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Effect of Onion Extract on the Carbon Tetrachloride-induced Liver Injury in Mouse

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Abstract

The protective effects of onion extract (OE), onion powder extracted in ethanol for 2 days, on carbon tetrachloride (CCl_4)-induced hepatotoxicities and the possible mechanisms involved in this protection were investigated in mice. Pretreatment with OE prior to the administration of CCl_4 significantly reduced the increase in serum alanine and aspartate aminotransferase activities and hepatic lipid peroxidation in a dose-dependent manner. In addition, pretreatment with OE significantly prevented the depletion of reduced glutathione content in the liver of CCl_4-intoxicated mice. CCl_4-induced hepatotoxicity was also prevented, as indicated by a liver histopathologic findings. The effects of OE on the cytochrome P450 (P450) 2E1, the major isozyme involved in CCl_4 biotransformation were investigated. Treatment of mice with OE resulted in a significant decrease in P450 2E1-dependent *p*-nitrophenol and aniline hydroxylation in a dose-dependent manner. Consistent with these observations, the P450 2E1 expressions were also decreased, as determined by immunoblot analysis. OE also exhibited antioxidant effects in $FeCl_2$-ascorbate induced lipid peroxidation in rat liver homogenates and in superoxide radical scavenging activity. These results show that the protective effects of OE against the CCl_4-induced hepatotoxicity may be due to its ability to block bioactivation of CCl_4, mainly by inhibiting the expression and activities of P450 2E1 and by scavenging free radicals.

Key words: onion extract (OE), carbon tetrachloride (CCl_4), cytochrome P450 2E1, antioxidant

INTRODUCTION

Many hepatotoxicants including carbon tetrachloride (CCl_4), nitrosamines, and polycyclic aromatic hydrocarbons that enter the body tissue are subject to metabolism (Phase I) especially by liver cytochrome P450 (P450) enzymes, to form reactive, toxic metabolites, that in turn produce liver injury in experimental animals and humans (1). CCl_4, a well-known model compound for the induction of chemical hepatic injury, requires biotransformation by hepatic microsomal cytochrome P450 (P450) to produce its hepatotoxic metabolites, trichloromethyl free radicals ($CCl_3^{\cdot}$ and/or $CCl_3OO^{\cdot}$) (2). Trichloromethyl free radicals can react with sulfhydryl groups, such as glutathione (GSH) and protein thiols. The covalent binding of trichloromethyl free radicals to cell protein is considered the initial step in a chain of events, which eventually leads to peroxidation of membrane lipids and finally cell necrosis (2-4). Although several isoforms of P450 may metabolize CCl_4, attention has been largely focused on the P450 2E1 isoform, which is ethanol-inducible (5,6). Alternations in the activity of P450 2E1 affect the susceptibility to hepatic injury from CCl_4 (7,8). Natural compounds that inhibit chemical activating enzymes have great potential for protection against chemically induced toxicities. P450 2E1 is well recognized for its role in the activation of many chemicals to toxic and carcinogenic agents (5,6).

Onion (*Allium cepa.* Linn), one of the important *Allium* species, is grown and consumed worldwide. It has been regarded for centuries as beneficial for health, and is recommended for curing or preventing a wide variety of disease. The beneficial effects of onion have only recently been validated in many experimental studies. Several medicinal effects have been described, such as anti-inflammatorty, anti-asthmatic, antimicrobial and cardiovascular protective effects (9). Moreover, onion was an effective antioxidant against the oxidative damage caused by nicotine (10) and the antimutagenic activites of onion against several ultimate carcinogens were closely related to their

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ability to induce phase II enzymes (glutathione, glutathione-S-transferase) in the rat liver (11). The preventive effects of onion have been clearly demonstrated in anti-hepatotoxicity studies. However, the mechanisms by which OE protects against CCl_4-induced hepatotoxicity are not fully understood. The present study was undertaken to evaluate the protective effects of OE on CCl_4-induced hepatotoxicity and to elucidate the mechanisms underlying these protective effects in mice.

