大韓獸醫學會誌 (2012) 第 52 卷 第 4 號
Korean J Vet Res(2012) 52(4) : 275~280

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Anti-oxidant activities of kiwi fruit extract on carbon tetrachloride-induced liver injury in mice

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(Received: February 16, 2012; Revised: November 5, 2012; Accepted: November 27, 2012)

Abstract : The kiwi (*Actinidia deliciosa*) is well known to contain anti-oxidants. In this study, we investigated the anti-oxidant effects of kiwi extract on carbon tetrachloride (CCl_4) induced liver injury in BALB/c mice. The radical scavenging effect of 80% methanol extract of Halla-Gold kiwi was observed. For the animal study, mice were randomly divided into four groups: normal group, CCl_4-induced model group, kiwi extract administered group, and silymarin treated group. The kiwi extract was provided daily for 10 days. At the 24 h after last administration, CCl_4 was injected. The kiwi extract showed strong inhibitory effect of DPPH radicals and superoxide scavenging. In animal study, administration of CCl_4 resulted in significantly elevated plasma levels of ALT and AST but they decreased in kiwi-extract pretreated group. Anti-oxidant enzymes such as GSH-px and GSH-rd were restored in the kiwi extract treatment group. Histopathological degeneration was also prevented in the kiwi extract treated group compared with of the control group, which exhibited CCl_4-induced hepatotoxicity. On the basis of the obtained results, it can be concluded that kiwi extract showed protective effects, not only as anti-oxidant effects, but also in the protection of hepatotoxicity in CCl_4-intoxicated mice.

Keywords : antioxidant, carbon tetrachloride, cytochrome P450 2E1, hepatotoxicity, kiwi fruit

Introduction

Liver injury has increased as a result of exposure to higher levels of environmental toxins, as the liver plays an important role in the detoxification of most environmental toxins entering the body [10]. It is well known that the hepatotoxic effect of carbon tetrachloride (CCl_4) is due to oxidative damage by free radical generation, and the antioxidant properties of hepato-protective drugs are their primary functionalities. Because of the similarity of cirrhotic responses induced by CCl_4 in animals, as compared to humans [16, 21, 23], CCl_4 is commonly used as a hepatotoxin in experimental studies [13]. Kiwi fruits contain abundant vitamins, polyphenols, and lipophilic constituents, and are used for the treatment of many different types of cancers like stomach, lung and liver cancer [4, 17]. Several studies have shown that extracts of kiwi fruits inhibit cancer cell growth and exhibit cell protection against oxidative DNA damage in *vitro* [17]. However, it is not known whether kiwi fruits can prevent or alleviate liver injury induced by CCl_4, and the mechanisms by which kiwi fruits may protect against CCl_4-induced hepatotoxicity are unclear. In this study, we aimed to investigate the effects of kiwi fruits, especially Halla-Gold kiwi fruit produced in Jeju island in South Korea, on CCl_4-induced oxidative stress.

Materials and Methods

Preparation of the kiwi extract

Korean kiwifruit (*Actinidis chinensis* var. "Halla Gold") a new kiwifruit cultivar bred by the Jeju Rural Development Administration. The pulp of the kiwi fruits were extracted in 80% MeOH after freeze-drying, and were thereafter referred to as kiwi extract. The kiwi extracts were freeze-dried and were used for *in vitro* experiments and for animal treatment studies.

In vitro assay of antioxidant activity

Diphenylpicrylhydrazyl (DPPH) radical scavenging assay: The scavenging activity of kiwi extract was measured using the stable radical DPPH. Briefly, the kiwi extract was added to 0.2 mM DPPH solution (100 μL) at various concentrations (0~10 mg/mL) (100 μL), and was incubated at 37°C for 30 min. The absorbance was measured at 490 nm and vitamin C and E were used as reference compounds. The inhibition percentage (%) of radical scavenging activity was calculated as $(1 - A_s / A_0) \times 100$, where A_0 and A_s are the absorbance of the control and sample, respectively.

