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Protective Effects of *Chlorella vulgaris* Extract on Carbon Tetrachloride-induced Acute Liver Injury in Mice

Hyun-Kyung Kim[1], Li Li, Hyeong-Seon Lee, Mi-Ok Park[2], Dinesh Bilehal, Wei Li, and Yong-Ho Kim*

Department of Smart Foods and Drugs, Graduate School, Inje University, Gimhae, Gyeongnam 621-749, Korea
[1]Department of Biomedical Laboratory Science, College of Health, Gimcheon University, Gimcheon, Gyeongbuk 740-704, Korea
[2]Department of Pathology, Ulsan General Hospital, Ulsan 682-714, Korea

Abstract The purpose of this study was to evaluate the protective effects of *Chlorella vulgaris* extract (CVE) against carbon tetrachloride (CCl_4)-induced hepatotoxicity in mice. The mice received silymarin (100 mg/kg), intragastrieally (i.g.) and CVE (50, 100, and 200 mg/kg, i.g.), respectively, every other day, for 4 weeks before CCl_4 administration. Twenty-four hr after the administration of CCl_4, the serum and liver were analyzed. Our study found that in the CVE groups, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels had decreased significantly and the tissue injury was notably diminished compared to the CCl_4 group. The antioxidant activities of CVE groups, such as superoxide dismutase (SOD), catalase, and glutathione (GSH), were significantly increased and the activity of nitric oxide synthase (NOS) was remarkably increased in a CVE concentration-dependent manner. In the CVE groups, cytochrome P450 2B1/2B2 (CYP2B1/2) content was decreased. These results indicate that CVE has protective effects against CCl_4-induced hepatotoxicity via stimulation of the antioxidant activity and nitric oxide (NO) production, and through inhibition of CYP2B1/2.

Key words: *Chlorella vulgaris* extract, carbon tetrachloride (CCl_4), antioxidant, nitric oxide synthase (NOS), cytochrome P450 2B1/2B2

Introduction

The liver is an organ that plays a major role in metabolizing endogenous and exogenous materials. Acute and chronic liver diseases, wherein sufficient detoxification of harmful substances is not carried out, lead to pathological health problems. Carbon tetrachloride (CCl_4) is a well-known potent hepatotoxicant and has primarily been used as a chemical to assess hepatotoxicity in animal experiments (1,2). CCl_4 metabolism is initiated by the specific isoenzymes of the CYP450 system, such as CYP2E1 and CYP2B1/2, in the liver endoplasmic reticulum, transforming CCl_4 into trichloromethyl radical ($CCl_3^{\bullet}$). There, the $CCl_3^{\bullet}$ interacts with oxygen (O_2) to form trichloromethylperoxy radical ($CCl_3OO^{\bullet}$)(3-5). These free radials formed by the activation of CYP450 isoenzymes can bind to cellular molecules, including nucleic acid, protein, and lipid and can initiate membrane lipid peroxidation, which leads to cell necrosis (5).

Macrophages, which are Kupffer cells in the liver tissue, are activated by CCl_4 metabolism. These activated macrophages release many inflammatory mediators such as cytokines [e.g., interleukins (ILs), tumor necrosis factor (TNF)-α] and reactive oxygen species (ROS, e.g., $^{\bullet}O_2^-$, H_2O_2, and $^{\bullet}OH$) including nitric oxide (NO) and they participate in hepatotoxicity (6,7). NO is a highly reactive oxidant that plays a role in various physiological processes such as neurotransmission, vasodilation, and immune responses; it is produced from L-arginine by either constitutive or inducible nitric oxide synthase (NOS) (8,9). In xenobiotic-induced hepatotoxicity, NO produced during inflammation may play a beneficial role in decreasing the liver injury via regulation of inflammatory mediators and free radicals (10-13). However, it can damage the cell because excess NO leads to the formation of peroxynitrite ($ONOO^-$), cytotoxic oxidant, through the reaction of NO and $^{\bullet}O_2^-$ (14-17).

The tissue injury caused by the cytotoxic products formed from the metabolic processes of CCl_4 can be protected by the removal of these products via the activation of enzymatic and molecular antioxidants, including superoxide dismutase (SOD), catalase, and thiol-containing glutathione (GSH) (18-20). However, overproduction of ROS by contaminants such as CCl_4 causes oxidative stress and, finally, cell damage and death (1,2,16).

