The Journal of Applied Pharmacology, **14**, 207-215(2006)

Protective Effect of Sachungwhan against CCl_4-induced Hepatotoxicity

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(Received 1 December, 2006; Accepted 21 December, 2006)

Abstract – Sachungwhan reduced hepatotoxicity induced by carbon tetrachloride (CCl_4). Improved liver function was observed by measuring the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine (CRE), total cholesterol (TCHO), triglyceride (TG), low density lipoprotein cholesterol (LDL-CHO), high density lipoprotein cholesterol (HDL-CHO), total protein (TP), albumin (ALB) and total bilirubin (BIL) in serum. Hepatic parameters monitored were levels of cholesterol (CHO), triglyceride (TG), malondialdehyde (MDA), content of cytochrome P450 (CYP), level of glutathione (GSH), and activities of NADPH-CYP reductase, superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx). The histopathological examination showed that the treatment of Sachungwhan relieved the ballooning degeneration of hepatocytes which had been generated by CCl_4. The results suggested that hepatoprotective effects of Sachungwhan possibly are due to their promising antioxidative activity.

Keywords □ Sachungwhan, CCl_4, free radical, lipid peroxidation, hepatotoxicity

INTRODUCTION

Sachungwhan is the extract of oriental herbal composition. It is reported that Sachungwhan affects blood deficiency, blood circulation, clearing heat, draining fire, anti-algesthesis, anti-convulsion(Kwon *et al.*, 2002).

Silymarin is a standardized mixture of antioxidant flavonolignans (silybin and silibinin) extracted from the medicinal plant *Silybum marianum*. It is a free-radical scavenger and a membrane stabilizer that prevents lipoperoxidation and its associated cell damage in some experimental models(Soto *et al.*, 1998). Silymarin was proved to have a protective effect against experimental hepatotoxicity by regulating the actions of the ultrastructures of the liver cells, and improving the activities of hepatic enzymes and bile production(Hagymasi *et al.*, 2002; Lucena *et al.*, 2002).

Carbon tetrachloride (CCl_4) is an extensively used xenobiotic to induce lipid peroxidation and toxicity. CCl_4 is metabolized by cytochrome P450 2E1 (CYP2E1) to the trichloromethyl radical ($CCl_3\cdot$), which is assumed to initiate free radical-mediated lipid peroxidation leading to the accumulation of lipid-derived oxidation products that cause liver injury (Recknagel *et al.*, 1989; Poli *et al.*, 1987). Also, CCl_4 in a large dose damages the endoplasmic reticulum (ER), induces accumulation of lipids, and reduces protein synthesis and mixed function oxidase activity (Recknagel, 1967). Polyunsaturated fatty acids (PUFAs) in membrane lipids are especially susceptible to free radical-initiated peroxidation (Svingen *et al.*, 1979). PUFAs in phospholipids of the ER were decreased following in vivo CCl_4 administration (James *et al.*, 1982).

Aerobic organisms generate superoxide anion radical ($O\cdot_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) of which the latter initiates lipid peroxidation in tissues (Oruc *et al*, 2000). The sensitivity of cells to oxidants can be prevented to some extent by antioxidant enzymes such as SOD, GPx, CAT, GR and glucose-6-phosphate dehydrogenase. These antioxidant enzymes allow only a relatively low rate of production and propagation of the reactive and harmful ·OH.

Pesticide and environmental chemicals have been reported to induce oxidative stress leading to generation of free radicals with alteration of the action of antioxidants or oxygen free radical (OFR) scavenging enzyme system (Ray *et al*, 1998; Koner *et al.*, 1998; Ahmed *et al.*, 2000).

The aim of this work was to determine if the action mecha-

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nism of Sachungwhan(Sa) on CCl_4-induced liver damage occurs by preventing lipid peroxidation.

MATERIALS AND METHODS

Materials

Silymarin, 1.1.3.3-tetraethoxypropa- ne, cytochrome C, xanthine oxidase, NADPH, Na-K tartrate, GSH, 1-chloro-2,4-dinitrobenzene, glutathione reductase, cumene hydroperxide, oxidized glutathione, 5.5'-Dithiobis(2-nitrobenzoic acid), bovine serum albumin, hydrosulfite, phosphoric acid, thiobarbituric acid, tris acetate, tris base, tris HCl, EDTA, glycerol, foline ciocalteu's phenol reagent, xanthine, sodium dithionite, metaphosphoric acid, xylene, ALT kit, AST kit, ALP kit, BIL kit, ALB kit, TP kit, BUN kit, CRE kit. Above all chemicals were supplied by Sigma-Aldrich. Sodium phosphate dibasic dihydrate was from Merck. Carbon tetrachloride was from Duksan. CHO kit and TG kit were from BCS. All the chemicals used were of analytical reagent grade quality.