O_2^- scavenging assay: The O_2^- scavenging activity was measured by monitoring the O_2^- induced reduction of

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nitroblue tetrazolium chloride (NBT) to the blue chromogen diformazan [7]. O_2^- was generated by the phenazine methosulphate (PMS)/nicotinamide adenine dinucleotide (NADH) system. The reaction mixtures in the sample wells contained the following reagents: 0.5 mM NBT (20 μL), 0.5 mM NADH (60 μL), 0.1mM PMS (20 μL) and kiwi extract at various concentrations (0~10 mg/mL) (20 μL). All reagents and kiwi extract were dissolved in 30 mM Tris-HCl, pH 8.0. After the mixtures were incubated at 37°C for 10 min, the absorbance was measured at 560 nm and was compared with the vitamin E content.

Animals and treatment

6-week-old, male BALB/c mice were purchased from Orient (Korea). All mice were allowed free access to rodent chow (Charles River, USA) and tap water, maintained in a controlled environment at 22 ± 2°C and 50 ± 5% relatively humidity with a 12 h dark/light cycle, and acclimated for at least 1 week before use. All animal experiments were carried out in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Jeju National University (approval No. 20080010). Halla-Gold 80% MeOH extract were prepared in saline, and silymarin (Sigma, USA) was prepared by suspension in 5% carboxyl methylcellulose as test compound. Mice were randomly divided four groups of 10. One group of mice was given saline (0.05 mL/10 g/day, p.o.) as a control and a second group of mice were given saline (0.05 mL/10 g/day, p.o.) as a CCl_4 control. The third group of mice received Halla-gold 80% MeOH (100 mg/kg/day, p.o.) and the forth group of mice received silymarin (100 mg/kg/day, p.o.). All groups were treated once daily for 10 consecutive days. 24 h after the final treatment, the first group of the mice were treated with olive oil (Sigma) (0.1 mL/10g, i.p.) and the other groups of mice were treated with CCl_4 (20 mg/kg, i.p., dissolved in olive oil). 24 h after administration of olive oil and CCl_4, the mice were sacrificed with 95% CO_2 and 5% O_2 gas, and blood was collected from the caudal vena cava. The cranial lobes of livers were separated and were fixed in 10% buffered formaldehyde solution. The remainder of the livers were frozen quickly in dry ice and were stored at –80°C for future study.

Measurement of plasma alanine aminotransferase and aspartate aminotransferase

Plasma alanine amino transferase and aspartate amino transferase were measured by automatic blood analyzer Pronto evolution (BPC, Italy) using commercial reagents (Asan pharm, Korea).

Measurement of NOx concentration and antioxidant activity

Analysis of NOx concentration in plasma: The plasma level of NO metabolites (NOx; nitrite and nitrate) was measured with an automated NO analyzer (Eicom, Japan). Briefly, nitrite and nitrate in plasma were separated by a reverse-phase separation column and the nitrate was reduced to nitrite in a reduction column. Nitrite was then mixed with Griess reagents (sulfanilamide and naphthalene-ethylene diamine dihydrochloride), and the absorbance at 540 nm was measured by a flow-through spectrophotometer.

Assay of glutathione peroxidase activity in RBC: Total glutathione peroxidase (GSH-Px) activity in RBC was assayed using a commercial kit (Cayman chemical, USA). Briefly, assay buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM EDTA) and a co-substrate mixture containing nicotinamide adenine dinucleotide phosphate (NADPH) were mixed and were added to RBC lysates. The reaction was started by the addition cumene hydroperoxide as quickly as possible, and was monitored by continuous spectrophotometry at 340 nm and 25°C.

Assay of glutathione reductase activity in liver: Total glutathione reductase (GSH-Rd) activity in liver was assayed using commercial kit (Cayman chemical, USA). Briefly, after assay buffer (50 mM potassium phosphate, pH 7.5, containing 1 mM EDTA) and oxidized glutathione (GSSG) were mixed, added to liver homogenates. The reaction was started by the addition NADPH as quickly as possible, and monitored by a continuous spectrophotometry at 340 nm and 25°C.