The *Chlorella* species are a unicellular green algae widely used in food supplements and as a health food in many countries (21,22). Numerous studies related to the effects of *Chlorella* have demonstrated its diverse beneficial effects in improving health. *Chlorella* has various functions that detoxify toxic materials such as dioxin, copper, and lead, stimulate the immune system, and modulates hypertension (23-29). *Chlorella vulgaris* extract (CVE) from *C. vulgaris* for clinical use is a glycoprotein containing protein, carbohydrates, and other nutrients (30). Previous studies on the effect of CVE have proved that CVE ameliorates physiological health problems, enhancing antioxidant activity, immune defense mechanism, and antitumor activity (31-37). Although many studies on CVE have been undertaken, there is still little known concerning the hepatoprotective effect of CVE against xenobiotic agent, such as CCl_4. Therefore, in this study, we examined the changes in various biochemical markers related to hepatotoxicity in groups pretreated with CVE after inducing

*Corresponding author: Tel.: +82-55-320-3481; Fax: +82-55-334-3426
E-mail: mlskimyh@inje.ac.kr
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liver damage by CCl_4 administration and evaluated the degree of liver damage. Silymarin, being used clinically for cure of liver diseases, was employed in this study to compare the effect of CVE. From these experiments, we elucidate that CVE has a hepatoprotective effect against CCl_4-induced acute liver injury.

Materials and Methods

Materials and chemicals *Chlorella vulgaris* extract (CVE) extracted from *C. vulgaris* and silymarin purified from milk thistle were supplied from Daesang Co., Ltd. (Seoul, Korea). CVE was prepared using a hot-water extraction method. Corn oil, carbon tetrachloride (CCl_4), a GSH assay kit, and a catalase assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). A SOD assay kit was purchased from Fluka Chemical Co. (Buchs, Switzerland). A NOS activity assay kit was purchased from Oxford Biomedical Research, Inc. (Oxford, MI, USA). Mouse monoclonal antibodies against CYP2B1/2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other chemicals were of analytical grade.

Animals and treatment Five-week-old male ICR mice (30-40 g, Hyo-Chang Science Co., Daegu, Korea) were used in this experiment. Animals were allowed free access to Purina Rodent Chow and tap water. They were maintained in a controlled environment at 22±2°C and 60±5% relatively humidity with a 12 hr dark/light cycle, and acclimatized for 1 week before the experiment. The mice were divided into 6 groups of 6 animals each. Group I (Control) and Group II (CCl_4) mice received distilled water intragastrically (i.g.), Group III mice received silymarin (100 mg/kg, i.g.), and Groups IV through VI mice received CVE (50, 100, and 200 mg/kg, i.g.), respectively, every other day for 4 weeks. Three hr after the final treatment, CCl_4 dissolved in corn oil (20 mg/kg of body weight) was administrated intraperitoneally (i.p.) to each group, except Group I. The food and water were removed from the cage 12 hr after the administration of CCl_4, and the mice were anesthetized with diethyl ether 24 hr after the administration of CCl_4. Blood samples were collected from the hepatic portal vein, and the livers were quickly excised from the mice. The blood samples were centrifuged to obtain serum at 3,000×g for 15 min at 4°C. The excised livers were washed with cold phosphate buffered saline (PBS, pH 7.4), and pieces of the liver samples were fixed in 10% formalin for histopathological examination. The remnants of the livers were stored at −80°C until the experiment.

Serum analysis The serum activity of AST and ALT was measured to evaluate hepatotoxicity. An autoanalyzer (200FR; Toshiba, Tokyo, Japan) was used in the experiments.

Homogenate preparation Liver tissues were homogenized with a sucrose buffer [0.25 M sucrose, 10 mM Tris. 1 mM ethylenediamide tetraacetic acid (EDTA), pH 7.4 1:9, w/v] and centrifuged at 10,000×g for 30 min in a high speed centrifuge (Combi-514R; Hanil, Incheon, Korea) at 4°C. The supernatants were collected to determine SOD and NOS activities. Samples for the catalase activity assay were prepared from liver tissue homogenization with 50 mM of a phosphate buffer (pH 7.0). Protein concentration was measured using the Bradford method and bovine serum albumin (BSA, Santa Cruz Biotechnology, Inc.) (38).