MATERIALS AND METHODS

Materials

Onion (*Allium cepa*) powder was obtained from Mooan's onion farms. The powder was prepared from grown in the plain of Mooan within 2 days of harvesting. Onions were washed and sliced before dehydrating in a conventional hot-air oven (Temperature gradient from 85 to 45 °C) until the product retained less than 6% moisture. Carbon tetrachloride (CCl_4), olive oil, diagnostic kits for serum ALT and AST, thiobarbituric acid, dithionitrobenzoic acid, *L*-ascorbic acid, xanthine, xanthine oxidase, phenylmethoxysulfonyl fluoride, reduced GSH, ferreous sulfate, ferreous chloride and hydrogen peroxides were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of the highest grade commercially available.

Animals and treatment

Male ICR mice (6 weeks, 20 g±2 g) were obtained from KFDA (Seoul, Korea). The animals were allowed free access to Purina Rodent Chow and tap water, maintained in a controlled environment at 21±2°C and 50±5% relatively humidity with a 12 hr dark/light cycle, and acclimatized for at least 1 week before use. OE in saline was administered intragastrically at 10~100 mg/kg once daily for 3 days in treatment groups and saline without OE was administered to control animals. Three hours after the final treatment, rats were treated with CCl_4 (20 mg/kg, intraperitoneally, dissolved in olive oil). Eighteen hours after the administration of CCl_4, rats were anesthetized with CO_2, blood was removed by cardiac puncture to determine the serum ALT and AST activities, and the animals were decapitated. After bleeding, livers were weighed and a thin slice preserved in a buffered formalin solution for obtaining histological sections. The remaining livers were frozen quickly in dry ice/methanol and stored at -70°C for GSH content and lipid peroxidation analysis.

Hepatotoxicity studies

Hepatotoxicities were assessed by quantifying the serum activities of ALT and AST, and by determining hepatic lipid peroxidation. Serum ALT and AST activities were measured with a spectrophotometric diagnostic kit obtained from the Sigma Chemical Co. Hepatic lipid peroxidation was measured by the formation of the thiobarbituric acid-reactive material, malondialdehyde (MDA) (12).

Hepatic GSH determination

Mice were killed by cervical dislocation. Livers were excised quickly, washed in ice-cold EDTA solution (0.02 M), blotted dry, dissected to remove connective tissues and weighed. Non-protein liver GSH was estimated by a colorimetric method using Ellman's reagent as described by Sedlak and Lindsay (13). Briefly, a weighed portion of the tissue was homogenized in 0.02 M EDTA. Protein was precipitated with 5% trichloroacetic acid, and the supernatant mixed with 2 volumes of tris buffer (0.4 M, pH 8.9), containing 0.2 M EDTA. Color was developed by adding dithionitrobenzoic acid prepared in methanol, and absorbance was measured at 412 nm. The GSH level was quantified using a standard curve prepared by plotting data from different concentrations of reduced GSH.

Histological examinations

Fresh liver tissues, previously trimmed to approximately 2 μm thickness, were placed in plastic cassettes and immersed in neutral buffered formalin for 24 hr. Fixed tissues were processed routinely, and then embedded in paraffin, sectioned, deparaffinized, and rehydrated. The extent of carbon tetrachloride-induced necrosis was evaluated by assessing morphological changes in liver sections stained with hematoxylin and eosin (H&E).

