approval of the local ethics committee.

Experimental design

Rats were randomly divided into 6 groups of ten each. Group I, normal control rats with i.g. saline pretreatment; group II, model group with i.g. saline and CCl_4 treatments; group III, low-dose BRE group with i.g. 100 mg/kg BRE and CCl_4 treatments; group IV, high-dose BRE group with i.g. 200 mg/kg BRE and CCl_4 treatments; group V, silymarin group with i.g. 50 mg/kg silymarin and CCl_4 treatments; group VI, 200 mg/kg BRE pretreatment alone. Silymarin or BRE suspension for intra-gastric administration was prepared by suspending in saline. All rats were pretreated with saline, silymarin or BRE once daily for a period of 7 days. At 1 h after the last pretreatment, 50% CCl_4 in olive oil was given intraperitoneally to rats of groups II–V at a dose of 1 ml/kg of body weight while olive oil was injected to groups I and VI. The dose of BRE treatment was selected based on previous reports (Yin et al., 2000; Shen and Yin, 1999), as well as its efficacy in a D-galactoasmine-induced hepatotoxicity model (Li et al., 2001).

Blood and liver sample preparation

Sixteen hours after administration of CCl_4, rats were anesthetized with sodium pentobarbital (50 mg/kg of body weight, i.p.), and then sacrificed by cervical decapitation. Blood was allowed to clot at room temperature and then centrifuged at 1000*g* for 10 min to obtain serum.

The whole liver was quickly excised, divided into portions and stored at −70 °C until needed. The frozen liver slices were washed in ice-cold 20 mM EDTA solution, blotted, dissected to remove connective tissues, and homogenized in 50 mM phosphate buffer saline (pH 7.2) in an ice bath. The homogenate was centrifuged at 600*g* for 10 min at 4 °C and the supernatant was used for the hepatic biochemical assays and western blot analysis of iNOS and COX-2. Liver microsome for analysis of CYP2E1 and HO-1 was prepared as described previously (Funae and Imaoka, 1985). Liver homogenate was centrifuged at 9000*g* for 20 min and the resulting supernatant was further centrifuged at 105,000*g* for 60 min. The resulting pellet (microsome) was resuspended in 50 mM sodium phosphate buffer (pH 7.4). All procedures were carried out under cold conditions.

Biochemical analysis

Serum marker enzymes, albumin (ALB) and TNF-α assays

The serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and ALB were determined in accordance with methods provided by the diagnostic kits (Nanjing Jiancheng Bioengineering Institute, China). The serum TNF-α was measured using a rat TNF-α ELISA kit (R&D Systems, USA) according to the corresponding protocol. Total protein level was measured by a method modified from that of Lowry et al. using bovine serum albumin as the standard (Lowry et al., 1951). Triplicate assays were performed in each measurement and the average counts were obtained from each individual sample.

Liver oxidative damage assay

Liver lipid hydroperoxide (LOOH) was determined enzymatically using a kit according to the manufacturer's instruction (Kyowa Medex Company Ltd., Japan). Malondialdehyde (MDA), a degrading product of lipid peroxidation known as thiobarbituric acid-reactive substance (TBARS), was determined according to the thiobarbituric acid method using a MDA test kit (Nanjing Jiancheng Bioengineering Institute, China).

Hepatic antioxidative defense potentials

Hepatic antioxidative defense enzymes including SOD, catalase (CAT), glutathione peroxidase (GPx), glutathione-*S*-transferase (GST) and glutathione reductase (GR) were assayed using commercial test kits (Nanjing Jiancheng Bioengineering Institute, China). The activity of SOD resulting in 50% inhibition of the reduction of nitroblue tetrazolium to blue formazon/min/mg of liver protein was defined as 1 unit. One unit of CAT activity was equal to μmol of H_2O_2 released per minute. The GPx activity was defined as μmol of GSH consumed/min/mg of liver protein while GR activity was expressed as μmol of NADPH oxidized/min/mg of liver protein. The GST activity was defined as μmol of 1-chloro-2,4-dinitrobenzene (CDNB) conjugated/min/mg of liver protein. The liver GSH was assayed using commercial test kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