Antioxidant enzymes activity SOD activity was determined by using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1), a highly water-soluble tetrazolium salt (Dojindo, Kumamoto, Japan), which produces a water-soluble formazan dye upon reduction with a superoxide anion (39). The amount of SOD causing a 50% inhibition of the production of superoxide anion/unit protein in 1 mL volume is expressed as 1 unit of SOD activity, and displayed as Unit/mg protein.

Catalase activity was measured by the hydrogen peroxide (H_2O_2) remaining after catalyzation. Catalase catalyses the H_2O_2 to water and oxygen (40). After decomposition of H_2O_2, this reaction is stopped with sodium azide and the amount of remainig H_2O_2 was colorimetrically determined. One unit of catalase decomposed 1.0 μL of H_2O_2 to water and oxygen/min at a substrate concentration of 50 mM of H_2O_2.

GSH content The sample was ground with a pestle and was deproteinized in 3 volumes (w/v) of 5% 5-sulfosalicylic acid (SSA) solution, and another 7 volumes of the 5% SSA solution was added and was homogenized. After standing for 10 min at 4°C, the sample was centrifuged at 10,000×g for 10 min. The supernatant was used to measure the level of GSH. A kinetic method was used in this assay in which catalytic amounts of GSH caused a continuous reduction of 5,5-dithiobis (2-nitrobenzoic acid)(DTNB) to TNB. Absorbance of the yellow product was measured at 412 nm (41). The amount of GSH was expressed as an nM of GSH/mg of homogenate protein.

NOS activity NOS converts L-arginine to NO and L-citrulline in an NADPH-dependent reaction, and the NO that is produced rapidly degrades to nitrite and nitrate in an aqueous solution (8,9,42). In this study, we employed an ultrasensitive colorimetric method that involves the use of nitrate reductase (NaR); this enzyme converts nitrate to nitrite, and nitrite is quantified using the Griess reagent (42,43). The absorbance value was read at 540 nm (Synergy HT, Biotek, VT, USA).

Hepatic microsomal preparation Microsomes were prepared as described previously, with minor modification (3). The liver was homogenized in 3 volumes (w/v) of 0.1 M Tris-KCl buffer (pH 7.4). The liver homogenate was centrifuged at 10,000×g for 30 min in a high speed centrifuge (Combi-514R; Hanil) at 4°C. After centrifugation, the supernatant was centrifuged at 105,000×g for 60 min (Optima L-100XP; Beckman, Fullerton, CA, USA). The pellet was washed and resuspended in a buffer containing 0.1 M sodium pyrophosphate and 1 mM EDTA (pH 7.4). The suspension was centrifuged again at 105,000×g for 60 min. The pellet was homogenized in 50 mM Tris acetate buffer (pH 7.4). The homogenate was defined as a microsome and was stored at −80°C until use.

Western blot analysis for CYP2B1/2 Ten μg of protein from the extracted microsome in each group was loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% polyacrylamide gels) for electrophoresis (Mini Format 1-D Electrophoresis Systems, Bio-Rad, Hercules, CA, USA). After electrophoresis, the separated proteins were transferred to a PVDF membrane using a transfer kit (Tetra Blotting Module, Bio-Rad). The membrane was blocked for 1 hr with 3% BSA buffer and was incubated with the primary antibody for 1 hr using a 1:200 dilution of mouse monoclonal CYP2B1/2 antibody (Mw 50 kDa, Santa Cruz Biotechnology, Inc.). After washing the membrane with buffer, it was incubated with the secondary antibody (1:10,000 dilution, Sigma-Aldrich) for 1 hr, which was conjugated to alkaline phosphatase. The membrane was washed in the buffer for 5 min, and detected with a mixture of 5-bromo-4-chloroindoly-phosphate (BCIP) and nitroblue tetrazolium (NBT). The immunoreactive band of CYP2B1/2 protein in each sample was quantified by densitometry analysis using PDQuest software (version 7.0, Bio-Rad) and expressed as the relative intensity compared to the control group.

Histopathological examination The fixed liver sample was embedded in paraffin and cut into 5 μm thick sections, and stained with hematoxyline-eosin (H-E). The stained tissue sample was examined under a light microscope to evaluate CCl_4-induced histopathological changes.