Microsome and cytosol isolation

Mice were treated with OE, pyridine (100 mg/kg, P450 2E1 inducer) or vehicle once daily for 3 days. Eighteen hours after the last treatment, the mice were sacrificed by cervical dislocation. The livers were quickly removed, weighed and perfused with ice-cold 0.15 M KCl, and then homogenized with 4 vol. (w/v) of 10 mM Tris-HCl (pH 7.4) containing 0.15 M KCl, 0.1 mM EDTA, 1.0 mM dithiothreitol and 0.01 mM phenylmethoxysulfonyl fluoride in a Potter-Elvehjem homogenizer. Hepatic microsomal and cytosolic fractions were prepared by differential centrifugation, as described previously (14). All preparations were stored at -70°C until use.

p-Nitrophenol and aniline hydroxylase assay

The hydroxylation of *p*-nitrophenol to 4-nitrocatechol was determined spectrophotometrically as described previously (15). Aniline hydroxylase activity was determined by measuring *p*-aminophenol formation. Microsomal protein was determined by the method of Bradford (16), using bovine serum albumin as a standard. For the inhibition study, OE was dissolved in saline and added

to the incubation mixture.

Immunoblot analysis

Immunochemical detection of P450 2E1 was performed according to the method of Jeong and Yun (17). Briefly, electrophoretic separation of microsomal proteins (10 mg) was performed using 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and then electrotransfered to nitrocellulose membranes and immunoblotted with anti rat-P450 2E1 antibody. Alkaline phosphatase-labeled rabbit anti-rat lgG was used as the secondary antibody and color developed using mixture of 5-bromo-4-chloro-indolylphosphate and nitroblue tetrazolium.

$FeCl_2$-ascorbic acid stimulated lipid peroxidation in liver homogenate

Young male SD rats weighing 250±20 g were killed by decapitation and their livers were quickly removed. A 2 g portion of liver tissue was sliced and then homogenized with 10 mL of 150 mM KCl-Tris-HCl buffer (pH 7.2). The protein content was determined by the method of Bradford (16). The reaction mixture consisted of 0.25 mL of liver homogenate, 0.1 mL of Tris-HCl buffer (pH 7.2), 0.05 mL of 0.1 mM ascorbic acid, 0.05 mL of 4 mM $FeCl_2$ and 0.05 mL of various concentrations of OE. Products of lipid peroxidation were measured by the formation of the thiobarbituric acid-reactive material, MDA (13). 1,1,3,3-Tetraethoxypropan was used as a standard for calibration of malondialdehyde. Appropriate controls were performed to eliminate any possible interference with the thiobarbituric acid assay.

Assay of superoxide scavenging activity "*in vitro*"

Superoxide was generated by xanthine (100 μM) and xanthine oxidase (0.02 U) with or without various concentrations of added OE in 1 mL of 10 mM KH_2PO_4-KOH buffer, pH 7.4, and was detected using nitroblue tetrazolium (100 μM) and quantified spectrophotometrically at 550 nm (18). Superoxide dismutase (100 U/mL) was used as a reference inhibitor.

Statistical analysis

All experiments were repeated at least three times. Results are reported as means±SD. A Dunnet's 't' test was used to compare the means of two specific groups, with $p<0.01$ considered significant.

RESULTS

Effect of OE on carbon tetrachloride (CCl_4)-induced hepatotoxicity

The effects of pretreatment with OE on the CCl_4-induced elevation of serum ALT and AST activities are shown in Table 1. Pretreatment with OE (100 mg/kg, o.p., 3 days) resulted in no changes in serum ALT or AST activities, compared to the control. A single dose of CCl_4 (20 mg/kg) caused hepatotoxicity in mice, as indicated by the increase in ALT and AST serum levels. OE pretreatment prevented the CCl_4-induced elevation serum ALT and AST serum, in a dose-dependent manner (Table 1). Low doses of OE (10 mg/kg) partially prevented the elevation of serum levels of ALT and AST. Medium or higher doses of OE (100 mg/kg) almost completely prevented hepatotoxicity.

Effects of GA on hepatic lipid peroxidation and GSH levels

In order to evaluate the effect of pretreatment with OE on CCl_4-induced liver lipid peroxidation, we monitored the levels of MDA, an indicator of oxidative damage, and one of the principal products of lipid peroxidation. As shown in Table 1, the production of MDA in the CCl_4-treated group increased 3.4-fold when compared with the control. Consistent with the serum levels of ALT and AST, pretreatment with OE significantly decreased CCl_4-induced hepatic lipid peroxidation in a dose-dependent manner (Table 1).