Sachungwhan herbal medicine is composed as followed:
Angelicae Radix-3.750 g
Cnidii Rhizoma-3.750 g
Angelicae Koreane Radix-3.750g
Phelloptori Radix-3.750g
Rhei Rhizoma-3.750g
Gardeniae Fructus-3.750g
Gentianae Scabrae Radix-3.750g
total 26.250g

Sachungwhan (26.250g) was added in round bottom flask with tab water 2,500 mL. It was concentrated 3 hours at 500 W heater with rotary vacuum evaporator to 240 mL.

Animals and treatment

Male Sprague-Dawley rats weighing 150~200 g were divided into four groups of 6 animals each.

The animals were housed in the temperature and humidity controlled room with a 12 hours (h)-light/dark cycle and with free access to Samtaco pellet diet and drinking water.

Control group received the vehicle only. Sa+CCl_4 group was orally administrated Sachungwhan (4.3 g/kg/day) and Sily (30 mg/kg/day) for 4 days, respectively. CCl_4 (0.45 mL/kg/10 mL in olive oil) was intraperitoneally injected at 3 hours after the last treatment with agent.

At 18 h after the last treatment, the animals were sacrificed in ether anesthesia. The liver tissues were quickly excised, rinsed in PBS and used immediately or stored frozen at -70°C until analysis.

Biochemical analysis

Blood samples were obtained from the cardiac puncture. AST, ALT, ALP, BUN, CRE, TCHO, TG, LDL-CHO, HDL-CHO, TP, ALB and BIL in serum were measured using commercially available kits.

Histological observation

Fresh liver tissues, previously trimmed to approximately 0.2 mm thickness, were placed in plastic cassettes and immersed in 10% formalin for 24 h. Fixed tissues were processed routinely, and then embedded in paraffin, sectioned, deparaffinized, and rehydrated using standard techniques. The extent of CCl_4-induced necrosis was evaluated by assessing morphological changes in liver sections stained with hematoxylin and eosin, using standard techniques.

Measurement of CHO and TG in liver tissue

Rats were sacrificed for the assay at 18 hours after CCl4 treatment. Partial liver was put into phosphate buffer (pH 7.0) and was made into 10% liver homogenate. Then, CHO and TG in it were analyzed by the kit.

Fractionation microsome and cytosol

The rat liver was homogenized in 3 volume of 0.1 M Tris-KCl buffer (0.1 M Tris acetate, 0.1 M KCl, 1 mM EDTA, pH 7.4 with Tris base) on ice. The homogenate was centrifuged at 10,000 *g* for 30 min. This supernatant was ultra centrifuged at 100,000 *g* for 90 min to obtain the cytosol. The pellet was resuspended in 0.1 M sodium pyrophosphate buffer (0.1 M sodium pyrophosphate and 1 mM EDTA) and ultra centrifuged at 100,000 *g* for 60 min to obtain the microsome. The microsome was resuspended in 50 mM Tris acetate buffer (50 mM Tris acetate, 20% glycerol, pH 7.4 with Tris base) and till use the fraction was stored at -70°C. Above examination was performed under 4°C.

Measurement CYP

Microsome with (protein 1~2 mg/mL) was put into 0.1 M phosphate buffer (pH 7.4). This solution was divided into two reference cells and sample cell. In semimicrocuvette, after adding sodium dithionite to 1 mL of the above solutions respectively, we set criteria at 400~500 nm. After gas bubbled in cell by 1 bubble/sec for 1 minute, we measured absorbance of

CYP-CO binding complex spectrophotome- trically at 400~500 nm. CYP was calculated by differences of absorbance between 450 nm and 490 nm which CYP-CO complex formation was before or behind (molecular coefficient; 91 $mM^{-1}cm^{-1}$) (Omura *et al*, 1964).