Western blot

For immunoblotting, liver tissues were homogenized in buffer containing 5 mM Tris-HCl pH 7.4, 320 mM sucrose, supplemented with fresh 1 mM phenylmethylsulfonyl fluoride (PMSF).

Liver homogenates were centrifuged at 10,000 × g for 20 min at 4°C, and proteins in supernatants were estimated using the modified Bradford assay [1]. 40 μg proteins were resolved using 10% (v/v) SDS-PAGE and were transferred onto PVDF membrane (Millipore, USA) for 90 min at 110 V. They were then incubated in blocking buffer TTBS (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% Tween-20) supplemented with 5% (w/v) skimmed milk for 90 min, washed in TTBS for 30 min and incubated with the following antibodies: cytochrome P4502E1 (1 : 5,000; Abcam, UK), anti-4-hydroxylnonenal (1 : 1,000; R&D systems, USA) and β-actin (1 : 2,500; Sigma) diluted in TTBS overnight at 4°C. Following 50 min washes in TTBS the membranes were incubated with anti-rabbit IgG or anti-mouse IgG HRP-linked secondary antibodies (Santa Cruz Bitotechnology, USA) at 1 : 10,000 or 1 : 5,000 in TTBS for 40 min. Reactive bands were revealed using enhanced chemiluminesence reagents (Intron Biotechnology, USA).

Histological examinations

Livers were isolated from mice and were fixed in 10% formaldehyde. The fixed tissues were then embedded in paraffin and were sectioned (4.0 μm). The sections were then

Table 1. Effects of kiwi 80% MeOH extract on radical scavenging

	DPPH radical IC_{50} (mg/mL)	Superoxideradical IC_{50} (mg/Ml)
Vit C	0.01 ± 0.001	–
Vit E	0.01 ± 0.001	0.24 ± 0.03
Halla-gold kiwi	$1.91 \pm 0.49^{***}$	$1.86 \pm 0.04^{***}$
Zespri	$2.37 \pm 0.39^{***}$	$7.3 \pm 0.8^{***\#\#\#}$

Values are mean ± SD. $^{***}p < 0.001$ *vs.* VitE, $^{\#\#\#}p < 0.001$ *vs.* Halla-Gold kiwi.

stained with hematoxylin and eosin and were examined under light microscope to determine histological changes.

Statistical analysis

All results are presented as the mean ± SEM. Comparisons among the results were carried out using LSD tests and one way analysis of variance (ver. 12.0; SPSS, USA). *P* values < 0.05 were considered significant.

Results

Anti-oxidative effects of Halla-Gold kiwi fruit extract

An investigation of the anti-oxidant effects *in vitro* demonstrated that Halla-Gold kiwi fruit extract had stronger anti-oxidant effects compared with green kiwi extract (Table 1).

Effect of Hall-Gold kiwi fruit extract on CCl_4-induced hepatotoxicity

Serum hepatic and lipid biochemical data for the evaluation of CCl_4-induced hepatotoxicity are showed in Fig. 1, respectively. There was a significant increase of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the CCl_4-treated group, as compared to the control, indicating CCl_4-induced damage to the hepatic cells ($p < 0.001$). However, treatment with kiwi extract prior to the CCl_4 challenge was observed to reverse the CCl_4-induced alteration of ALT and AST ($p < 0.001$). These results suggest that kiwi extract provides protection against CCl_4-induced liver injury in mice.