Hepatic GSH levels were determined 18 hr after the CCl_4 administration. Whereas, administrations of CCl_4 alone significantly depleted GSH levels, pretreatment with

Table 1. Effects of OE on the serum levels of ALT, AST and hepatic MDA content

Treatment	Serum ALT (U/liter)	Serum AST (U/liter)	Liver lipid peroxidation (MDA, *n*mole/g wt.)
Control	62 ± 7^{c}	32 ± 4^{c}	10.5 ± 1.3^{c}
OE (100 mg/kg)	64 ± 8^{c}	35 ± 4^{c}	10.8 ± 1.2^{c}
CCl_4	$2,636\pm346^{a,b}$	$1,984\pm231^{a,b}$	$35.8\pm4.7^{a,b}$
OE (10 mg/kg)+CCl_4	$2,312\pm262^{a,b}$	$1,595\pm218^{a,b}$	$33.2\pm4.1^{a,b}$
OE (50 mg/kg)+CCl_4	$1,825\pm213^{a,b,c}$	$1,092\pm162^{a,b,c}$	$26.5\pm3.2^{a,b,c}$
OE (100mg/kg)+CCl_4	$752\pm96^{a,b,c}$	$563\pm97^{a,b,c}$	$16.8\pm2.2^{a,b,c}$

Serum ALT, AST and hepatic MDA contents were measured as described in Materials and Methods. Each value represents the mean±SD of five mice.
[a]Significantly different from control.
[b]Significantly different from OE.
[c]Significantly different from carbon tetrachloride.

OE significantly reduced the GSH depletion produced by CCl_4 (Fig. 2). A dose-dependent protective effect from GSH depletion was observed.

Pathological histology of the liver

Histopathological studies showed that CCl_4, compared to the control, induces degeneration in hepatocytes and hepatic cords and focal necrosis (Fig. 1). By microscopic examination, the severe hepatic lesions induced by CCl_4 were remarkably reduced by the administration of OE, and this was in good agreement with the results of the serum aminotransferases activities and hepatic lipid peroxidation levels. Necrosis, which is a more severe form of injury, was either markedly prevented or minimized by pretreatment with OE. OE (100 mg/kg) treatment alone did not cause a change in the liver histology (data not shown).

Effects of OE on CCl_4 bioactivation-related P450 2E1 activity and expression

In mice, OE pretreatment showed a dose-dependent protective effect against CCl_4-induced hepatotoxicity. It is known that CCl_4 requires P450 2E1-associated bioactivation to produce liver injury. Therefore, the effects of OE on hepatic microsomal P450 2E1-specific microsomal monooxygenase activities were examined. As shown in Table 2, hepatic microsomal fractions from mice treated with OE had significantly decreased hydroxylation activities for the two P450 2E1-specific substrates, *p*-nitrophenol and aniline, in a dose-dependent manner. The inhibitory activity of OE on the hepatic microsomal P450 2E1-specific microsomal monooxygenase activities were conformed in P450 2E1 inducer pyridine-induced hepatic

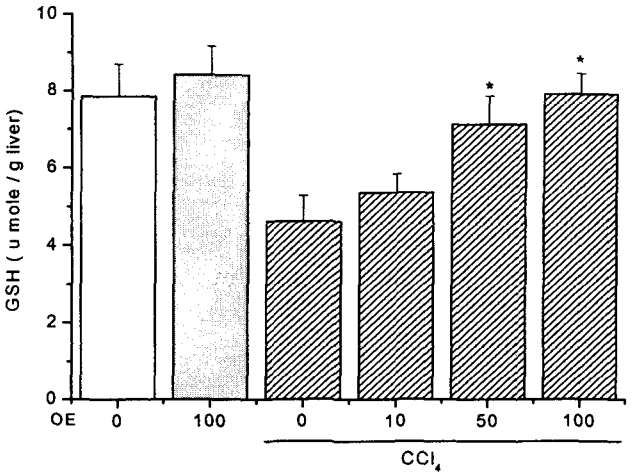

Fig. 2. Protective effect of onion extract (OE) on the CCl_4-induced depletion of hepatic cellular glutathione. Hepatic cellular glutathione (GSH) contents were measured as described in Materials and Methods. Each bar represents the mean ± SD for five mice. *Significantly different from CCl_4 at $p < 0.01$.