Statistical analysis The results were expressed as mean± standard deviation (SD). A Student's *t*-test was used to compare the means of the remaining data. A value of $p<0.05$ was accepted as statistically significant.

Results and Discussion

Effects of CVE on CCl_4-induced hepatotoxicity Serum AST, ALT, and histopathological changes were examined to evaluate CCl_4-induced hepatotoxicity. AST and ALT have been widely used as markers for liver injury: the increase of these markers in bloodstream indicates that liver tissue was damaged by cytotoxic products formed during CCl_4 intoxication (2,13). Figure 1 shows the activities of AST and ALT for each group. In this study, the activities of AST and ALT notably increased in the CCl_4 group, indicating the damage of liver tissue by CCl_4-induced hepatotoxicity. But, in the silymarin and CVE groups, the increase of AST and ALT activities were significantly inhibited ($p<0.05$) and the decrease in CVE groups was in a dose-dependent manner. Figure 2 presents the histopathological change of each group. The liver tissue of the CCl_4 group (Fig. 2B) showed severe hepatocyte necrosis with extensive inflammatory cell infiltration around the central vein. However, the groups pretreated with CVE showed that liver injury remarkably minimized in a dose-dependent manner. In CVE 50 mg/kg group (Fig. 2D), hepatocyte necrosis was mildly diminished, and disappeared in the CVE 100 and 200 mg/kg group (Fig. 2E and 2F), showing severe to moderate ballooning degeneration of hepatocytes.

Based on these results of AST, ALT, and histopathological changes, CVE is considered to have a hepatoprotective effect against CCl_4 intoxication.

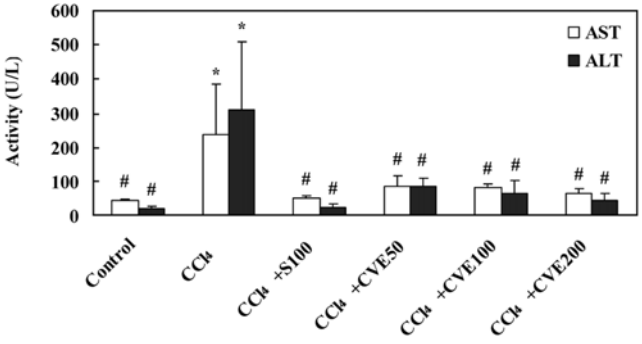

Fig. 1. Effects of CVE on AST and ALT activities in CCl_4-treated mice. Data are mean±SD. *Significantly different from the control group at $*p<0.05$ and from the CCl_4 group at $^{\#}p<0.05$.

Effects of CVE on hepatic SOD activity, catalase activity, and GSH content Overproduction of ROS through CCl_4 metabolism triggers oxidative stress accompanied by cellular damage (5). In the liver, cytotoxic reactive molecules produced by CCl_4 intoxication can be protected by enzymatic and molecular antioxidants such as SOD, catalase, and GSH.

SOD has been known to convert $^{\bullet}O_2^-$ into H_2O_2 and O_2, and has been shown to suppress apoptosis in cell cultures and *in vivo*. NO reacts to $^{\bullet}O_2^-$ and leads to the formation of $ONOO^-$, which contributes to hepatotoxicity due to its cytotoxic property (14). NO competes with SOD for $^{\bullet}O_2^-$. Therefore, the decrease of SOD enhances $^{\bullet}O_2^-$, which can lead to augmentation of $ONOO^-$ production by binding of NO and $^{\bullet}O_2^-$, and induce lipid peroxidation, while the increase of SOD decreases $ONOO^-$ production and promotes NO activity by removing $^{\bullet}O_2^-$ (44,45).

Figure 3A shows hepatic SOD activity. In our result, hepatic SOD activities of the group that received CCl_4 alone was significantly decreased ($p<0.01$) compared to the control group. On the other hand, the SOD activities of the silymarin (100 mg/kg) and CVE groups (50, 100, and 200 mg/kg) were significantly increased compared to the control and CCl_4 group. In the CVE groups, it increased in a dose-dependent manner, and SOD activities of CVE 200 mg/kg group was about 2 fold compared to the CCl_4 group and the SOD level was even higher than in the control group, which suggests that the $ONOO^-$ production would be inhibited by interaction of SOD and $^{\bullet}O_2^-$, and the removal of $^{\bullet}O_2^-$ increases NO bioavailability, thus reducing hepatotoxicity (44,45).