Measurement NADPH-CYP reductase activitiy

To 300 μM potassium phosphate (pH 7.7) 1 mL with 40 nM cytochrome C, we added microsome 50 μL and mixed 0.1 μM NADPH 1 mL. Immediately, absorbance at 550 nm at 30°C for 5 min was measured every minute. Molecular coefficient is 21 $mM^{-1}cm^{-1}$ (Strobel *et al*, 1978).

Measurement of lipid peroxidation, SOD and CAT

Lipid peroxidation (LPO) in the liver homogenate was measured as MDA production, and was assayed in the thiobarbituric acid reaction as described by Mihara and Uchiyama (1978), using the spectrophotometer (Agilent 89090 A, 8453). The results are expressed as "nmol" MDA/g tissue weight.

SOD activity was determined by an indirect method involving the inhibition of cytochrome C reduction. In this method, SOD competes with cytochrome C for O_2^-, generated by hypoxanthine and xanthine oxidase action. The reduction of cytochrome C by O_2^- was monitored by the absorbance increase at 550 nm (Fridovich, 1995)

CAT activity was measured by the decrease in absorbance at 240 nm due to H_2O_2 consumption (extinction coefficient; 43.6 $mM^{-1}cm^{-1}$). The reaction volume was 1 mL, which contained 50 mM phosphate buffer, pH 7.0, 50 mM H_2O_2 (Aebi, 1974). The reaction was started by the addition of the sample.

Measurement of GSH content

GSH level was estimated in the deproteinized supernatant fraction of liver homogenate using 5,5-dithiobis(2-nitrobenzoicacid), recording absorption at 412 nm (Ellman, 1959). Results are expressed as "mol" GSH/g tissue.

Measurement of GST activity

Spectrophotometric method was used to determine the activity of GST according to the method of Habig *et al* (1974). The assay was performed in 0.1 M potassium phosphatate buffer, pH 6.5, at 25°C using 100 •ĭL GSH and 100 μL CDNB as substrates.

Measurement of GR activity

0.2 M potassium phosphate buffer (pH 7.5) 0.5 mL, 2 mM NADPH 50 μL, and 20 mM disulfide GSH 50 μL including 2.5 mM ethylene diamine tetraactic acid were added to liver cytosol 50 μL including 100 μL protein and volume of final reaction was made 1 mL with distilled water. Immediately, the reaction showed the decrease in absorbance at 340 nm (Carlberg *et al*, 1985).

Measurement of GPx activity

Cytosol 100 μL containing 1.0 mg/mL protein was incubated for 5 min at 37°C with stock solution (0.25 mM glutathione, 0.12 mM NADPH, and 1 unit/mL glutathione reductase) in a final volume of 1.55 mL. 50 μL of cumene hydroperoxide (1.0 mg/mL) was added to start the reaction, and the absorbance at 340 nm was monitored for the rate of disappearance of NADPH in a thermostated spectrophotometer with a recoder (extinction coefficient; 6.22 $mM^{-1}cm^{-1}$) (Tappl, 1978).

Protein quantitation

Microsomal and cytosolic protein were estimated by the method of (Lowry *et al.* 1951), using bovine serum albumin as the standard.

Statistical analysis

All results are expressed as the means±S.D. ANOVA Duncan; $p<0.05$ was considered statistically significant.

RESULTS

The effectiveness of Sachungwhan was evaluated through the normalization of CCl_4-induced biochemical parameters.

Biochemical analysis

Administration of CCl_4 serum significantly enhanced AST and ALT activities from 177.25±53.05 unit/L to 1065.62±100.96 unit/L and from 27.83±4.39 unit/L to 595.39±97.01 unit/L, respectively. AST and ALT activities were decreased by oral administration of Sachungwhan. Serum ALP activity was elevated from 191.83±26.88 unit/L to 271.49±46.60 unit/L in CCl_4 group. ALP was also decreased by oral administration of sachungwhan.

Serum BUN and CRE levels were from 18.81±3.79 mg/dL to 11.20±1.30 mg/dL and from 0.40±0.02 mg/dL to 0.48±0.04 mg/dL, respectively in CCl_4 group.

Serum CHO and TG levels were showed from 74.63±13.01 mg/dL to 80.85±4.06 mg/dL and from 29.96±2.58 mg/dL to 18.19±2.18 mg/dL, respectively in CCl_4 group. CHO and TG

were protected by oral administration of sachungwhan.