Liver nitrite level

Hepatic nitrite level was estimated enzymatically using commercial test kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

Western blot analysis of hepatic iNOS, COX-2, HO-1 and CYP2E1

The electrophoretic separation of the proteins was performed using 10% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) and then electrotransferred to nitrocellulose membranes (Schleicher and Schuell, Germany), which was immunoblotted with anti-rat CYP2E1, HO-1, COX-2, iNOS or anti-rabbit glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies. Alkaline phosphatase-labeled goat anti-rabbit IgG or horseradish peroxidase conjugated anti-rat IgG were used as the secondary antibodies and the color developed using a mixture of 5-bromo-4-chloro-indolylphosphate and nitroblue tetrazolium. The filter images were captured on a Gel Doc Image Analysis System (Kodak, Japan). The relative levels of protein expressions were normalized to GAPDH after quantitative estimation using NIH Image software (Bethesda, MD) and expressed in arbitrary units.

CYP2E1-specific monooxygenase activity

CYP2E1-specific monooxygenase activity was measured from the rate of oxidation of *p*-nitrophenol (PNP) to *p*-nitrocatechol in the presence of NADPH according to the method of Reinke and Moyer, (1985). The reaction was performed with 0.1 mg of liver microsomal protein for 30 min at 37 °C and the absorbance was determined at 510 nm after neutralization. The results are expressed as nmol/min/mg of protein.

Statistical analysis

Data from experiments were expressed as mean ± S.D. of 10 rats in each group. Statistical analysis was conducted by one-way analysis of variance followed by Tukey's post hoc test using Statistics Package for Social Science 11.5 software (SPSS Inc., USA). $P < 0.05$ was considered statistically significant.

Results

Effect of BRE on serum ALT, AST, ALP and ALB

The effect of BRE pretreatment on the CCl_4-induced modification in serum ALT, AST, ALP and ALB levels is shown in Table 1. A single dose of CCl_4 (0.5 mg/kg) caused hepatotoxicity in rats as indicated by an increase in serum ALT, AST and ALP activities and a decrease in ALB level after CCl_4 administration. Whereas, animals pretreated with BRE exhibited a significant decrease in the activities of these marker enzymes, combined with an elevation in ALB level. Silymarin administration also reversed the alterations of ALT, AST, ALP and ALB levels when compared with the model group. The group VI animals did not show any alteration indicating the nontoxic nature of the BRE.

Effect of BRE on serum TNF-α level

TNF-α is an important cytokine involved in hepatocyte damage in rats with CCl_4-induced fulminant hepatitis. The serum level of TNF-α was low in the normal control animals. However, the serum TNF-α level increased 2.5-fold in the CCl_4-treated animals after the CCl_4 treatment. This increase was reduced in both 200 mg/kg BRE and silymarin supplemented groups (Fig. 1).

Effect of BRE on hepatic oxidative damage

An expected increase of hepatic lipid peroxidative indices in CCl_4-treated model group confirmed that oxidative damage was

Table 1
Effect of BRE on serum ALT, AST, ALP and ALB levels.

Group	ALT (U/L)	AST (U/L)	ALP (U/L)	ALB (g/dl)
I	42.3 ± 12.4	101.2 ± 38.6	151.3 ± 60.1	4.98 ± 0.84
II	120.9 ± 24.7$^{\#}$	244.8 ± 36.7$^{\#}$	401.9 ± 75.2$^{\#}$	3.35 ± 0.55$^{\#}$
III	82.4 ± 14.7*	205.1 ± 56.9	210.5 ± 62.3	3.74 ± 0.57
IV	53.1 ± 12.9*†	191.4 ± 53.7*	178.4 ± 60.4*	4.52 ± 0.74*
V	71.8 ± 20.2*	179.1 ± 48.6*	163.5 ± 53.8*	4.05 ± 0.71
VI	44.1 ± 13.3‡	99.4 ± 30.4‡	159.9 ± 49.6‡	4.83 ± 1.20‡

Data are presented as mean ± SD of ten rats per group. $^{\#}P < 0.05$, significantly different from group I; $^{*}P < 0.05$, significantly different from group II; $^{\dagger}P < 0.05$, significantly different from group III; ‡Nonsignificantly different from group I. Group I, normal control; group II, model (CCl_4 alone); group III, CCl_4+100 mg/kg BRE; group IV, CCl_4+200 mg/kg BRE; group V, CCl_4+50 mg/kg silymarin and group VI, 200 mg/kg BRE alone.

