

Hepatoprotective effect of polysaccharides from *Boschniakia rossica* on carbon tetrachloride-induced toxicity in mice

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The purpose of this study was to investigate the protective effect of polysaccharides from *Boschniakia rossica* against hepatotoxicity induced by carbon tetrachloride (CCl₄). *Boschniakia rossica* polysaccharides was administered intragastrically once daily for 7 days. One hour after the final treatment, mice were treated intraperitoneally with 80 mg/kg of CCl₄. CCl₄-induced hepatotoxicity was manifested by increased levels of serum marker enzymes and hepatic lipid peroxidation, and by decreased potential of hepatic antioxidative defense system. CCl₄ challenge also resulted in elevated serum tumor necrosis factor- α and hepatic nitric oxide level, and up-regulation of inducible nitric oxide synthase and cyclooxygenase-2 proteins of liver tissue. Pretreatment of mice with *Boschniakia rossica* polysaccharides reversed these altered parameters of mice with liver injury induced by CCl₄. Furthermore, caspase-3 cleavage and activities, and DNA fragmentation of liver in mice treated with *Boschniakia rossica* polysaccharides were decreased than mice treated with CCl₄ alone. Hepatoprotective effect of *Boschniakia rossica* polysaccharides was further demonstrated by histopathological examination of liver sections. The results indicate that *Boschniakia rossica* polysaccharides play a protective role in CCl₄-induced acute liver injury and the hepatoprotective effect of *Boschniakia rossica* polysaccharides may be due to elevated antioxidative defense potentials, suppressed inflammatory responses and apoptosis of liver tissue.

Key Words: *Boschniakia rossica*, polysaccharides, carbon tetrachloride, hepatotoxicity, mice

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 treatment of liver diseases.⁽¹⁾

Boschniakia rossica Fedtsch. et Flerov (*Orobanchaceae*) is a parasitic plant growing on the root of plants of the genus *Alnus* (*Betulaceae*).⁽²⁾ It is widely used in Chinese traditional medicine as a substitute for *Cistanchis Herba*, and is called an “anti-senility herb”. Although the crude extracts of *Boschniakia rossica* have been reported to display a variety of pharmacological activities,⁽²⁻⁷⁾ the tonic principle has not been well elucidated. So far, the chemical constituent research had led to the isolation and identification of three major groups of compounds, namely phenylpropanoids, iridoids and polysaccharides,⁽⁸⁻¹²⁾ and the antioxidative and hepatoprotective effects of the first two constituents have been demonstrated in animal models in our previous work.^(2,5)

Nowadays, plant polysaccharides have also drawn a great deal of attention of the pharmacologist for their various biological properties. Polysaccharides from *Boschniakia rossica* (BRPS) had been reported to have free-radical scavenging activity,⁽¹³⁾ anti-tumor and immunologic function,⁽⁷⁻¹²⁾ and have characteristics of non-toxicity.⁽¹⁴⁾ Based on the data from our laboratory and others, we suggested that BRPS may be effective in protecting the liver against the acute carbon tetrachloride (CCl₄) toxicity.⁽⁷⁻¹⁴⁾ However, the hepatoprotective function of BRPS in animal model of liver injury is not well understood so far.

CCl₄, a well-known model compound for producing chemical hepatic injury, requires biotransformation by hepatic microsomal cytochrome P450 to produce the trichloromethyl free radicals,^(15,16) which can react with sulfhydryl groups, such as reduced glutathione (GSH) and protein thiols. Covalent binding of trichloromethyl free radicals to cell proteins is considered to be the initial step in a chain of events that eventually lead to membrane lipid peroxidation and finally to cell apoptosis and necrosis.⁽¹⁵⁻¹⁷⁾ High levels of reactive oxygen species (ROS) damage cells and are involved in several human pathologies, including liver cirrhosis and fibrosis. Therefore, the use of compounds with antioxidant properties may prevent or alleviate many diseases associated with ROS. The second phase of CCl₄-induced hepatotoxicity involves the activation of Kupffer cells, which is accompanied by the production of proinflammatory mediators.⁽¹⁸⁾ Several microarray studies have been reported describing gene expression changes caused by acute CCl₄ toxicity,⁽¹⁹⁾ although the significance of these changes has not been fully understood.

In the present study, we investigated the protective effect of BRPS against CCl₄-induced hepatotoxicity and sought to determine how BRPS protects mouse liver from CCl₄-induced damage. The effect of BRPS on liver injury was compared to that of silymarin, which is used clinically in Europe and Asia for the treatment of liver diseases. Various studies indicate that silymarin exhibits a strong antioxidant activity,⁽²⁰⁾ and protects against hepatic toxicity by inhibiting lipid peroxidation.⁽²¹⁾ For evaluation of the hepatoprotective mechanisms of BRPS, liver oxidative damage and antioxidant defense potential, proinflammatory mediators such as serum tumor necrosis factor- α (TNF- α), hepatic nitric oxide (NO), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), as well as caspase-3 activation were determined in this study.

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Materials and Methods

Reagents and chemicals. Olive oil and CCl₄ were obtained from Sigma Chemical Company, St. Louis, MO, and TNF- α ELISA kit was from BD Bioscience, San Jose, CA. Diagnostic kits to measure serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), malondialdehyde (MDA), reduced GSH, glutathione reductase (GR), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione-S-transferase (GST) and NO were obtained from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. A Wizard genomic DNA purification kit and caspase-3 activity assay kit were obtained from Kaiji Biotechnology Institute (Nanjing, China). Antibodies against mouse COX-2, caspase-3, rabbit glyceraldehyde 3-phosphate dehydrogenase (GAPDH), horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, antibody against mouse iNOS was from Transduction Laboratories, Franklin Lakes, NJ. All other chemicals were of analytical grade.