Table 2. Effects of OE on hepatic microsomal *p*-nitrophenol and aniline hydroxylation activities *in vivo*

Treatment (OE, mg/kg)	Activity (unit: *n*mole/mg protein/min)	
	AH	PNPH
Control	0.68 ± 0.08	2.47 ± 0.29
10	0.54 ± 0.06	2.01 ± 0.23
50	0.48 ± 0.05*	1.63 ± 0.20*
100	0.32 ± 0.04*	1.06 ± 0.15*

Mice were treated with onion extract (OE 10, 50 or 100 mg/kg, o.p.) once daily for 3 days. Control mice were given saline. Each value represents the mean ± SD of five mice. PNPH: *p*-Nitrophenol hydroxylation, AH: Aniline 4-hydroxylation.
*Significantly different from control at $p < 0.01$.

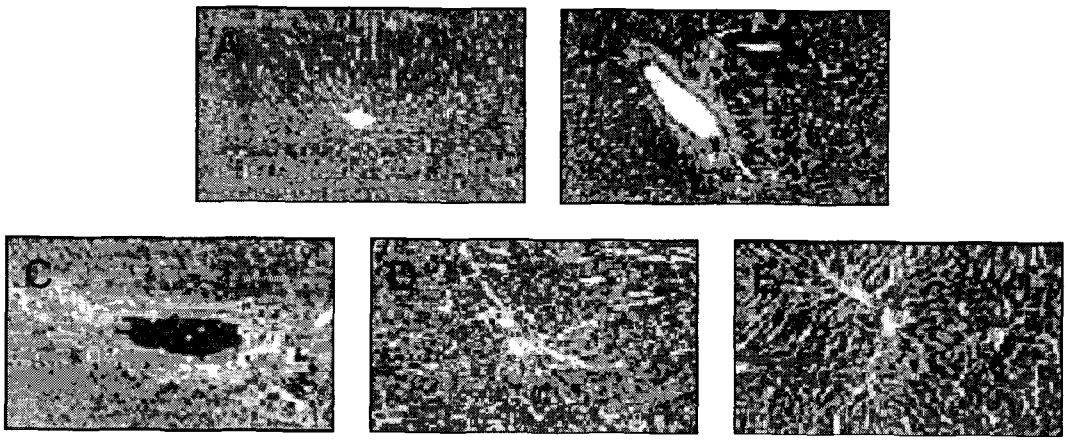

Fig 1. Effects of OE pretreatment on carbon tetrachloride-induced liver damage in mice. The mice were pretreated with OE (10, 50 or 100 mg/kg, o.p.) once daily for 3 days. Control mice were given saline. Three hours after the final treatment, mice were treated with carbon tetrachloride (CCl_4 20 mg/kg, i.p.). The mice were sacrificed 18 hr after the CCl_4 administration. (A) Liver from mouse treated with saline; (B) liver from mouse treated with CCl_4; (C) liver from mouse treated with OE (10 mg/kg) plus CCl_4; (D) liver from mouse treated with OE (50 mg/kg) plus CCl_4; (E) liver from mouse treated with OE (100 mg/kg) plus CCl_4.

microsomal incubations. Immunoblot analysis was performed to examine the effect of OE on the P450 2E1 protein expression. The hepatic microsomes from OE-treated mice were resolved by SDS-PAGE and immunoblotted with anti-P450 2E1. Immunoblot for P450 2E1 protein is shown in Fig. 3. P450 2E1 expression was suppressed by treatment with OE in a dose-dependent manner. This result is consistent with the decreases of microsomal *p*-nitrophenol and aniline hydroxylation activities (Table 2).