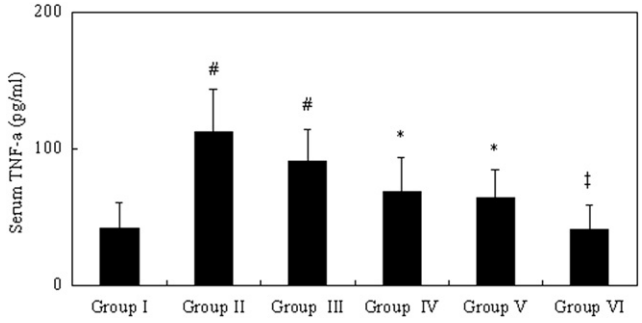

Fig. 1. Effect of BRE on serum TNF-α level. Data are presented as mean ± SD of ten rats per group. $^{\#}P < 0.05$, significantly different from group I; $^{*}P < 0.05$, significantly different from group II; ‡Nonsignificantly different from group I. Group I, normal control; group II, model (CCl_4 alone); group III, CCl_4+100 mg/kg BRE; group IV, CCl_4+200 mg/kg BRE; group V, CCl_4+50 mg/kg silymarin and group VI, 200 mg/kg BRE alone.

induced (Fig. 2). When CCl_4 was injected into rats that were pretreated with BRE or silymarin, levels of LOOH and MDA in liver were significantly reduced as compared with the model group. The observed dose-dependent suppression of oxidative damage in CCl_4-injured liver by BRE administration suggests that BRE is antioxidative and hepatoprotective.

Effect of BRE on hepatic antioxidative defense system

Change was observed in the concentration of GSH, which plays a pivotal role in the non-enzyme antioxidative defense system. Fig. 3 shows the hepatic GSH level was reduced following CCl_4 injection. However, supplementation with 200 mg/kg BRE or silymarin restored the decreased level of GSH caused by CCl_4 injection similar to the normal level, leading to the conclusion that BRE and silymarin helped maintain the same level of GSH despite CCl_4 injection. Beyond the GSH depletion, CCl_4 also induced a substantial modification in the hepatic antioxidative enzyme activities (Table 2). Data showed that the decreased levels of hepatic SOD and GPx activities as the result of CCl_4 injection elevated dose-dependently in BRE and silymarin groups. The high-dose BRE (200 mg/kg) group showed an even higher GPx level than the normal control group. In addition, although the observed differences did not reach statistical significance, the elevated GR and GST activities in BRE groups indicated that, BRE was antioxidative and beneficial for the liver recovery from acute injury. CAT activity also tended to decrease to a small extent after CCl_4 injection, although the difference was not statistically significant as compared with the normal group. Interestingly, CAT activity of BRE and silymarin groups increased remarkably as compared with the CCl_4-treated group to an even higher level than the normal control group.

Effect of BRE on hepatic nitrite level and iNOS protein expression

Hepatic nitrite levels were significantly elevated in all groups of rats receiving CCl_4 compared with normal rats (Fig. 4). However, a less increase in hepatic nitrite level was found in rats treated with BRE or silymarin than in rats receiving CCl_4 alone. CCl_4-induced hepatotoxicity is also accompanied by the production of proinflammatory mediator iNOS. Western blot analysis revealed that the amount of hepatic iNOS protein increased considerably 16 h after the CCl_4 administration. However, the increase in iNOS protein level was attenuated to some extent by BRE (Fig. 5).

Effect of BRE on hepatic COX-2 and HO-1 protein expression

The protein expression of COX-2 and HO-1 in liver also increased after CCl_4 administration. Interestingly, the level of HO-1 protein expression was further elevated by treatment of BRE while COX-2 was downregulated to near-normal level when compared to animals administered with CCl_4 alone. BRE treatment alone did not alter the protein levels of COX-2 and HO-1 (Fig. 5).