Serum LDL-CHO and HDL-CHO levels were showed in CCl_4 group from 22.53±2.59 mg/dL to 35.22±5.51 mg/dL and from 43.04±5.14 mg/dL to 19.77±3.09 mg/dL, respectively. LDL-CHO and HDL-CHO were protected by oral administration of sachungwhan. HDL-CHO/T-CHO in CCl_4 group was showed from 0.59±0.11 to 0.39±0.05.

TP and ALB levels were showed in CCl_4 group from 6.10±0.32 g/dL to 5.25±0.32 g/dL and from 3.23±0.22 g/dL to 2.93±0.07 g/dL, respectively. They were protected by oral administration of sachungwhan.

Serum BIL level was showed in CCl_4 group from 0.10±0.06 unit/L to 0.14±0.03 unit/L. BIL level was protected by oral administration of sachungwhan (Table I).

Histopathological examination

The histopathological examination showed that the treatment of Sachungwhan relieved the ballooning degeneration of hepatocytes which had been generated by CCl_4 (Fig. 1).

Contents of CHO and TG

Hepatic CHO and TG levels were increased from 9.65±1.13 mg/g Liver to 23.51±0.71 mg/g Liver and from 21.95±1.68 mg/g Liver to 33.25±3.07 mg/g Liver in CCl_4 group compared with the control group, respectively. However, the CCl_4-induced increase was prevented by oral administration of sachungwhan (Fig. 2).

Change of oxidative damage

Activity of CYP was decreased to 0.11±0.01 μmol/mg protein in CCl_4 group compared with 0.22±0.02 μmol/mg protein in control group, and was increased in sachungwhan group compared with CCl_4 group (Fig. 3).

Activity of NADPH-CYP reductase was decreased to 159.66±16.04 nmol/min/mg protein in CCl_4 group compared with 278.84±43.22 nmol/min/mg protein in control group, and

Table I. Effects of Sachungwhan on serum in CCl_4 treated rats

Parameters \ Treatment Dose(mg/kg, p.o.)	Control -	CCl_4 -	Sily+CCl_4 30	Sa+CCl_4 4,300
AST (unit/L)	177.25±53.05	1065.62±100.96	624.58±117.35***	681.83±50.30***
ALT (unit/L)	27.83±4.39	595.39±97.01	291.51±69.62***	284.83±52.77***
ALP (unit/L)	191.83±26.88	271.49±46.60	221.12±24.86*	224.31±30.55*
BUN (mg/dL)	18.81±3.79	11.20±1.30	17.60±3.22***	17.57±2.88***
CRE (mg/dL)	0.40±0.02	0.48±0.04	0.41±0.04*	0.47±0.03**
CHO (mg/dL)	74.63±13.01	50.85±4.06	61.56±3.23*	60.44±5.58*
TG (mg/dL)	29.96±2.58	18.19±2.18	26.65±2.86***	27.42±4.20***
LDL-CHO (mg/dL)	22.53±2.59	35.22±5.51	28.35±2.67*	28.43±3.6*
HDL-CHO (mg/dL)	43.04±5.14	19.77±3.09	26.96±4.41*	30.16±4.84**
HDL-CHO/ T-CHO	0.59±0.11	0.39±0.05	0.44±0.07	0.50±0.12
PRO (g/dL)	6.10±0.32	5.25±0.32	5.95±0.60**	5.83±0.21*
ALB (g/dL)	3.23±0.22	2.93±0.07	3.22±0.29*	3.16±0.08*
T-BIL (unit/L)	0.10±0.06	0.14±0.03	0.11±0.04	0.14±0.05

Sily, Silymarin; Sa, Sachungwhan
CCl_4(0.45mL/kg) (i.p.)
Significantly different from CCl_4 treated group.
*p<0.05, **p<0.01, ***p<0.001 (n=6)