Effects of OE on $FeCl_2$-ascorbic acid stimulated lipid peroxidation and superoxide scavenging activity

In order to determine the antioxidant effects of OE in terms of the mechanism of its hepatoprotective effect, anti-lipid peroxidation in liver homogenate and the superoxide scavenging activity of OE were investigated (Table 3). Consistent with the results of CCl_4-induced hepatic lipid peroxidation, OE showed a dose-dependent inhibition of $FeCl_2$-ascorbic acid stimulated lipid peroxidation, with

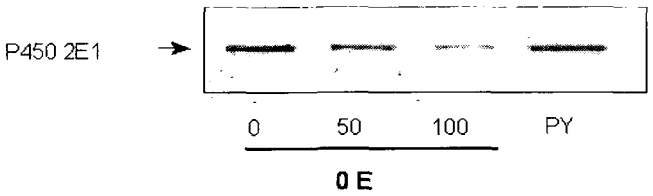

Fig. 3. Immunoblot analysis of P450 2E1. Liver microsomes were obtained from mice treated with onion extract (OE) once daily for 3 consecutive days as described in Materials and Methods. Microsomal proteins (15 μg) were electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antibody against P450 2E1. Lane 1, Control; Lane 2, OE 50 mg/kg; Lane 3, OE 100 mg/kg; Lane 4, pyridine (5 μg).

Table 3. Inhibitory effects of OE on $FeCl_2$-ascorbic acid stimulated lipid peroxidation and superoxide scavenging activity

Inhibition of lipid peroxidation[1)]		Inhibition of scavenging activity[2)]	
Addition (mg/mL)	(%)	Addition (mg/mL)	(%)
OE 0.01	9.7±1.4[3)]	OE 0.01	5.1±1.6
OE 0.1	36.8±4.8	OE 0.1	14.3±2.4
OE 0.5	43.5±7.2*	OE 0.5	53.9±4.4
OE 1.0	72.8±9.3*	OE 1.0	87.8±6.7*

[1)]Rat liver homogenates were stimulated with $FeCl_2$-ascorbic acid in the presence or absence of onion extract (OE) and lipid peroxidation was measured as described in Materials and Methods.
[2)]Superoxide was generated by oxidation of xanthine/xanthine oxidase in the presence or absence of OE and scavenging activity was measured as described in Materials and Methods.
[3)]Values are presented as the mean of the percentage inhibition ±SD for three independent experiments, performed in triplicate.
*Significantly different from control at $p<0.01$.

an IC_{50} value of 0.63 mg/mL in liver homogenate. OE also showed superoxide scavenging activity with an IC_{50} value of 0.48 mg/mL.

DISCUSSION

Liver injury induced by carbon tetrachloride (CCl_4) is one of the best characterized systems of xenobiotic-induced hepatotoxicity and is a commonly used model for screening drugs for anti-hepatotoxic/hepatoprotective activity (3,4,19). The rise in the serum levels of lactate dehydrogenase, AST and ALT was brought to rise in the serum levels (2,19). The present results demonstrate that pretreatment of mice with onion extract (OE) protected mice against CCl_4-induced hepatotoxicity, as evidenced by decreased serum aminotransferase activity and hepatic lipid peroxidation in a dose-dependent manner (Table 1). Moreover, these results were found to be dose-dependent, specifically, OE pretreatment at dosages from 10 to 100 mg/kg was found to significantly decrease CCl_4-induced hepatotoxicity. Whileas in histological findings, the degree of damage was dependent to the dose of OE. This phenomenon was also confirmed by histological observation (Fig. 1). It is now generally accepted that the hepatotoxicity of CCl_4 is the result of reductive dehalogenation, which is catalyzed by P450 and forms the highly reactive trichloromethyl free radical which readily interacts with molecular oxygen to form the trichloromethyl peroxy radical (3,4,19,20). Our results support this hypothesis, in that there is a good correlation between the decreased P450 2E1 enzyme activity in OE-treated hepatic microsomes in vivo and the level of protection against CCl_4-induced hepatotoxicity in mice (Table 1, 2 and Fig. 1). The hydroxylation of *p*-nitrophenol and aniline, as used in the present study, has been used extensively to probe the activity of P450 2E1 (5,7,12,15,21). In the immunoblot analysis, OE reduced the expression of P450 2E1 (Fig. 3). The decreased levels of P450 2E1 by OE were consistent with the results of these monooxygenase activities. The above observations suggest that the inhibition of P450 2E1 by OE in mice plays an important role in the OE-induced hepatoprotection against CCl_4. Thus, the inhibition of P450 2E1 by OE not only plays an important role by protecting against the hepatotoxicity of CCl_4, but also may play a role in modulating the toxicity of other xenobiotics, by acting as a chemopreventive agent by decreasing metabolic activation.