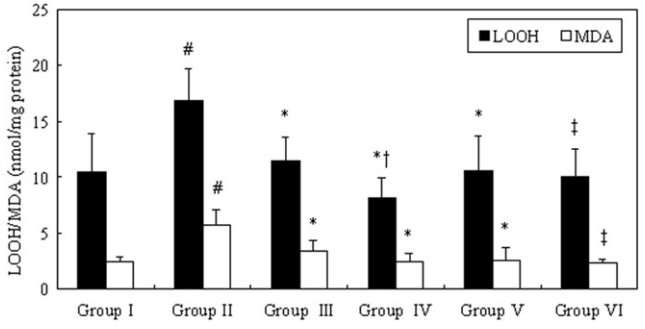

Fig. 2. Effect of BRE on CCl_4-induced hepatic lipid peroxidation. Data are presented as mean ± SD of ten rats per group. $^{\#}P < 0.05$, significantly different from group I; $^{*}P < 0.05$, significantly different from group II; $^{\dagger}P < 0.05$, significantly different from group III; ‡Nonsignificantly different from group I. Group I, normal control; group II, model (CCl_4 alone); group III, CCl_4+100 mg/kg BRE; group IV, CCl_4+200 mg/kg BRE; group V, CCl_4+50 mg/kg silymarin and group VI, 200 mg/kg BRE alone.

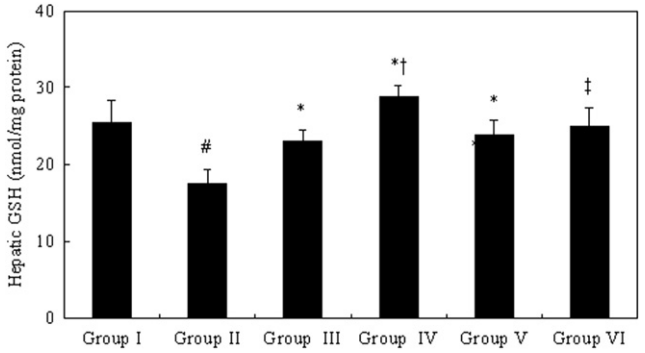

Fig. 3. Effect of BRE on hepatic GSH level. Data are presented as mean ± SD of ten rats per group. $^{\#}P < 0.05$, significantly different from group I; $^{*}P < 0.05$, significantly different from group II; $^{\dagger}P < 0.05$, significantly different from group III; ‡Nonsignificantly different from group I. Group I, normal control; group II, model (CCl_4 alone); group III, CCl_4+100 mg/kg BRE; group IV, CCl_4+200 mg/kg BRE; group V, CCl_4+50 mg/kg silymarin and group VI, 200 mg/kg BRE alone.

Table 2
Effect of BRE on CCl_4-induced alterations of hepatic antioxidant enzyme activities.

Group	SOD	GPx	GR	GST	CAT
I	9.9 ± 2.1	7.9 ± 1.2	6.7 ± 0.5	0.72 ± 0.05	8.0 ± 1.3
II	3.9 ± 1.0$^{\#}$	5.3 ± 0.9$^{\#}$	4.2 ± 0.9$^{\#}$	0.34 ± 0.12$^{\#}$	7.2 ± 1.2
III	6.8 ± 1.3*	7.5 ± 1.0*	5.1 ± 1.4	0.49 ± 0.12	9.8 ± 1.5*
IV	9.6 ± 1.6*†	10.1 ± 1.3*†	5.7 ± 1.3	0.53 ± 0.14	12.5 ± 1.7$^{\#*\dagger}$
V	9.1 ± 2.0*	7.6 ± 0.8*	6.6 ± 1.4*	0.60 ± 0.07*	11.3 ± 1.8*
VI	9.4 ± 1.9‡	7.7 ± 1.0‡	6.8 ± 0.7‡	0.71 ± 0.17‡	8.2 ± 1.1‡

Data are presented as mean ± SD of ten rats per group. Values are expressed as U/mg of protein for SOD, GPx, GR, GST and CAT. $^{\#}P < 0.05$, significantly different from group I; $^{*}P < 0.05$, significantly different from group II; $^{\dagger}P < 0.05$, significantly different from group III; ‡Nonsignificantly different from group I. Group I, normal control; group II, model (CCl_4 alone); group III, CCl_4+100 mg/kg BRE; group IV, CCl_4+200 mg/kg BRE; group V, CCl_4+50 mg/kg silymarin and group VI, 200 mg/kg BRE alone.