Effect of Hall-Gold kiwi fruit extract on anti-oxidative enzymes and NO_X levels in CCl_4-induced hepatotoxicity

GSH-px activity in RBC, and plasma NOx concentrations are shown in Figs. 2 and 3. GSH is a non-enzymatic antioxidant in the detoxification pathway, which reduces the toxic metabolites of CCl_4. GSH-px activity determined in the CCl_4 group was significantly lower ($p < 0.01$) than that in the control. Mice treated with kiwi extract showed significantly increased GSH-px activity ($p < 0.05$). In addition, a high production of NO has been suggested as a cause of tissue injury. Mean NO_X levels among the groups were not found to differ in this study. The activity of GSH-rd in liver was showed in Fig. 4. GSH-rd activity in the CCl_4 group was reduced, but

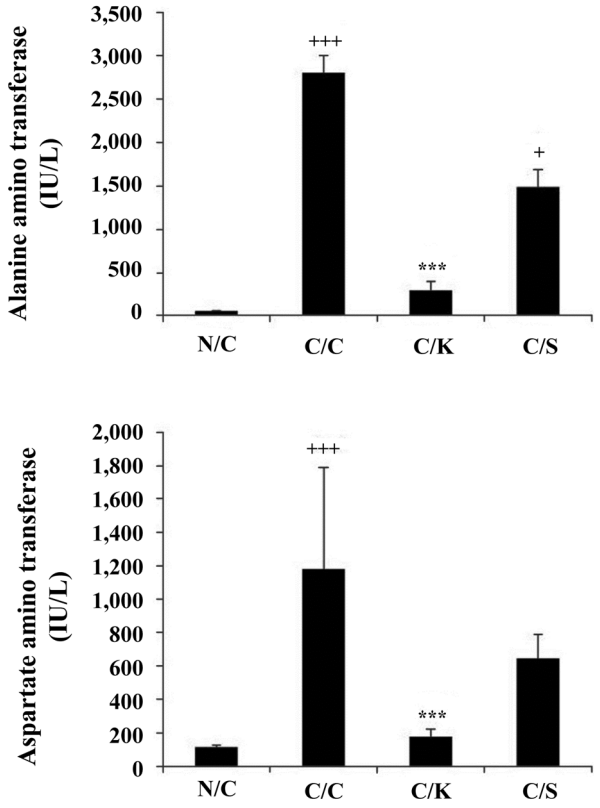

Fig. 1. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. (A) ALT and (B) AST levels in serum decreased by treatment of kiwi extract. N/C: normal mice, C/C: CCl_4 control, C/K: injected with CCl_4 after treatment of Halla-Gold kiwi fruits, C/S: injected with CCl_4 after treatment of silymarin. Values are mean ± SEM. $^{+}p < 0.05$, $^{+++}p < 0.001$ *vs.* N/C; $^{***}p < 0.001$ *vs.* C/C.

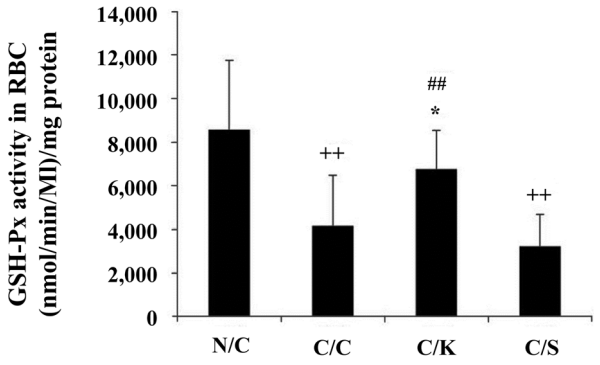

Fig. 2. Glutathione peroxidase activity in RBC. N/C: normal mice, C/C: CCl_4 control, C/K: injected with CCl_4 after treatment of Halla-Gold kiwi fruits, C/S: injected with CCl_4 after treatment of silymarin. Values are mean ± SEM. $^{++}p < 0.01$ *vs.* N/C; $^{*}p < 0.05$ *vs.* C/C; $^{\#\#}p < 0.01$ *vs.* C/S.

kiwi extract and syrimarin prevented inhibition of enzyme activity.