In contrast to the toxic activation of CCl_4 *via* the P450 2E1 pathway, the detoxification pathway involves glutathione (GSH) conjugation of the trichloromethyl radical, a P450 2E1-mediated CCl_4 metabolite. Previous studies on the mechanism of CCl_4-induced hepatotoxicity have

shown that GSH plays a key role in the detoxification of the reactive toxic metabolites of CCl_4 and that liver necrosis begins when GSH stores are markedly depleted (4,19). Our results show that pretreatment with OE significantly inhibits lipid peroxidation (Table 1) and significantly reduces CCl_4-induced hepatic GSH depletion (Fig. 2). This is attributed to the decreased bioactivation of CCl_4 as a result of OE pretreatment (Table 3). The previous report showed that OE significantly increased hepatic GSH levels and cytosolic glutathione-S-transferase activity (11). These results showed that the protection afforded by OE against CCl_4-induced hepatotoxicity may, at least in part, be related to the increase of cellular GSH content or glutathione-S-transferase activity.

Lipid peroxidation (LPO) is accepted to be one of the principal causes of CCl_4-induced liver injury, and is mediated by the production of free radical derivatives of CCl_4. The initiation of LPO is carried out in most cases by free radicals such as superoxide and hydroxyl radicals, and other reactive oxygen species like H_2O_2, causing cellular injury by the inactivation of membrane enzymes and receptors, depolymerization of polyscharides as well as protein cross-linking and fragmentation. Thus, antioxidant activity and/or the inhibition of free radical generation are important in terms of protecting the liver from CCl_4-induced damage (22). *In vitro* lipid peroxidation in a liver homogenate can proceed in a non-enzymatic manner. The process is induced by ascorbate in the presence of Fe^{2+}/Fe^{3+}, and it has been reported that Fe^{2+} and ascorbic acid stimulate lipid peroxidation in rat liver microsomes and mitochondria. In order to clarify the mode of action of OE, *in vitro* lipid peroxidation experiments were carried out. The results indicated that OE inhibited the $FeCl_2$-ascorbic acid-stimulated lipid peroxidation in liver homogenate (Table 3). Moreover, our experimental results show that OE exercised a free radical scavenging activity upon the superoxide radical generated using the xanthine-xanthine oxidase system (Table 3), and may therefore act by scavenging free radicals and reactive oxygen species formed during CCl_4 metabolism. Hepatoprotective effects of flavonoids in OE on the CCl_4-induced liver injury have been reported to be due to its antioxidative actions (23,24).

In conclusion, the results of this study demonstrate that OE has a potent hepatoprotective action against CCl_4-induced liver damage in mice. These results show that the hepatoprotective effects of OE may be due to its ability to block the bioactivation of CCl_4 by inhibition of P450 2E1 activity and its expression, which results in decreased formation of trichloromethyl radicals, and also by its antioxidant activity in combination with its ability to scavenge free radicals and inhibit lipid peroxidation, all of which are capable of hepatocellular injury.

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