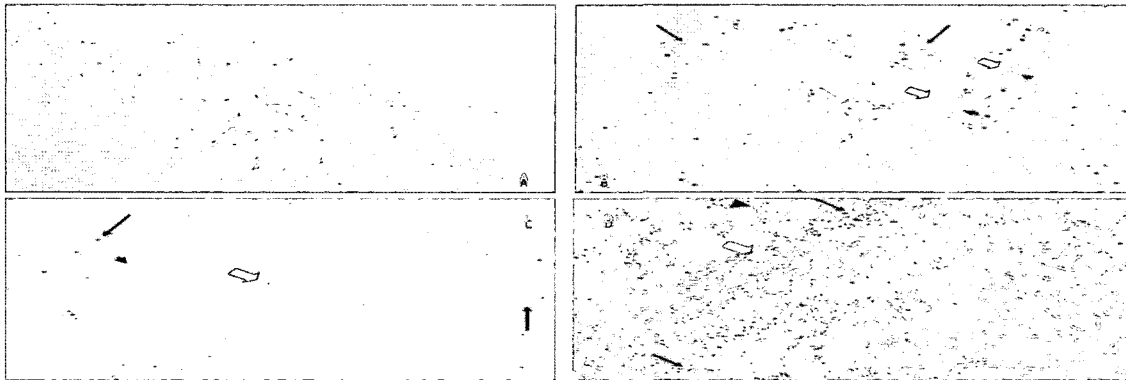

Fig. 1. Histologic effects of Sachungwhan treatment on CCl_4-induced hepatotoxic rats. Hematoxilin and eosin-stained agent treatment. Hematoxylin and eosin-stained sections were photographed at ×200; Well-preserved lobular architecture was observed in rat of control (A). Note moderate individual cell necrosis (arrows) and marked ballooning degeneration (arrowheads) and infilteration of inflammatory cells around the central vein in the CCl_4 treated rat (B). Ballooning degeneration of hepatocyte with necrosis was relived in the Silymarin treated rat (C). Ballooning degeneration of hepatocyte with necrosis was relived in the Sachungwhan treated rat (D).

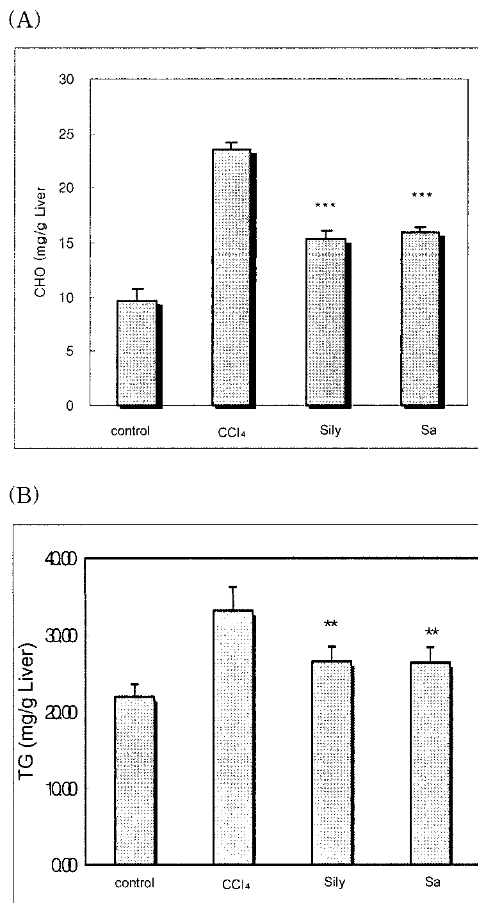

Fig. 2. Effects of Sachungwhan treatment on hepatic cholesterol (A) and triglyceride (B) levels in CCl_4 treated rats.
Sily, Silymarin; Sa, Sachungwhan
Sa (4.3 g/kg/day), Silyr (30 mg/kg/day), CCl_4 (0.45 mL/kg).
Significantly different from CCl_4 treated group.
***p<0.001 (n=6)