Catalase acvtivity is presented in Fig. 3B. Catalase is an antioxidant enzyme that catalyses toxic H_2O_2 into H_2O and O_2, and protects against liver injury by the removal of H_2O_2 (18,40). The catalase activity of the CCl_4 group decreased significantly ($p<0.01$) compared to the control group. In comparison, it increased significantly in both the silymarin and CVE groups, which imply that CVE has a stimulating effect in catalase activity.

GSH is tripeptide in the sulfhydryl group. GSH reduces H_2O_2 and reacts with trichoromethyl radicals, preventing the binding of trichloromethyl radicals to cell proteins (20,46). Therefore, the decrease in these antioxidants may

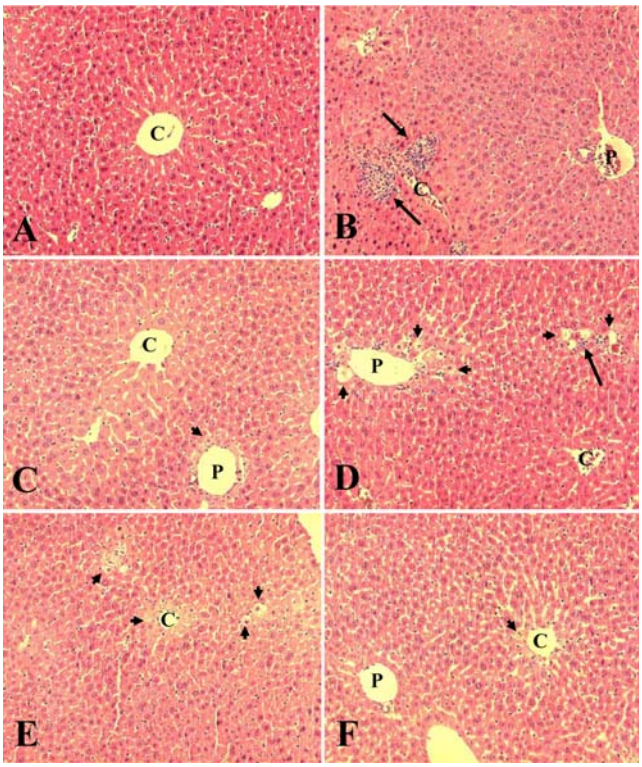

Fig. 2. Effects of CVE on histopathological changes by CCl_4. Liver sections were stained with hematoxyline-eosin (H-E, ×200). (A) Liver tissue of a control mouse, (B) liver tissue of a mouse treated with CCl_4, presenting severe hepatocyte necrosis (long arrow), (C) liver tissue of a mouse pretreated with silymarin (100 mg/kg, i.g.), showing moderate ballooning degeneration (short arrow), (D) liver tissue of a mouse pretreated with CVE (50 mg/kg, i.g.), showing mild hepatocyte necrosis (long arrow) and severe ballooning degeneration (short arrow), (E) liver tissue of a mouse pretreated with CVE (100 mg/kg, i.g.), showing severe ballooning degeneration (short arrow), and (F) liver tissue of a mouse pretreated with CVE (200 mg/kg, i.g.), showing mild ballooning degeneration (short arrow).