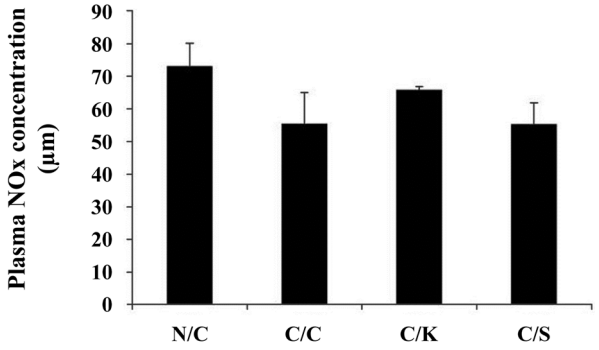

Fig. 3. Plasma nitrite and nitrate (NOx) concentration. N/C: normal mice, C/C: CCl_4 control, C/K: injected with CCl_4 after treatment of Halla-Gold kiwi fruits, C/S: injected with CCl_4 after treatment of silymarin. Values are mean ± SEM.

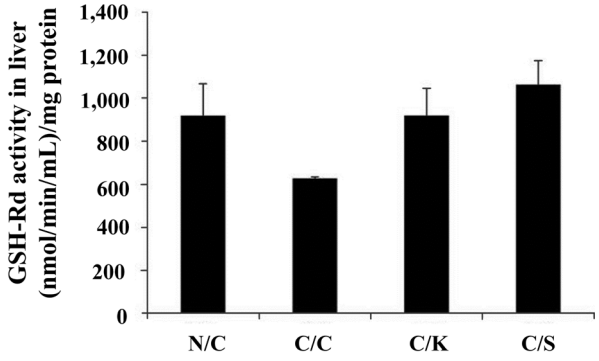

Fig. 4. Glutathione reductase activity in liver. N/C: normal mice, C/C: CCl_4 control, C/K: injected with CCl_4 after treatment of Halla-Gold kiwi fruits, C/S: injected with CCl_4 after treatment of silymarin. Values are mean ± SEM.

Effect of Hall-Gold kiwi fruit extract on 4-HNE and CYP450 2E1 expressions

4-hydroxynonenal (4-HNE) and CYP450 2E1 expressions in mouse liver homogenates are shown in Figs. 5 and 6. 4-HNE, an aldehyde product of lipid peroxidation, is relatively stable and is able to diffuse into the cytosol from the site of production in the membrane [5, 6]. 4-HNE expression in the CCl_4 group was significantly increased ($p < 0.01$) compared to that in the control. Pretreatment of kiwi extract significantly prevented up regulation of 4-HNE expression by CCl_4 challenge. To investigate the possible mechanisms by which kiwi extract prevented the hepatic injuries induced by CCl_4, hepatic CYP2E1 expression was assessed. CCl_4 treatment significantly suppressed hepatic CYP2E1 expression, as compared to the control ($p < 0.01$). In contrast, kiwi extract increased ($p < 0.05$) hepatic CYP2E1 expression significantly.

Histopathological examination

At the microscopic level, liver architecture and hepatocytes of normal mice had no degenerative and lipomatous changes (Fig. 7). In the CCl_4-treated group, fatty degenera-

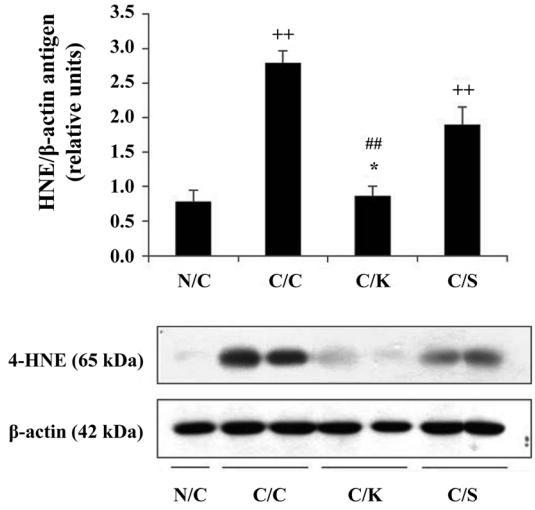

Fig. 5. 4-hydroxynonenal expression in liver. N/C: normal mice, C/C: CCl_4 control, C/K: injected with CCl_4 after treatment of Halla-Gold kiwi fruits, C/S: injected with CCl_4 after treatment of silymarin. Values are mean ± SEM. $^{++}p < 0.01$ *vs.* N/C; $^{**}p < 0.01$ *vs.* C/C; $^{\#\#}p < 0.01$ *vs.* C/S.