Preparation of test substance. The whole plant of *Boschniakia 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. The preparation of BRPS was performed as previously described.^(11,12) Briefly, BRPS was extracted by hot water from the whole plants of *Boschniakia rossica*, followed by ethanol precipitation. The precipitate collected by centrifugation was further separated with ethanol fractionation process, and washed with absolute ethanol, acetone and ether, respectively. Then the precipitate was deproteinated by a combination of proteinase digestion and the Sevag method, followed by exhaustive dialysis with water. The concentrated dialyzate was lyophilized to give BRPS with a yield of 4.37% (w/w) in terms of the whole plant. The total carbohydrate and protein contents of BRPS were determined by the phenol-sulfuric acid method and kjeldahl method, respectively.^(8,16) BRPS contained 67.3% total sugar and 3.11% protein.

Animals and treatment. Male ICR mice weighing 18–20 g were obtained from the animal house section of Yanbian University Health Science Center, China. Animals were housed in a controlled environment at 22 \pm 2°C and a humidity of 50 \pm 10% with 12 h light cycle. Food and tap water were supplied *ad libitum*. The food and water intake was recorded daily throughout the experiment. The composition of the mineral mixture was according to AIN-93G MX and that of the vitamin mixture to AIN-93G VX.⁽²²⁾ 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), which are in agreement with the Helsinki declaration, with the approval of the local ethics committee.

Mice were randomly assigned to 6 groups of 10 each. Group I, the normal control mice with an intragastric (i.g.) saline pretreatment; group II, the model group with an i.g. saline and CCl₄ treatment; group III, the low-dose BRPS group with an i.g. 100 mg/kg BRPS and CCl₄ treatment; group IV, the high-dose BRPS group with an i.g. 200 mg/kg BRPS and CCl₄ treatment; group V, the silymarin group with an i.g. 50 mg/kg silymarin and CCl₄ treatment; group VI, 200 mg/kg BRPS pretreatment alone. The BRPS and silymarin suspension for intragastric administration was prepared by suspending in saline. All mice were pretreated with saline, BRPS and silymarin suspension once daily for a period of 7 days. The dose of BRPS treatment were selected based on our previous experiment and other reports.^(2,5–7,18) CCl₄ injection was performed on day 3 and 7, respectively. On test days, at 1 h after the pretreatment, CCl₄ dissolved in olive oil (1% V/V,

5 ml/kg) was given intraperitoneally (i.p.) to mice of group II-V at a dose of 80 mg/kg of body weight while olive oil was injected to group I and VI. Eight, sixteen or twenty-four hours after CCl₄ administration, mice were anesthetized with ether, and then sacrificed by cervical decapitation. The liver was quickly excised, divided into portions and stored at –80°C until needed.

Serum biochemistry. ALT, AST and ALP were determined in accordance with the methods provided by the diagnostic kits. The serum TNF- α was measured using a mouse TNF- α ELISA kit according to the corresponding protocol.

Histopathological examinations. Fresh liver tissues, trimmed to a thickness of 3 mm, were placed in plastic cassettes and immersed in neutral buffered formalin for 20 h. The fixed tissues were processed routinely, and then embedded in paraffin, sectioned, deparaffinized, and rehydrated using the standard techniques. The extent of the carbon tetrachloride-induced necrosis was evaluated by assessing the morphological changes in the liver sections stained with hematoxylin and eosin (HE), using the standard techniques.

Preparation of hepatic homogenate. The liver slices were washed in ice-cold 20 mM EDTA solution, blotted, dissected to remove connective tissues, and homogenized in 50 mM phosphate-buffered 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. The total protein level was measured by the Bradford method with bovine serum albumin as the standard.⁽²³⁾

Determination of hepatic lipid peroxidation, antioxidant defense potential and NO level. A degrading product of lipid peroxidation known as thiobarbituric acid-reactive substance (TBARS) was determined according to the thiobarbituric acid methods using an MDA test kit. Hepatic antioxidative enzymes including SOD, CAT, GPx, GST and GR, as well as the liver GSH were assayed by using commercial test kits. The activity of SOD resulting in 50% inhibition of the reduction of nitro blue tetrazolium to blue formazon/min/mg of liver protein is defined as 1 unit. One unit of CAT activity is equal to μ mol of H₂O₂ released per minute. The GPx activity is defined as μ mol of GSH consumed/min/mg of liver protein while GR activity is expressed as μ mol of oxidized glutathione (GSSG) reduced/min/mg of liver protein. The GST activity was defined as μ mol of 1-chloro-2,4-dinitrobenzene conjugated/min/mg of liver protein. Hepatic NO level was also estimated enzymatically using commercial test kits according to the manufacturer's instructions.

Western blot analysis of hepatic iNOS, COX-2 and cleaved caspase-3. 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-mouse COX-2, iNOS, cleaved caspase-3 or anti-rabbit GAPDH antibodies. Horseradish peroxidase conjugated goat anti-rabbit IgG or anti-mouse 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 Kodak Gel Doc Image Analysis System (Tokyo, Japan). The relative levels of protein expressions were normalized to GAPDH after quantitative estimation by using NIH Image software (Bethesda, MD) and expressed in arbitrary units.

Determination of activation of hepatic caspase-3. Activation of caspase-3 in liver tissue was quantified by colorimetric assay, using commercial test kits according to the manufacturer's instructions. The relative ratios of caspase-3 activity were normalized to normal control.