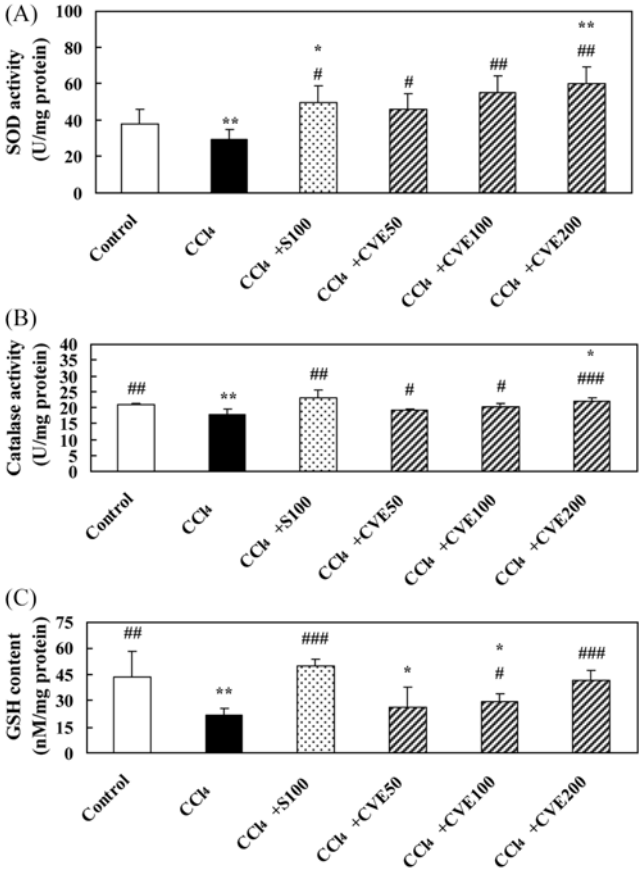

Fig. 3. Effects of CVE on hepatic SOD activity (A), catalase activity (B), and GSH content (C) in CCl_4-treated mice. The data are mean±SD. Significantly different from the control group at $^{*}p<0.05$, $^{**}p<0.01$ and from the CCl_4 group at $^{\#}p<0.05$, $^{\#\#}p<0.01$, and $^{\#\#\#}p<0.001$.

lead to oxidative stress and cellular damage. GSH content is presented in Fig. 3C. In mice receiving CCl_4 alone, the GSH level was decreased by 50% when compared to the control group ($p<0.01$), however, GSH levels of the silymarin and CVE groups were significantly increased, and GSH level of the silymarin and CVE 200 mg/kg group was 2 times more than that in the CCl_4 group.

In previous studies, hepatic SOD, catalase, and GSH levels decreased after CCl_4 administration, resulting in liver damage (2,13,47). These results suggest that antioxidants can be consumed and decreased during CCl_4 intoxication, which is consistent with our results. In the CCl_4 group in our study, antioxidant activity decreased, on the other hand, we observed that antioxidant activity in the CVE groups markedly increased compared to the CCl_4 group. In addition, in the CVE groups, the increase of antioxidant activities was in a dose-dependent manner.

Effects of CVE on hepatic CYP2B1/2 content The CYP450 superfamily is heme-thiolate monooxygenases that play an important role in metabolizing drugs and chemicals (48). CYP2B1/2, members of the CYP450 superfamily, are phenobarbital-induced enzymes and able to attack CCl_4 as well as CYP2E1 (3-5). Although CYP2B1/2 also participates in metabolizing CCl_4, previous studies about CCl_4-induced acute liver injury have dealt mainly with CYP2E1 (47). Our study focused on the metabolism of CCl_4 by CYP2B1/2 and investigated the change in CYP2B1/2 content. To evaluate the effect of CVE on CYP2B1/2 content, Western blot analysis was performed using hepatic microsomes.

In our study, as shown in Fig. 4, the CYP2B1/2 content slightly increased in mice that received CCl_4 alone, compared to the control group. On the other hand, CYP2B1/2 content was markedly decreased in mice that received silymarin (100 mg/kg, i.g.) and CVE (50, 100, and 200 mg/kg, i.g.) before CCl_4 treatment, compared to the CCl_4 group. In addition, CYP2B1/2 content in the CVE groups decreased in a dose-dependent manner.

The slight increase of CYP2B1/2 content in CCl_4-treated mice is in agreement with the results reported by Jia *et al.* (3). Those results showed that in the case of the CYP450 superfamily, the expression levels were different, and those of 2B1and 2B2 were up-regulated following CCl_4 administration. The differences in the expression levels of CYP450 superfamily may be due to the differences in the

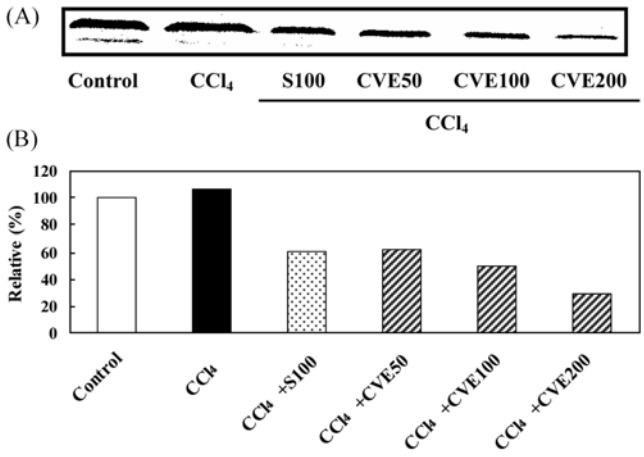

Fig. 4. Effects of CVE on hepatic CYP2B1/2 content in CCl_4-treated mice. (A) Western blot analysis of hepatic microsomal CYP2B1/2 content in each group. (B) Densitometry analysis of the Western blot for each group. The value was expressed as the relative intensity compared to that of the control group.