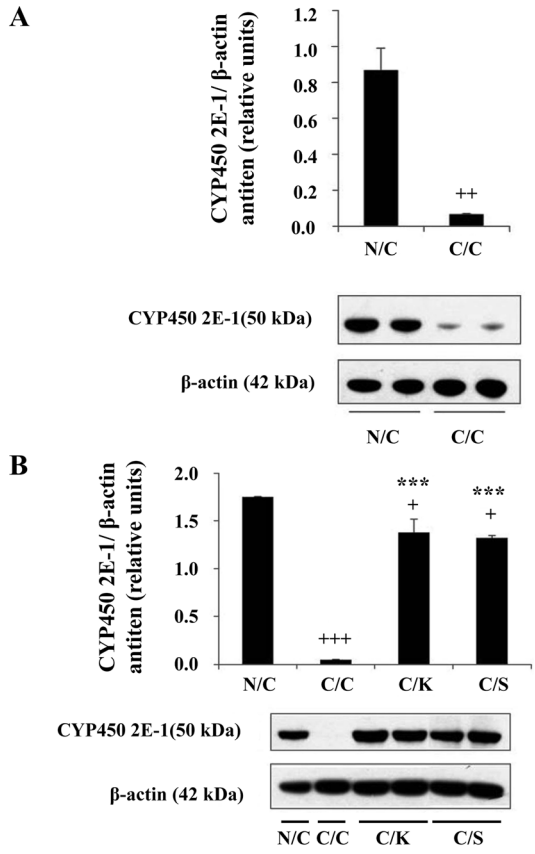

Fig. 6. Cytochrome450 2E1 expression in liver. (A) Cytochrome450 2E1 expression reduced by CCl_4 treatment. (B) Kiwi extract and syrimarin restored Cytochrome450 2E1 expression. N/C: normal mice, C/C: CCl_4 control, C/K: injected with CCl_4 after treatment of Halla-Gold kiwi fruits, C/S: injected with CCl_4 after treatment of silymarin. Values are mean ± SEM. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ *vs.* N/C; $^{***}p < 0.001$ *vs.* C/C.

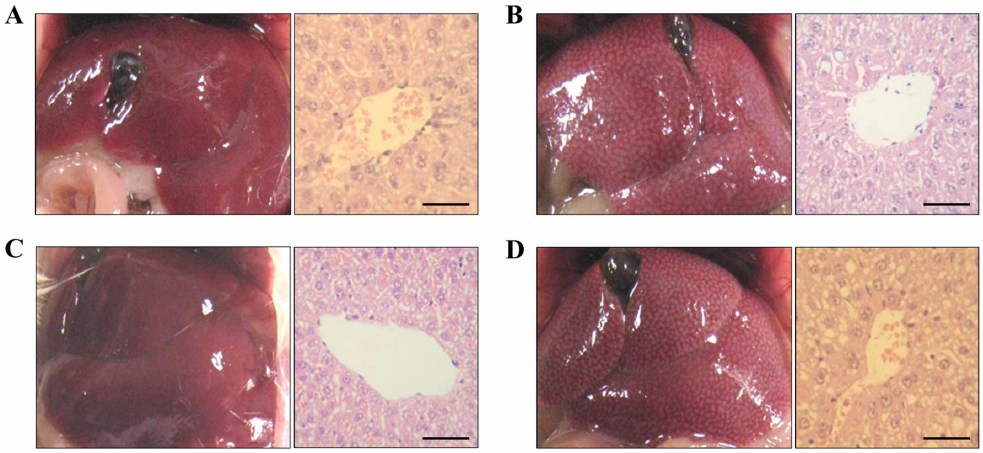

Fig. 7. Histopathology in liver. (A) normal mice, (B) CCl_4 control, (C) injected with CCl_4 after treatment of Halla-Gold kiwi fruits, (D) injected with CCl_4 after treatment of silymarin. Scale bars = 50 μm.

tion, especially that of hepatocytes, settled in the central zone was clearly identified. In the kiwi extract group, the liver and hepatocytes were in good condition, and were similar to those observed in control animals.