Detection of DNA fragmentation. The DNA fragmentation was assayed by agarose gel electrophoresis. Genomic DNA was extracted from liver tissues using a genomic DNA purification kit according to the manufacturer's protocol. Extracted DNA

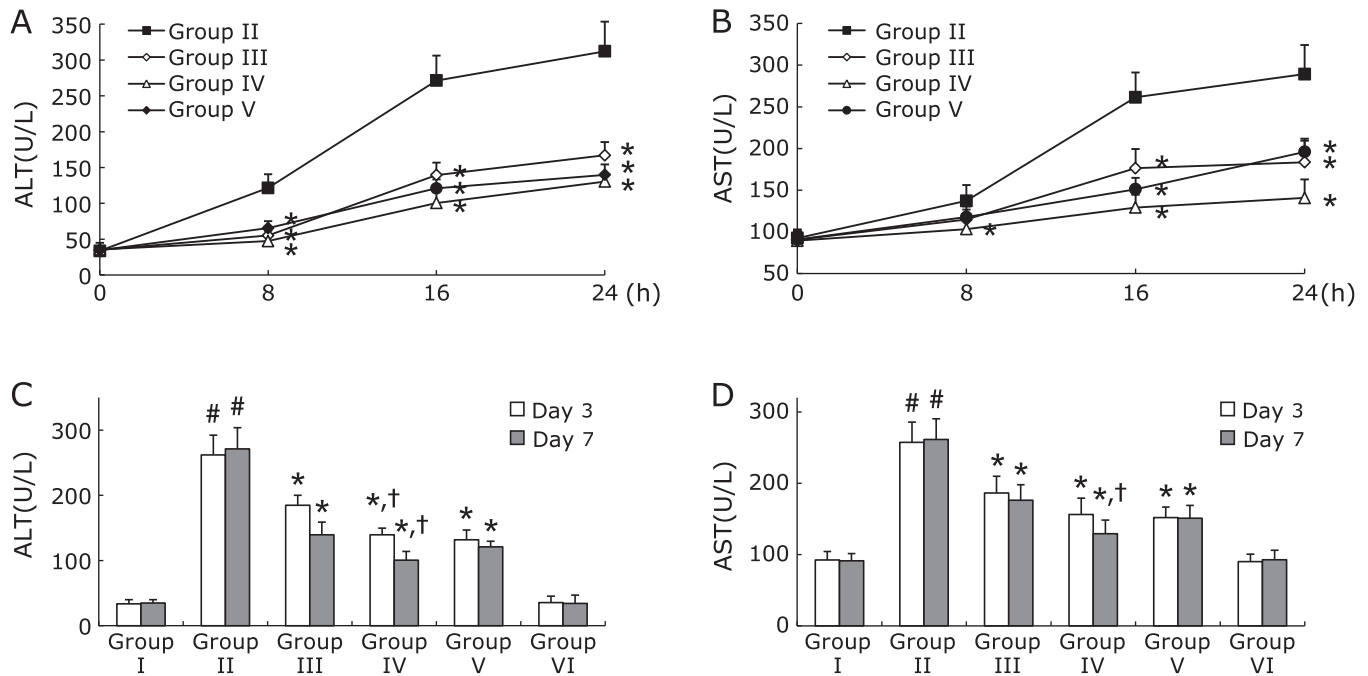


Fig. 1. BRPS alleviated CCl₄-induced hepatotoxicity dose- and time-dependently. The animals were pretreated with BRPS, silymarin or a vehicle. Blood samples were harvested at 8 h, 16 h or 24 h after CCl₄ exposure on day 7, and the serum levels of ALT (A) and AST (B) were determined. In another set of animals, BRPS or silymarin were administered on a daily basis for 3 and 7 days, respectively. Blood samples were harvested at 16 h after CCl₄ exposure and the serum levels of ALT (C) and AST (D) were determined. #*p*<0.05, significantly different from group I; **p*<0.05, significantly different from group II; †*p*<0.05, significantly different from group III. Group I: normal control; group II: model (CCl₄ alone); group III: CCl₄ plus BRPS (100 mg/kg); group IV: CCl₄ plus BRPS (200 mg/kg); group V: CCl₄ plus silymarin (50 mg/kg); group VI: BRPS (200 mg/kg) alone.

was subjected to electrophoresis on 1.5% agarose gel containing 0.5 mg/ml ethidium bromide. DNA fragmentation pattern was photographed under ultraviolet illumination.

Statistical analysis. Data from experiments are expressed as mean ± SEM of 10 mice in each group. Statistical analysis was conducted by a one-way analysis of variance followed by Tukey's post hoc test using SPSS ver. 11.5 (SPSS Inc., Chicago, IL). The significance level for all analyses was set at probability of less than or equal to 0.05.

Results

As expected, serum ALT and AST levels, which are quantitative biochemical markers of hepatocellular damage, increased obviously after CCl₄ exposure. A time-course study, which measured serum ALT and AST levels at 8, 16 or 24 h after the CCl₄ injection on the 7th day, indicated that serum levels of ALT and AST were the highest about 16 to 24 h after the CCl₄ injection (Fig. 1 A and B). Therefore, mice were sacrificed 16 h after CCl₄ injection to obtain blood to determine biochemical parameters in the experiment.

To compare the effect of BRPS feeding time on CCl₄-induced liver injury, BRPS was administered via intragastric route on a daily basis for 3 and 7 days, respectively. In mice of group III and IV, oral administration of BRPS at doses of 100 and 200 mg/kg prior to CCl₄ challenge was observed to dose-dependently reverse the CCl₄-induced alterations of AST by 34% and 54%, of ALT by 43% and 61% on day 3, and of AST by 52% and 72%, of ALT by 56% and 78% on day 7, respectively (Fig. 1 C and D). Since the hepatoprotective effect of BRPS was becoming more pronounced at later time point (day 7), we performed the remainder of our studies according to 7-day feeding regimen.

Fig. 2B showed a typical feature of CCl₄-induced hepatic injury, such as steatosis, invasion of lymphocytes, and necrosis of

centrilobular hepatocytes. However, the hepatic lesions induced by CCl₄ were remarkably alleviated or prevented by the administration with BRPS or silymarin, which is in good agreement with the results of the serum marker enzymes. BRPS treatment alone did not cause any alterations of serum biomarkers of liver injury or a change in the liver histology, indicating the nontoxic nature of the BRPS.