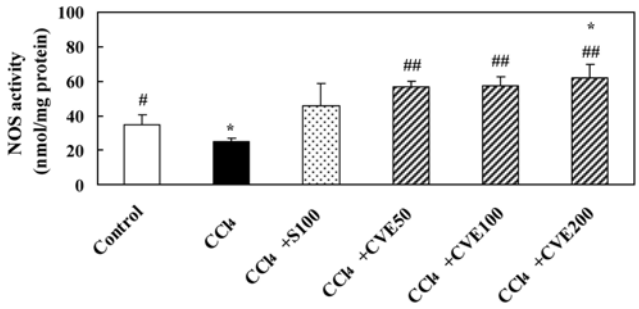

Fig. 5. Effects of CVE on hepatic NOS activity in CCl_4-treated mice. The data are mean±SD. Significantly different from the control group at $^{*}p<0.05$ and from the CCl_4 group at $^{\#}p<0.05$, $^{\#\#}p<0.01$.

half-life of each enzyme. During steady-state expression, the half-life of CYP2B1 was about 2 times greater than that of CYP2E1 (19).

In previous studies, CYP2B inhibitor decreased the CCl_4-induced lipid peroxidation and further, CYP2B1/2-dependent hepatic damage was such that the number of necrotic hepatocytes was more than that of ballooned hepatocytes, and necrotic hepatocytes were found extensively. However, the CYP2E1-induced damage was characterized by ballooned hepatocytes that were restricted to the centrilobular area (49,50). These results suggest that the increase of CYP2B1/2 activity may cause the increase of liver injury and lead to more serious tissue damage than an increase in CYP2E1 activity. From these results, we suppose that the inhibition of CYP2B1/2 activity would protect the liver against CCl_4 intoxication and CVE would have a hepatoprotective effect through CYP2B1/2 inhibition.

Effects of CVE on hepatic NOS activity NO is produced when L-arginine is converted to L-citrulline by NOS and participates in various physiological processes (8,9). NOS can be divided into 3 different isoforms. NOSI in neurons and NOSIII in endotherial cells are constitutively expressed and their activity is regulated by Ca^{2+}/calmodulin. NOSII is inducible and its activity is Ca^{2+}/calmodulin-independent. Inducible NOS (iNOS, NOSII) is expressed in macrophages by the stimulation of inflammatory mediators such as cytokines and lipopolysaccharides (9,51). NOS activity is shown in Fig. 5. Our results showed that NOS activity decreased by 30% in the CCl_4 group compared to the control group; this result differed from other research that revealed an increase of NOSII expression after CCl_4 administration (15-17), however, this may be due to differences in the concentration of CCl_4, a different time course, and/or different expression of NOS isoforms following CCl_4 administration. NOSII expression revealed in a CCl_4 concentration-dependent manner. Twenty-four hr after CCl_4 administration, NOSII expression of the group that received high concentration of CCl_4 was evident, however, that of the group that received low concentration of CCl_4 did not appear (10). Moreover, NOSII activity increased, while NOSI+III activity decreased by CCl_4 intoxication, and the increase of NOSII activity reached a maximum around 20 hr after CCl_4 administration and decreased after that time (10,11). In addition, Ye *et al.* (52) reported that NOS activity remained unchanged after CCl_4 administration of the higher dose than in this work. In the silymarin and CVE groups, the NOS activity increased significantly compared to the CCl_4 group, and the difference was more pronounced in the CVE groups. The NOS activity of the CVE groups increased 2 times more than in the CCl_4 group, from 50 to 200 mg/kg ($p<0.01$).