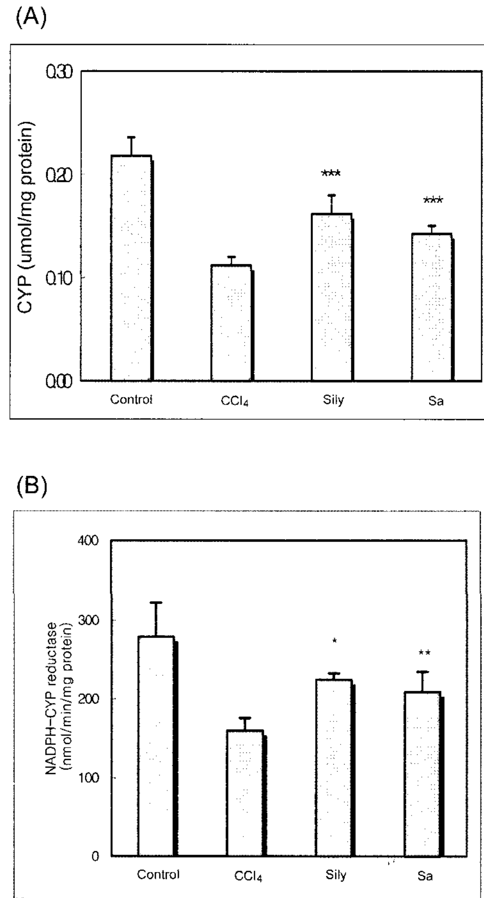

Fig. 3. Effects of Sachungwhan treatment on cytochrome P450 (A) and NADPH-cytochrome P450 (B) activities in microsomal liver of CCl_4 treated rats.
Sily, Silymarin; Sa, Sachungwhan
Sa (4.3 g/kg/day), Silyr (30 mg/kg/day), CCl_4 (0.45 mL/kg).
Significantly different from CCl_4 treated group.
*p<0.05, ***p<0.001 (n=6)

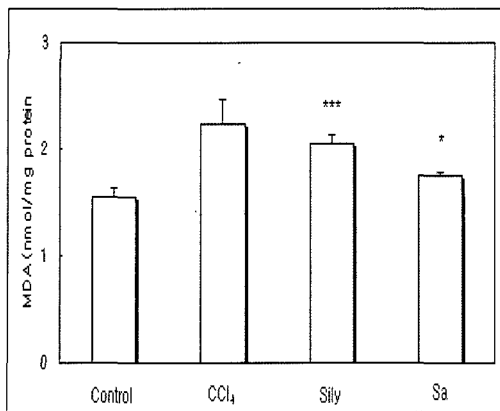

Fig. 4. Effect of Sachungwhan treatment on malondialdehyde level in liver of CCl_4 treated rats.
Sily, Silymarin; Sa, Sachungwhan
Sa (4.3 g/kg/day), Silyr (30 mg/kg/day), CCl_4 (0.45 mL/kg).
Significantly different from CCl_4 treated group.
*p<0.05, **<0.01 (n=6)

was increased in sachungwhan group compared with CCl_4 group (Fig. 3).

CCl_4 increased the MDA content in liver microsome. The control group showed MDA basal values of 1.54±0.08 nmol/mg protein. However, the CCl_4-induced increase in liver MDA content was prevented by oral administration of sachungwhan (Fig. 4).

Change of antioxidative activities

Activities of SOD and CAT were showed in control group as 5.64±0.30 unit/mg protein and 18.77±4.12 decreased H_2O_2 nmol/mg protein/min. In CCl_4 group, activity of CAT was decreased from 18.77±4.12 decreased H_2O_2 nmol/mg protein/min to 10.25±1.69 decreased H_2O_2 nmol/mg protein/min. Decreased activities were elevated by oral administration of Sachungwhan (Fig. 5).

Glutathione metabolizing enzymes

GSH is the major intracellular non-protein antioxidant and is present in virtually all mammalian tissues, where it plays a crucial role in the detoxification of free radicals.

GSH and GST in liver of CCl_4 group were decreased from 4.92±0.20 μmol/g liver to 3.58±0.15 μmol/g liver and from 1.92±0.23 nmol CDNB conjugated/min/mg protein to 0.67±0.17 nmol CDNB conjugated/min/mg protein compared with control group, respectively. Elevation of above two enzyme activities showed the protective effects of Sachungwhan (Fig. 6).