The protective effect of BRPS pretreatment on the CCl₄-induced elevation of serum ALP is presented in Fig. 3. A single dose of CCl₄ caused hepatotoxicity in mice, as indicated by the significant elevation of serum ALP activity. However, pretreatment with BRPS significantly prevented the elevation of serum ALP activity. Silymarin administration also reversed the alteration of ALP activity when compared with the model group.

Fig. 4 showed that the administration of CCl₄ induced hepatotoxicity with a marked increase in serum TNF-α level while vehicle-treated mice had a normal level. However, pretreatment with BRPS or silymarin reduced serum TNF-α level to 36%, 50% and 42%, respectively, of those in CCl₄-treated mice. There was no significant difference in the serum TNF-α levels in mice treated with BRPS alone when compared with the normal mice.

In order to evaluate the effect of BRPS pretreatment on CCl₄-induced liver lipid peroxidation, we monitored the concentration of TBARS, an end product of lipid peroxidation, in the mice. Consistent with the observed attenuation of CCl₄-induced increase in serum marker enzymes, BRPS also reduced CCl₄-induced TBARS levels to the control level (Fig. 5). The observed suppression of oxidative damage in the CCl₄-injured liver by BRPS administration suggests that BRPS was antioxidative and hepatoprotective. The effects were comparable with that of silymarin.

As the oxidative stress of tissue generally involves the GSH system, we measured the level of GSH in each group of livers. Whereas the administration of CCl₄ significantly decreased GSH, pretreatment with BRPS significantly and dose-dependently pro-

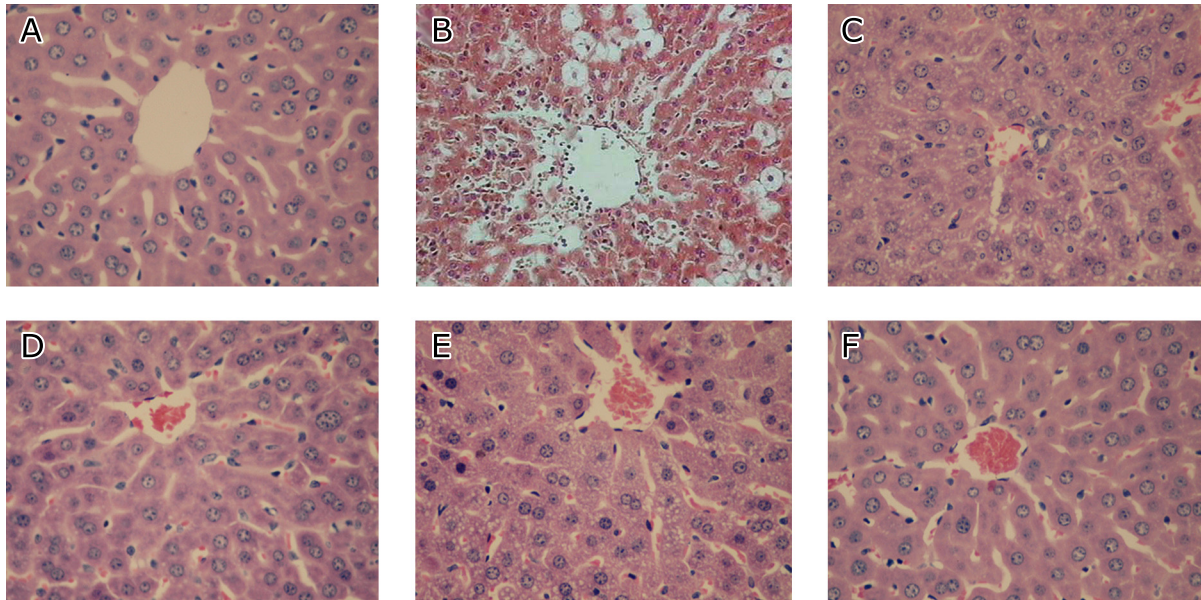


Fig. 2. BRPS improved liver histological alterations in CCl₄-challenged mice. Liver sections were stained with hematoxylin-eosin for morphological evaluation (×400). The animals were pretreated with BRPS, silymarin or a vehicle. CCl₄ was given i.p. on the 7th day of the treatment protocol, and the animals were sacrificed 16 h after CCl₄ administration. (A) Normal control; (B) CCl₄ model; (C) CCl₄ plus BRPS (100 mg/kg); (D) CCl₄ plus BRPS (200 mg/kg); (E) CCl₄ plus silymarin (50 mg/kg); (F) BRPS (200 mg/kg) alone.

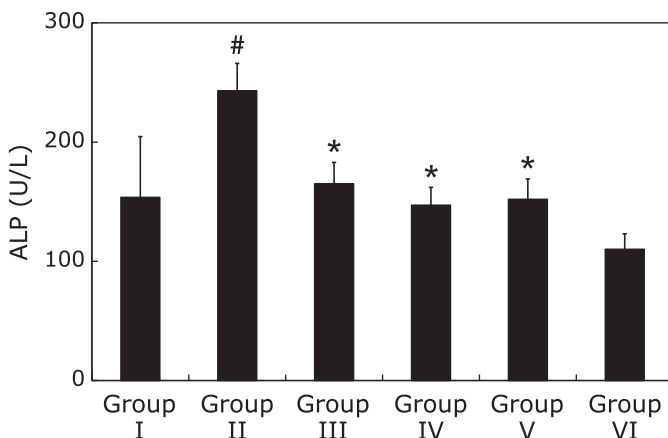


Fig. 3. BRPS reduced serum ALP activities in CCl₄ exposed mice. Data are presented as mean ± SEM of ten mice per group. The animals were pretreated with BRPS, silymarin or a vehicle. CCl₄ was given i.p. on the 7th day of the treatment protocol, and the animals were sacrificed 16 h after CCl₄ administration. [#]*p*<0.05, significantly different from group I; ^{*}*p*<0.05, significantly different from group II. Group I: normal control; group II: model (CCl₄ alone); group III: CCl₄ plus BRPS (100 mg/kg); group IV: CCl₄ plus BRPS (200 mg/kg); group V: CCl₄ plus silymarin (50 mg/kg); group VI: BRPS (200 mg/kg) alone.