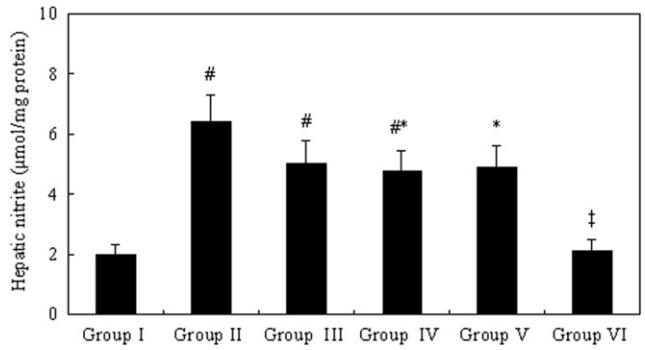

Fig. 4. Effect of BRE on hepatic nitrite level. Data are presented as mean ± SD of ten rats per group. $^{\#}P < 0.05$, significantly different from group I; $^{*}P < 0.05$, significantly different from group II; ‡Nonsignificantly different from group I. Group I, normal control; group II, model (CCl_4 alone); group III, CCl_4+100 mg/kg BRE; group IV, CCl_4+200 mg/kg BRE; group V, CCl_4+50 mg/kg silymarin and group VI, 200 mg/kg BRE alone.

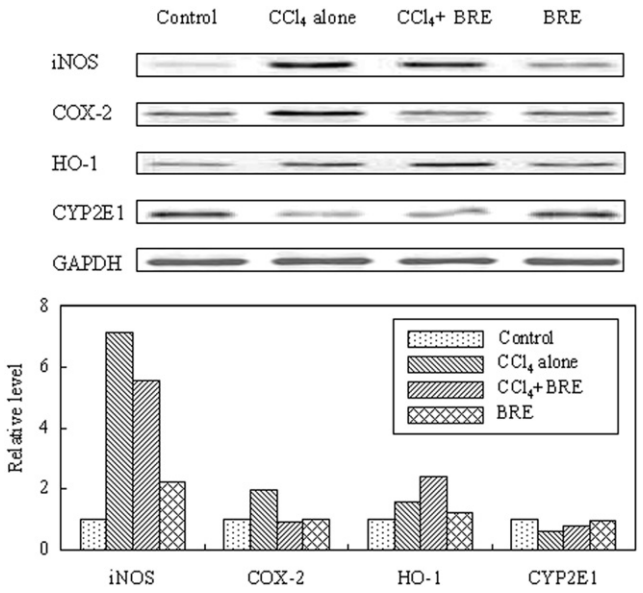

Fig. 5. Western blot analysis of hepatic iNOS, COX-2, HO-1 and CYP2E1. Data are presented as mean ± SD of four rats per group. Group I, normal control; group II, model (CCl_4 alone); group III, CCl_4+200 mg/kg BRE; group VI, 200 mg/kg BRE alone.

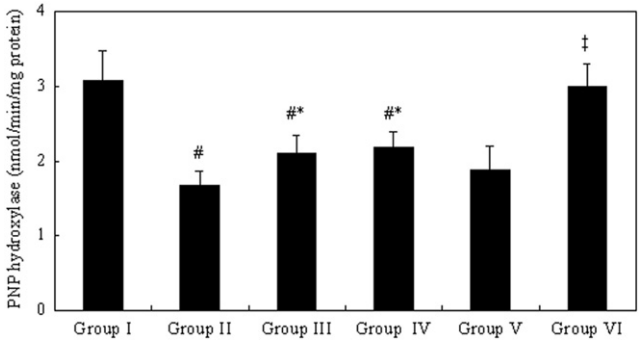

Fig. 6. Effect of BRE on hepatic CYP2E1-specific monooxygenase activity. Data are presented as mean ± SD of ten rats per group. $^{\#}P < 0.05$, significantly different from group I; $^{*}P < 0.05$, significantly different from group II; $^{\dagger}P < 0.05$, significantly different from group III; ‡Nonsignificantly different from group I. Group I, normal control; group II, model (CCl_4 alone); group III, CCl_4+100 mg/kg BRE; group IV, CCl_4+200 mg/kg BRE; group V, CCl_4+50 mg/kg silymarin and group VI, 200 mg/kg BRE alone.