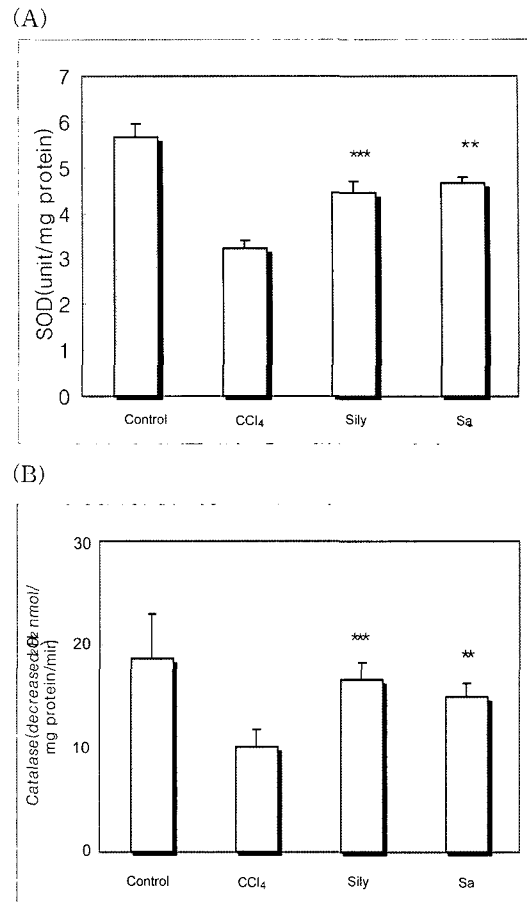

Fig. 5. Effects of Sachungwhan treatment on superoxide dismutase (A) and catalase (B) activities in liver of CCl_4 treated rats
Sily, Silymarin; Sa, Sachungwhan
Sa (4.3 g/kg/day), Silyr (30 mg/kg/day), CCl_4 (0.45 mL/kg).
Significantly different from CCl4 treated group.
*p<0.05, ***<0.001 (n=6)

CCl_4 group decreased the activities of GR and GST in liver. The control groups showed 21.16±1.56 nmol NADPH oxidized/min/mg protein and 59.66±4.49 nmol NADPH oxidized/min/mg protein, respectively. However, the CCl_4-induced decreases in contents of liver GR and GST were alleviated by oral administration of Sachungwhan (Fig. 7).

DISCUSSION

Free radical may play an important role in the origin of life and biological evolution, implicating their beneficial effects on the organism (McCord, 2000). For example, oxygen radicals exert critical actions such as signal transduction, gene transcription, and regulation of soluble guanylate cyclase activity in

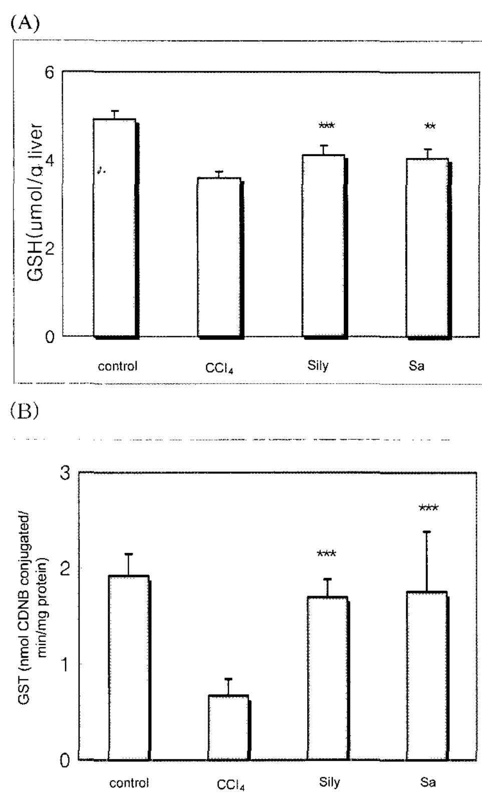

Fig. 6. Effects of Sachungwhan treatment on glutathione (A) and glutathione S-transferase (B) activities in liver of CCl_4 treated rats.
Sily, Silymarin; Sa, Sachungwhan
Sa (4.3 g/kg/day), Silyr (30 mg/kg/day), CCl_4 (0.45 mL/kg).
Significantly different from CCl_4 treated group.
**p<0.01, ***p<0.001 (n=6)