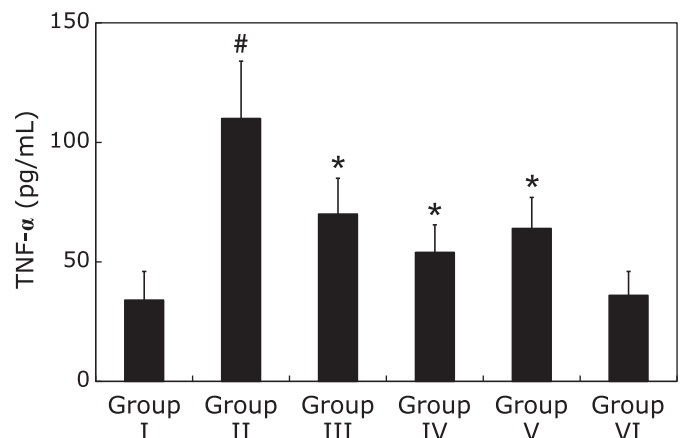


Fig. 4. BRPS reduced serum TNF-α contents in CCl₄ exposed mice. Data are presented as mean ± SEM of ten mice per group. The animals were pretreated with BRPS, silymarin or a vehicle. CCl₄ was given i.p. on the 7th day of the treatment protocol, and the animals were sacrificed 16 h after CCl₄ administration. [#]*p*<0.05, significantly different from group I; ^{*}*p*<0.05, significantly different from group II. Group I: normal control; group II: model (CCl₄ alone); group III: CCl₄ plus BRPS (100 mg/kg); group IV: CCl₄ plus BRPS (200 mg/kg); group V: CCl₄ plus silymarin (50 mg/kg); group VI: BRPS (200 mg/kg) alone.

ected against GSH depletion induced by CCl₄ (Table 1). Remarkable change was also observed in the activities of the hepatic antioxidative enzymes, except for GST (Table 1). The data show that the decreased levels of hepatic GR, SOD and GPx activities as the result of CCl₄ injection were significantly elevated in the BRPS and silymarin groups. The high-dose BRPS (200 mg/kg) group showed an even higher GPx level than the normal control group. CAT activity also tended to decrease to a small extent after the CCl₄ injection, though the difference was not statistically significant as compared with the normal group. However, CAT

activity of the BRPS and silymarin groups increased remarkably as compared with the CCl₄-treated group, to an even higher level than the normal control group. These results showed that the protection afforded by BRPS against CCl₄-induced hepatotoxicity may be related to the increased hepatic GSH content and antioxidative enzyme activities.

Fig. 6 shows that the hepatic NO was significantly elevated in all groups of mice receiving CCl₄ as compared to the normal mice. However, the increase in hepatic NO was attenuated to some extent by BRPS and silymarin. The protein expression of iNOS

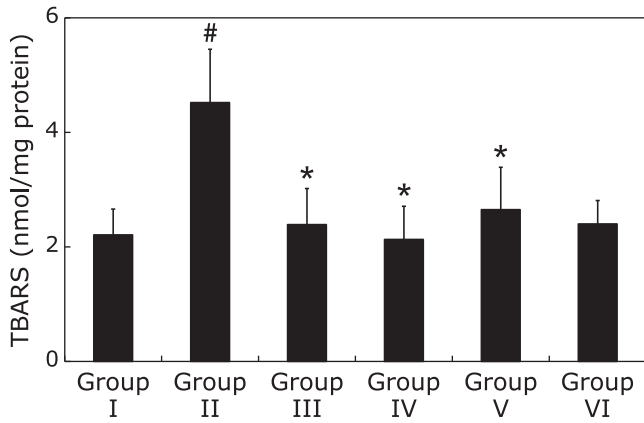


Fig. 5. BRPS reduced hepatic TBARS contents in CCl₄ exposed mice. Data are presented as mean \pm SEM of ten mice per group. The animals were pretreated with BRPS, silymarin or a vehicle. CCl₄ was given i.p. on the 7th day of the treatment protocol, and the animals were sacrificed 16 h after CCl₄ administration. [#]*p*<0.05, significantly different from group I; ^{*}*p*<0.05, significantly different from group II. Group I: normal control; group II: model (CCl₄ alone); group III: CCl₄ plus BRPS (100 mg/kg); group IV: CCl₄ plus BRPS (200 mg/kg); group V: CCl₄ plus silymarin (50 mg/kg); group VI: BRPS (200 mg/kg) alone.

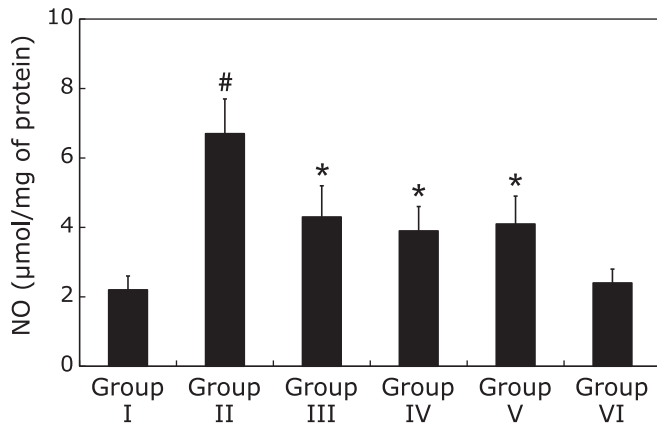


Fig. 6. BRPS reduced hepatic NO contents in CCl₄ exposed mice. Data are presented as mean \pm SEM of ten mice per group. The animals were pretreated with BRPS, silymarin or a vehicle. CCl₄ was given i.p. on the 7th day of the treatment protocol, and the animals were sacrificed 16 h after CCl₄ administration. [#]*p*<0.05, significantly different from group I; ^{*}*p*<0.05, significantly different from group II. Group I: normal control; group II: model (CCl₄ alone); group III: CCl₄ plus BRPS (100 mg/kg); group IV: CCl₄ plus BRPS (200 mg/kg); group V: CCl₄ plus silymarin (50 mg/kg); group VI: BRPS (200 mg/kg) alone.

and COX-2 in liver also increased after the CCl₄ administration, while it was down-regulated by BRPS or silymarin pretreatment (Fig. 7). BRPS treatment alone did not alter the levels of NO, iNOS and COX-2 contents of liver tissue.