Discussion

Liver damage induced by CCl_4 is a model commonly used to screen for the anti-hepatotoxic/hepatoprotective activity of drugs [2, 3]. The increased plasma levels of ALT and AST, which are released into the circulation after cellular damage, have been suggested to damage the structural integrity of the liver [19]. In the present study, pretreatment with kiwi extract effectively protected mice against CCl_4-induced hepatotoxicity, as evidenced by decreased plasma ALT and AST levels as compared to a positive control. Increased oxidative stress represents an imbalance between the intracellular production of free radicals and cellular defense mechanisms. Especially, lipid peroxidation is one of the most important markers of oxidative stress. Many studies have reported that CCl_4 increases hepatic levels of MDA, depletes GSH, and decreases the activities of antioxidant enzymes [12, 18, 28].

The expression of 4-HNE induced by treatment of CCl_4 in this study was as a result of the increase of lipid peroxidation. 4-HNE forms covalent cross-links with proteins via lysine, cysteine, and histidine residues [25, 26]. 4-HNE is normally detoxified by conjugation with GSH [11, 22, 27]. In this study, GSH levels were not measured, but the observed increase in the expression of 4-HNE could have been due to a depletion of GSH stores. The GSH system includes antioxidant enzymes such as GSH-px, GSH-rd and glutathione-S-transferase. The determination of these enzymes is appropriate for an assessment of the oxidative stress status in cells, and in CCl_4 treated rats, deletion of GSH-px, GSH-rd and GSH has been previously reported [15]. In this study, kiwi extract maintained the activity of GSH-rd and reduced 4-HNE expressions. Therefore, one of the protective mechanisms of kiwi extract against acute hepatotoxicity could be the restoration of anti-oxidative enzyme activity and prevention of lipid peroxidation.

Biotransformed metabolites of CCl_4 formed by cytochrome P450 (CYP) 2E1 include trichloromethyl radical (CCl_3) and trichloromethyl peroxyl radical (CCl_3O_2). The role of CYP2E1 in the initiation of CCl_4-induced hepatotoxicity has already been reported [29]. By using cyp2e1−/− mice, no hepatotoxicity of CCl_4 was observed in the absence of CYP2E1, unlike the significant liver damage observed in the wild type control mice. However, in the present study, the expression of CYP2E1 was dramatically decreased by CCl_4 treatment. This result was in agreement with the previous reports [24, 28], in which treatment of mice with CCl_4 caused a reduction in levels of CYP2E1 protein and mRNA. After CCl_4 is bioactivated, the resulting reactive radical binds covalently to CYP2E1, either at the heme group of CYP or at the active site of the enzyme, causing suicidal inactivation of the CYP pathway [8, 20]. It suggests that the other potential mechanism of the protective effect of kiwi extract may be mediated through a direct interaction with the reactive metabolite of CCl_4, since kiwi extract has novel radical scavenging activity.

Our study demonstrated the anti-oxidative activity of Halla-Gold kiwi extract. Kiwi fruit was previously reported to contain a number of anti-oxidant constituent, such as vitamin C and E, caffeic acid, naringenin, quercetin and epicathechin [9]. Overall, gold kiwi was evaluated to have better anti-oxidative effects than green kiwi, both of which having stronger anti-oxidative effects than other fruits [14], and this was further confirmed in the present study.

Therefore, we proposed the novel role of Halla-Gold kiwi fruit of Jeju Island in South Korea for the effective prevention of hepatotoxicity and further disease caused by excessive oxidation.

Acknowledgments

This study was carried out with the support of "Coopera-

tive Research Program for Agricultural Science & Technology Development (Project No. PJ006686)", Rural Development Administration, Republic of Korea.

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