Effect of BRE on hepatic CYP2E1 protein expression and function

The effect of BRE on the hepatic microsomal CYP2E1-specific monooxygenase activity was examined. As shown in Fig. 6, hepatic microsomal fractions from the rats pretreated with BRE significantly decreased the hydroxylation of PNP in a dose-dependent manner. Immunoblot analysis was performed to examine the effect of BRE on CYP2E1 protein expression. Compared to normal control rats, rats receiving CCl_4 alone showed a remarkable decrease in CYP2E1 protein level. In contrast, in rats pretreated with 200 mg/kg BRE, a little higher level of CYP2E1 was found as compared with rats intoxicated with CCl_4 alone (Fig. 6). This result was consistent with the elevated microsomal PNP hydroxylase activities in BRE groups. The restoration of CYP2E1 protein content and function by BRE supplementation implies that BRE could have a hepatoprotective effect, which led to rapid recovery from CCl_4-induced liver injury.

Discussion

The antioxidative and free-radical scavenging activities of many substances have been assessed, and many substances that possess anti-hepatotoxic activity also show strong antioxidative activity (Hwang et al., 2009). *B. rossica* exhibits a number of beneficial effects against various types of degenerative diseases in humans, largely because its major ingredients, phenylpropanoid glycosides and iridoid glucosides, have potent antioxidative activity (Konishi and Shoji, 1981; Yin et al., 1999; Lin and Chen, 2004). The present *in vivo* study has demonstrated the hepatoprotective potential of BRE rich in phenylpropanoid and iridoid compounds.

Liver injury induced by CCl_4 is a common model for screening the hepatoprotective activity of drugs because this chemical is a potent hepatotoxin and a single exposure can rapidly lead to severe hepatic necrosis and steatosis (Recknagel et al., 1989; Manibusan et al., 2007). Increases in serum ALT, AST and ALP levels by CCl_4 have been attributed to hepatic structural damage because these enzymes are normally localized to the cytoplasm and released into the circulation after cellular damage has occurred (Recknagel et al., 1989). Our study showed that serum ALT, AST, ALP and ALB levels rapidly increased in parallel with CCl_4 injection, indicating the induction of acute hepatotoxicity by CCl_4. However, serum ALT, AST and ALP activities significantly declined in BRE-supplemented groups while ALB was elevated, suggesting that BRE is beneficial for liver regeneration to reverse liver injury.

Lipid peroxidation is one of the principal causes of CCl_4-induced liver injury and is mediated by the free-radical derivatives of CCl_4. In addition, the antioxidative activity and/or the inhibition of free-radical generation are important in terms of protecting the liver from CCl_4-induced damage (Manibusan et al., 2007). Thus, antioxidative enzymes also play an important role in the detoxification of xenobiotics, catalyzing their conjugation with reduced GSH. In this study, exposure to CCl_4 caused GSH depletion and decreased activities of SOD, GPx, GST and GR, as well as an increased level of lipid peroxidation in liver, implying down-regulation of numerous antioxidative reactions in liver. BRE-pretreated animals showed a significant reduction in the levels of hepatic peroxidative markers with concomitant improvement in the hepatic antioxidative defense system. This suggests that BRE may be able to protect against the oxidation of hepatic cellular membrane damage *via* a free-radical scavenging property.