To further investigate the protective mechanism of BRPS on CCl₄-induced liver injury, we examined caspase-3 activation in liver tissues. As shown in Fig. 8 and 9, increases in levels of caspase-3 activities and cleaved caspase-3 protein resulted from CCl₄ administration. However, pretreatment with BRPS or silymarin significantly suppressed the CCl₄-induced cleavage and activation of caspase-3. No changes in caspase-3 cleavage and activation resulted from BRPS treatment alone.

DNA fragmentation was detected in liver tissues of the CCl₄ treated group, while a reduced level of DNA fragmentation were observed in the groups pretreated with BRPS or silymarin (Fig. 10). No DNA fragmentation was induced by BRPS in the absence of CCl₄ treatment.

Discussion

It has been recognized that oxidative stress and generation of free radicals play a critical role in CCl₄-induced liver injury. Therefore, some natural products with antioxidant activity have attracted great attention as potential functional ingredients to protect CCl₄-induced liver injury.^(5,20,21,25) Polysaccharides, important natural compounds widely existed in plants, animals and microorganism, have been demonstrated to possess potent anti-oxidative, anti-inflammatory activities and to protect liver injury induced by various chemicals.^(26–28) *Boschniakia rossica* exhibits a number of beneficial effects against various types of degenerative diseases in humans, largely because its major ingredients, such as polysaccharides, phenylpropanoids and iridoids, have potent antioxidant activity.^(2–5,13) Previous reports exhibited the potential prospects of BRPS as functional ingredient to prevent the inflammatory and ROS related diseases.^(6–8) Therefore, we considered that BRPS is useful in the prevention of liver injuries induced by oxidative stress. In the present study, the capability of BRPS to protect against CCl₄-induced hepatotoxicity was firstly investigated.

The liver injury induced by CCl₄ is the best characterized system for xenobiotic-induced hepatotoxicity and are commonly used models for screening of the anti-hepatotoxic and/or hepatoprotective activities of drugs.^(15,24) Our study showed that serum ALT, AST, and ALP activities and hepatic TBARS rapidly increased in parallel with CCl₄ injection, indicating the induction of acute hepatotoxicity by CCl₄. The acute hepatotoxic effects induced by CCl₄ administration were confirmed histopathologically, revealing hepatocellular degeneration and necrosis. The obtained results are in accordance with those of the previous reports.^(15,25–27) However, BRPS significantly lowered the CCl₄-induced serum activities of ALT, AST and ALP, and reduced hepatic lipid peroxidation. This phenomenon was further con-

Table 1. Effect of BRPS on CCl₄-induced alteration of hepatic antioxidant defence potential in mice

Group	GSH (nmol/mg protein)	GR (U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)	CAT (U/mg protein)	GST (U/mg protein)
I	29.3 \pm 2.5	6.5 \pm 0.5	140 \pm 15	15.4 \pm 1.1	5.4 \pm 0.7	0.34 \pm 0.05
II	21.4 \pm 1.9 [#]	3.9 \pm 0.8 [#]	114 \pm 21 [#]	11.3 \pm 1.3 [#]	4.8 \pm 1.0	0.24 \pm 0.06 [#]
III	26.8 \pm 1.8 [*]	5.4 \pm 1.3	162 \pm 25 [*]	14.5 \pm 1.4 [*]	6.8 \pm 0.7 [*]	0.25 \pm 0.08
IV	29.7 \pm 2.0 ^{*,†}	6.7 \pm 1.4 [*]	183 \pm 37 ^{*,†}	18.6 \pm 1.6 ^{*,†}	8.5 \pm 0.8 ^{*,†}	0.27 \pm 0.08
V	27.5 \pm 1.7 [*]	5.4 \pm 1.3 [*]	190 \pm 41 [*]	16.1 \pm 1.3 [*]	7.2 \pm 0.9 [*]	0.32 \pm 0.07 [*]
VI	28.8 \pm 2.3	6.6 \pm 0.7	142 \pm 23	14.7 \pm 1.0	5.6 \pm 0.8	0.35 \pm 0.06

Data are presented as mean \pm SEM of ten mice per group. The animals were pretreated with BRPS, silymarin or a vehicle. CCl₄ was given i.p. on the 7th day of the treatment protocol, and the animals were sacrificed 16 h after CCl₄ administration. [#]*p*<0.05, significantly different from group I; ^{*}*p*<0.05, significantly different from group II; [†]*p*<0.05, significantly different from group III. Group I: normal control; group II: model (CCl₄ alone); group III: CCl₄ plus BRPS (100 mg/kg); group IV: CCl₄ plus BRPS (200 mg/kg); group V: CCl₄ plus silymarin (50 mg/kg); group VI: BRPS (200 mg/kg) alone.