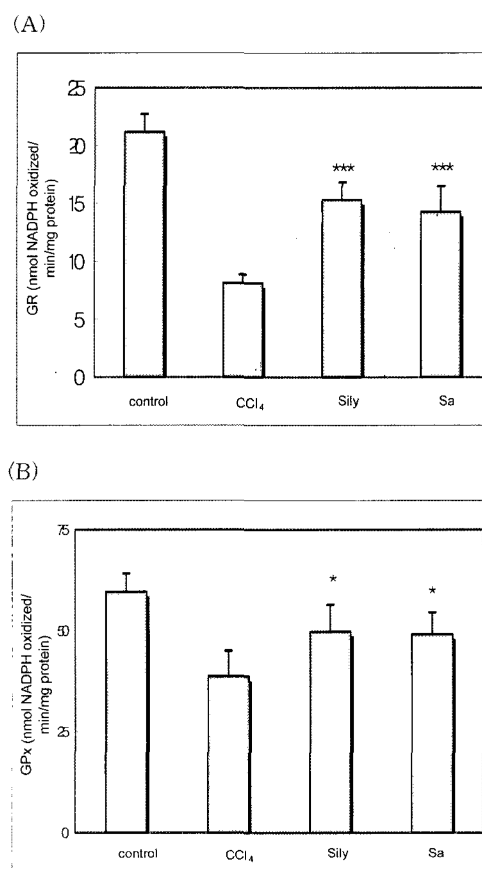

Fig. 7. Effects of Sachungwhan treatment on glutathione reductase (A) and glutathione peroxidase (B) activities in liver of CCl_4 treated rats.
Sily, Silymarin; Sa, Sachungwhan
Sa (4.3 g/kg/day), Silyr (30 mg/kg/day), CCl_4 (0.45 mL/kg).
Significantly different from CCl_4 treated group.
*p<0.05, ***p<0.001 (n=6)

cells. There are "Two faces" of free radicals in biology in that they serve as signaling and regulatory molecules at physiologic levels but as highly deleterious and cytotoxic oxidants at pathologic levels (Freidovich, 1999).

A wide variety of oxidizing molecules such as ROS and/or depleting agents can alter the glutathione redox state, which is normally maintained by the activity of GSH-depleting (GPx) and replenishing enzymes (GR) (Sun, 1996).

The importance of glutathione and related enzymes has been poorly investigated in sachungwhan.

This study investigated GSH content, the activities of GSH-dependent enzymes (GPx, GST, GR), SOD and CAT and MDA level in order to obtain antioxidant enzymes activities.

MDA level was significantly increased in CCl_4 group. Some studies reported increased MDA level CCl_4 hepatotoxicity (McCay *et al*, 1984; Recknagel, 1967).

In the data reported in the literature on antioxidant system, SOD and CAT acted as eliminating scavenger of free radical (Ho, 1988; Comporti, 1993). Our findings are consistent with those of theirs. GSH content and the activities of GST, GR and GPx activities are consistent with those of others. For instance, GSH (Antebi, 1984), GST (David, 1983), GR (Sies, 1984), GPx (Niki, 1993), respectively.

Observed significant decrease in CAT and SOD activities in CCl_4 group may have been due to the response to increased ROS production, which with elapsing time may be inadequate to detoxify high level of ROS (Kaynar, 2005).

The aim was to determine scavenging action of free radical in sachungwhan. In fact, it was observed that sachungwhan prevented the increase in LPO produced by CCl_4 and the liver damage induced by this agent.

1. Level of serum AST, ALT, ALP, total bilirubin and LDL-

CHO by CCl_4 was decreased in On and On+Cur groups. Decreased levels of serum alb, protein, BUN, CHO, TG and HDL-CHO by liver injury was increased in sachungwhan groups.

2. Sachungwhan decreased levels of CHO and TG in tissue.

3. Sachungwhan increased content of cytochrome P450 and activity of NADPH-cytochrome P450 reductase decreased by CCl_4-induced injury to metabolism in liver.

4. Sachungwhan decreased level of MDA and increased activities of SOD and CAT against CCl_4-induced injury to antioxidant defence system in liver.

5. Sachungwhan increased the activities of GSH, GST, GR and GPx against CCl_4-induced change of glutathione metabolism system in liver.

6. In histopathologic examination, there were observative changes. Ballooning degeneration of hepatocyte with necrosis was relieved in sachungwhan groups.

When sachungwhan was compared with Silymarin, we found sachungwhan was not likely bad.

According to observation level of MDA decrease, activities of SOD and CAT increase, and activities of GSH metabolism system increase, it is suggested that one of the mechanisms of hepatoprotection is scavenger of free radical.

These findings suggested that sachungwhan had hepatoprotective effects against hepatotoxicity.

ACKNOWLEDGEMENTS

This research was supported in part by the fund 2006, from Duksung Women's University.

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