The liver is a major inflammatory organ, and inflammatory processes contribute to a number of pathological events after exposure to various hepatotoxins. Kupffer cells release proinflammatory mediators either in response to necrosis or as a direct action by the hepatotoxin, activated, which are believed to aggravate CCl_4-induced hepatic injury (Badger et al., 1996). TNF-α, a pleiotropic proinflammatory cytokine, is rapidly produced by macrophages in response to tissue damage (Wu et al., 2007). While low levels of TNF-α may play a role in cell protection, excessive amounts cause cell impairment. It is known that TNF-α also stimulates the release of cytokines from macrophages and induces the phagocyte oxidative metabolism and nitric oxide production. Nitric oxide is a highly reactive oxidant that is produced through the action of NOS and plays an important role as a vasodilator, neurotransmitter and in the immunological system as a defense against tumor cells, parasites and bacteria (Lee et al., 2007). The role of nitric oxide in liver damage remains controversial. Although several studies have found that nitric oxide protected against CCl_4-induced liver injury (Morio et al., 2001), certain evidences have found that excessive nitric oxide production by iNOS may lead to hepatic damage (Lee et al., 2003; Nadler et al., 2001; Inoue et al., 2000). In addition, COX-2 is the rate-limiting enzyme and responsible for the catalysis of prostaglandin E2 (PGE2) from arachidonic acid (Chun and Surh,

2004). Chang et al. (2006) noted that the induction of COX-2 activity and subsequent generation of PGE2 are closely related to the NO production. In other words, the overproduction of PGE2 mediated by COX-2 has been linked to the development of inflammation and carcinogenesis (Park et al., 2004). The current study confirmed significant increases in hepatic nitrite, iNOS and COX-2 production as well as serum TNF-α level after CCl_4 administration. These alterations were attenuated by BRE treatment, which suggests that BRE suppressed TNF-α, iNOS and COX-2 protein secretion and/or enhanced the degradation of these proteins. Accordingly, the possible mechanism of protection against CCl_4-induced hepatotoxicity appears to be, at least in part, due to the suppressed inflammatory responses.

HO-1 is a rate-limiting enzyme in the catabolism of heme and a heat shock protein (HSP32). Excessive oxidative stress has been suggested as a reason for the upregulation of HO-1, as this enzyme is known to be readily inducible upon such stressors (Otterbein and Choi, 2000). By the equimolar production of the antioxidant bilirubin, free iron and vasodilative carbon monoxide, HO-1 represents a cytoprotective enzyme and, when expressed, produces therapeutic benefits in a number of different conditions and diseases, such as sepsis, inflammation and ischemia/reperfusion injury (Hwang et al., 2009). In line with this, HO-1 induction has been shown to confer protection in CCl_4-induced hepatotoxicity. In our study, the expression of HO-1 protein was significantly increased after CCl_4 treatment. This is in concordance to the results reported by Nakahira et al. (2003). Additionally, pretreatment with BRE further augmented HO-1 protein expression following CCl_4 treatment, which suggests that a strong inductive response of HO-1 by BRE is to protect liver cells from CCl_4-induced oxidative cellular injuries.

Generally, CCl_4 is metabolized to highly reactive trichloromethyl free radicals mainly by CYP2E1 (Recknagel et al., 1989). However, the trichloromethyl radicals may bind either at the heme group of CYP or at the active site of the enzyme near the heme group, leading to the inactivation CYP pathways (Fernandez et al., 1982). In the present study, a considerable decrease in hepatic CYP2E1 protein level and activity was found in rats treated with CCl_4 alone. In contrast, in rats pretreated with BRE, CYP2E1 protein content and function was less suppressed following CCl_4 administration. This result suggests that BRE exerted a beneficial effect on hepatic CYP2E1 restoration in this animal model. Similar observations regarding a decrease in CYP2E1 protein content in rats treated with CCl_4 (Lee et al., 2003) was reported previously. It is possible that BRE may restore the hepatic CYP2E1 function and lead to a rapid recovery from CCl_4-induced liver injury. However, it is also possible that administration of BRE caused a reduction of CCl_4-related hepatotoxicity that lead to an early recovery of CYP2E1 level in rats receiving BRE, which would lead to an elevation of the enzyme activity. Further studies may be needed to elucidate this phenomenon.

In summary, this study demonstrates that BRE had a protective effect against CCl_4-induced acute hepatic damage in rats. The hepatoprotective effect of BRE is likely due to its ability to restore the CYP2E1 function, induce the HO-1 expression and suppress the inflammatory responses, in combination with the ability to scavenge free radicals.

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