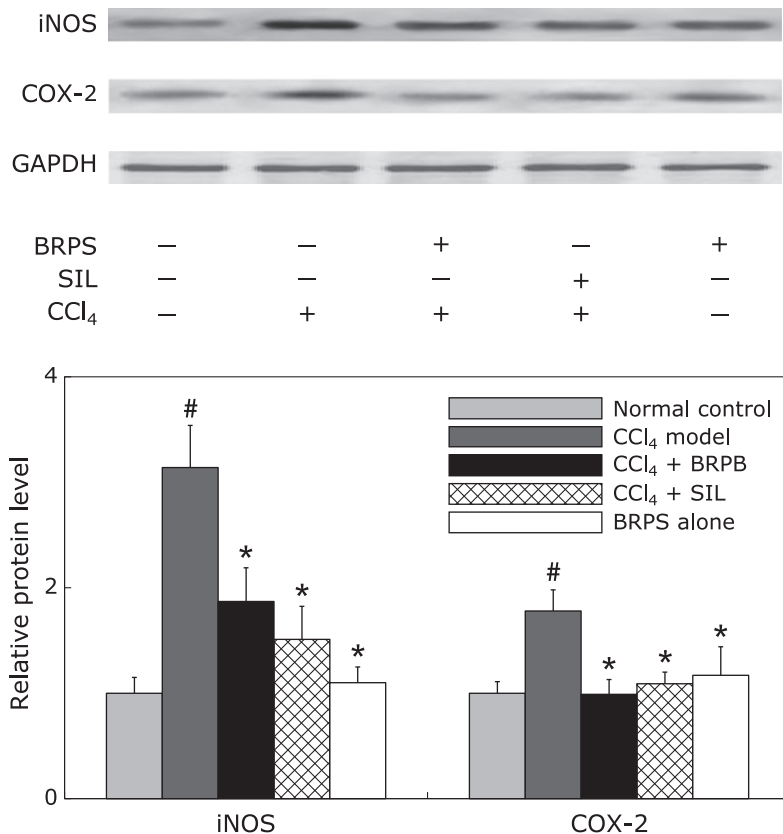


Fig. 7. BRPS down-regulated hepatic iNOS and COX-2 protein expression in CCl₄ exposed mice. Data are presented as the means of six mice per group. The animals were pretreated with BRPS (200 mg/kg), silymarin (50 mg/kg) or a vehicle. CCl₄ was given i.p. on the 7th day of the treatment protocol, and the animals were sacrificed 16 h after CCl₄ administration. GAPDH was used as an internal control and the relative level of protein expression was normalized to the control.

firming by the results of histopathological examination, as evidenced by decrease in the severity of hepatic lesions.

It has been accepted that lipid peroxidation of hepatocyte membranes is one of the principal causes of CCl₄-induced hepatotoxicity, and is mediated by the production of free radical derivatives of CCl₄.⁽²⁵⁾ Therefore, the antioxidative activity and/or the inhibition of free radical generation are important in terms of protecting the liver from CCl₄-induced damage.⁽²⁴⁾ Cells have a number of mechanisms to protect themselves from the toxic effects of free radicals, including free radical scavengers and chain reaction terminators such as SOD, CAT, GPx, GST and GR. GSH acts as a non-enzymatic antioxidant both intracellularly and extracellularly in conjunction with various enzymatic processes that reduce hydrogen peroxide and hydroperoxides and plays a primary role in the protection against trichloromethyl radical-induced liver damage. In this study, exposure to CCl₄ caused GSH depletion and decreased activities of SOD, GPx, GST and GR in the liver, implying down-regulation of numerous antioxidative reactions in liver. BRPS-pretreated animals showed a considerable improvement in the hepatic antioxidative defense potentials. This suggests that BRPS may be able to protect against hepatic 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.⁽²⁹⁾ TNF- α is a pleiotropic pro-inflammatory cytokine mainly produced by activated macrophages and monocytes and is involved in many different biological and pathologic processes including inflammation, autoimmune diseases and cancer.⁽³⁰⁾ It is also known that TNF- α

stimulates the release of cytokines from macrophages and induces the phagocyte oxidative metabolism and NO production. NO is a highly reactive oxidant that is produced through the action of iNOS, and plays an important role as a vasodilator, neurotransmitter and in the immunological system as a defense against tumor cells, parasites and bacteria.⁽³¹⁾ However, certain evidences have found that excessive NO production by iNOS may lead to hepatic damage.^(32,33) COX-2 is the enzyme responsible for the catalysis of prostaglandin E₂ from arachidonic acid⁽³⁴⁾ and the induction of COX-2 is also closely related to NO production.⁽³⁵⁾ The current study confirmed significant increases in serum TNF- α , hepatic NO, iNOS and COX-2 protein expression after the CCl₄ administration. These alterations were attenuated by BRPS treatment, which suggests that BRPS suppressed TNF- α , iNOS and COX-2 protein secretion and/or enhanced the degradation of these proteins. Accordingly, the possible mechanism of protection against CCl₄-induced hepatotoxicity appears to be, at least in part, due to the suppressed inflammatory responses.

In addition, TNF- α also induces apoptosis through the adaptor proteins of Fas-associated death domain and TNFR-associated death domain to its receptor, which in turn leads to the activation of caspase-8. The activated caspase-8 directly activates caspase-3 which plays a pivotal role in cell apoptosis when being activated.^(36,37) In our study, an obvious DNA fragmentation were observed in the mouse liver tissues obtained at 16 h after the CCl₄ treatment. However, little hepatocyte DNA fragmentation was observed in the livers from the mice pretreated with BRPS. Data also demonstrated that BRPS inhibited caspase-3 cleavage and caspase-3 activities which were elevated after the CCl₄