Whether the NO formed during inflammation plays a beneficial role or a harmful role as far liver injury in concerned is a controversial topic. The NO produced in large amounts from NOSII led to toxin-induced liver injury. Further, the inhibition of NOSII expression and NO production reduced the severity of liver injury (15-17). On the other hand, some studies have demonstrated that NO may have a beneficial effect in CCl_4-induced hepatotoxicity. The liver damage was found to increase in animal lacking NOS2 gene and with the inhibition of NO, while it decreased when NO was added and was increased (10-13). These results suggest that NO may protect the liver against CCl_4-induced liver injury through regulation of inflammatory mediators such as TNFα and, by modulating free radicals, carbohydrate metabolism, and collagen production. Our results showed that NO may have a protective effect in CCl_4-induced hepatotoxicity. NOS activity in the CVE groups increased compared to the control and CCl_4 groups and the groups that NOS activity was increased presented the decrease of liver damage. The increase of NOS activity in the CVE groups might have occurred because the CVE augmented macrophage activity. Liu *et al.* (6) reported that macrophage activation increased NO production and Hasegawa *et al.* (31) obtained evidence that the activation of macrophages is influenced by CVE. This report suggested that CVE may augment immune responses by activation of macrophages and enhances host defenses.

In addition, some studies have proposed that NO may play a role in the down-regulation of CYP2B1/2 activity. In the research related to CYP450 isoenzymes, NO was found to inhibit the CYP450-mediated activity, suggesting the possibility of suppression by the binding of NO to the

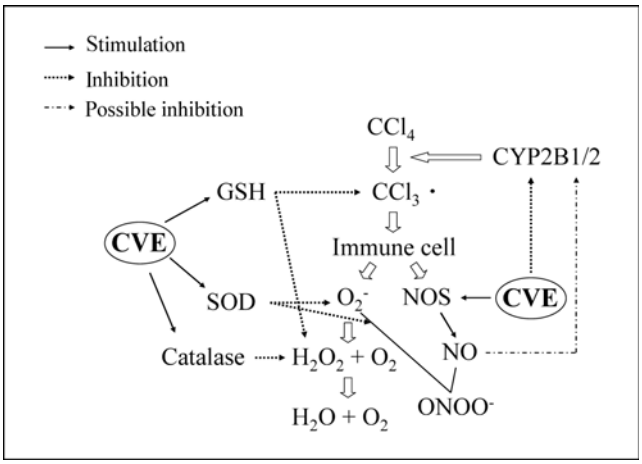

Fig. 6. Proposed model for protective mechanism of CVE against CCl_4-induced hepatotoxicity.

heme in CYP450 enzymes (54-56). There are conflict of opinions whether CYP450 isoenzymes is down-regulated by NO. CYP2C11, 2E1, and CYP3A2 expression were not affected by inhibition of NOS activity (53). In contrast, NO inhibited the activity of CYP2E1 and was found to inhibit the CYP2B1 in a NO concentration-dependent manner, which was more evident than in CYP1A1 (54,55). The increase in NO production was accompanied by a decrease in CYP2B1/2 and CYP3A2, and the cytokine-mediated decrease in CYP2B1/2 was prevented by the addition of the NOS inhibitor (56). Based on this and previous research related to CYP2B1/2 and NO, it is possible to suppose that NO may inhibit the expression of CYP2B1/2. In the present study, CYP2B1/2 content in CVE groups decreased in a NO concentration-dependent manner and the correlation coefficient between CYP2B1/2 content and NOS activity was -0.954 ($p<0.001$). This result implies that NO might have inhibited CYP2B1/2 activity.

Figure 6 exhibits proposed model to summarize results from this work. We found that CVE functions in several ways to have a protective effect against CCl_4-induced acute liver injury. CVE enhanced NOS activity and decreased CYP2B1/2 content in a dose-dependent manner, which implies the possibility that NO might have inhibited CYP2B1/2 activity. The antioxidant activity of CVE would reduce oxidative stress by their scavenging effect on the free radicals. In addition, SOD activity augmented by CVE may possibly diminish the cytotoxic $ONOO^-$ production, while promote NO activity due to the competition between NO and SOD for $^{\cdot}O_2^-$. Taken together, CVE would stimulate NO production, inhibit CYP2B1/2 activity, and augment antioxidant activity in animals treated with CCl_4, all of which may be able to exert protective effects in CCl_4-induced hepatotoxicity.

Acknowledgments

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