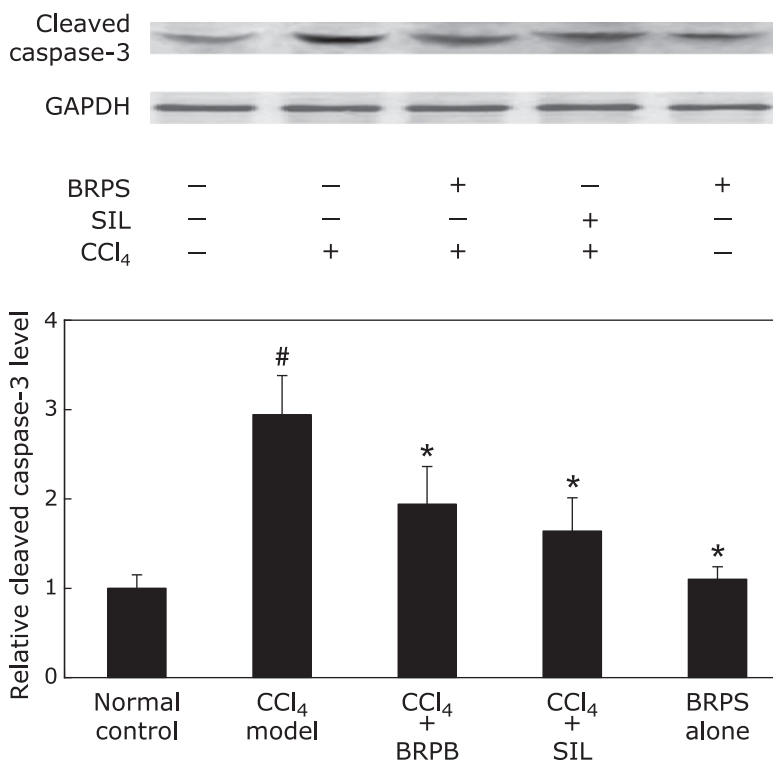


Fig. 8. BRPS suppressed hepatic caspase-3 cleavage in CCl₄ exposed mice. Data are presented as the means of six mice per group. The animals were pretreated with BRPS (200 mg/kg), silymarin (50 mg/kg) or a vehicle. CCl₄ was given i.p. on the 7th day of the treatment protocol, and the animals were sacrificed 16 h after CCl₄ administration. GAPDH was used as an internal control and the relative level of protein expression was normalized to the control.

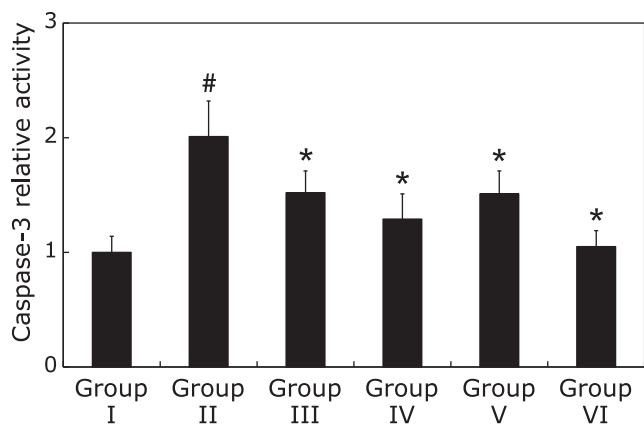


Fig. 9. BRPS reduced hepatic caspase-3 activities in CCl₄ exposed mice. Data are presented as mean \pm SEM of ten mice per group. The animals were pretreated with BRPS, silymarin or a vehicle. CCl₄ was given i.p. on the 7th day of the treatment protocol, and the animals were sacrificed 16 h after CCl₄ administration. * p <0.05, significantly different from group I; # p <0.05, significantly different from group II. Group I: normal control; group II: model (CCl₄ alone); group III: CCl₄ plus BRPS (100 mg/kg); group IV: CCl₄ plus BRPS (200 mg/kg); group V: CCl₄ plus silymarin (50 mg/kg); group VI: BRPS (200 mg/kg) alone.

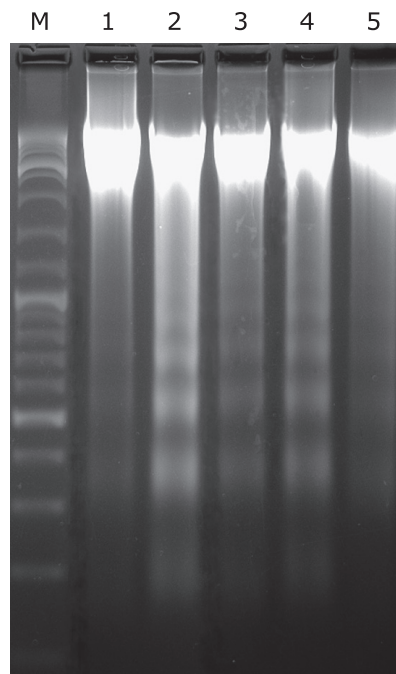


Fig. 10. BRPS suppressed hepatic DNA fragmentation in CCl₄ exposed mice. The animals were pretreated with BRPS (200 mg/kg), silymarin (50 mg/kg) or a vehicle. CCl₄ was given i.p. on the 7th day of the treatment protocol, and the animals were sacrificed 16 h after CCl₄ administration. Lane M: marker; Lane 1: normal control; Lane 2: model (CCl₄ alone); Lane 3: CCl₄ plus BRPS (200 mg/kg); Lane 4: CCl₄ plus silymarin (50 mg/kg); Lane 5: BRPS (200 mg/kg) alone.

injection. This result suggested that pretreatment with BRPS may inhibit the CCl₄-induced apoptosis and protect against hepatic damage via an anti-apoptotic function.

On the whole, it can be concluded that BRPS has a protective effect against CCl₄-induced acute liver injury in mice and the

hepatoprotective effect of BRPS may be due to the ability to suppress the inflammatory responses and apoptosis in combination with the ability to scavenge free radicals.

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Abbreviations

ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BRPS	<i>Boschniokia rossica</i> polysaccharides
CCl ₄	carbon tetrachloride
COX-2	cyclooxygenase-2
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione-S-transferase
HE	hematoxylin and eosin
iNOS	inducible nitric oxide synthase
MDA	malondialdehyde
NO	nitric oxide
ROS	reactive oxygen species
SOD	superoxide dismutase
TBARS	thiobarbituric acid-reactive substances
TNF- α	tumor necrosis factor- α

Conflict of Interest

No potential conflict of interests were disclosed.

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