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The Protective Effects of Isoflavone Extracted from Soybean Paste in Free Radical Initiator Treated Rats

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Abstract This study was performed to investigate the antioxidant effects of Korean soybean paste extracts (SPE) on 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced liver damage in rats. Thirty healthy Sprague Dawley rats were selected and divided into 5 groups. Isoflavone contents were measured using HPLC technique. The antioxidant activity was measured in the plasma and liver of the rats with the following results. Levels of isoflavone in fermented soy paste, red pepper paste and soy sauce were 28.9, 30.3 and 3.4 μg/g for daidzein and 244.3, 187.7 and 6.1 μg/g for genistein, respectively. The activities of glutamate oxaloacetic transaminase (GOT) and glutamate pyruvate transaminase (GPT) were significantly higher in the AAPH-treated group in the SPE-AAPH group ($p<0.05$). The thiobarbituric acid reactive substance (TBARS) production was significantly increased in the AAPH-treated liver tissue ($P<0.05$). Glutathione peroxidase (GPx), glutathione reductase (GR) and catalase in the liver were significantly ($p<0.05$) decreased by AAPH administration. The glutathione (GSH) concentration was higher in the SPE-treated (Ed- confirm) group than in the control and other groups ($p<0.05$). These results suggest that SPE led to increased antioxidative activities against AAPH-induced peroxyl radical.

Keywords: AAPH, soypaste, antioxidants, glutathione

Introduction

Nowadays, there is an increasing worldwide awareness of healthy and fermented foods. In this context, soy products have received much attention due to their potential to prevent or treat chronic diseases (1). Epidemiological studies have shown that people consuming diets high in soy have a lower risk for developing cardiovascular disease and cancer than those consuming lesser amounts of soybean in their diet (2-5). Isoflavones, major dietary components from soybeans (6), have recently attracted great attention because of their proposed health related and clinical benefits such as estrogen receptor binding (7), radical scavenging (8), and antiproliferative and growth inhibiting effects on cancer cells (9). Isoflavones and genistein have been demonstrated to show several kinds of biological activity (10). Genistein also has an inhibitory effect on angiogenesis (11), antioxidative potential (12), and phytoestrogenic activity (13, 14). The molecular structures of daidzein are shown in Fig 1. Isoflavones have direct free radical quenching ability, with genistein and daidzein being particularly effective (15-18). They may also decrease the oxidative damage in cells via indirect mechanisms (19). In vitro study has demonstrated that the isoflavone aglycone form has higher antioxidative activity than the glycoside form. Supplementation of the human diet with soy products has been shown to decrease the ease with which lipids can be oxidized (20, 21). Fermented soybean products are known to carry larger amounts of the isoflavone aglycones than unfermented soy products (22-25).

This study was performed to investigate the antioxidant effects of soybean paste extracts (SPE) containing larger amounts of the isoflavone aglycones administrated prior to AAPH treatment in rats. In this study on the biological implication of oxygen free radical, 2,2-azobis(2-amidino-propane) dihydro-chloride (AAPH) was used as a free radical initiator.

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Material and Methods

Chemicals Soybean paste was purchased from Daesang Tech. Ltd., (Seoul, Korea). Standard genistein and daidzein were purchased from Sigma Chemical Co. (St. Louis, MO. U.S.A.) for application as standard substances to estimate isoflavone contents. HPLC-grade solvents of methanol, acetic acid, and acetonitriles were processed from J.T. Baker (Phillipsburg, NJ, U.S.A.). L-(+)-Ascorbic acid was purchased from ACROS (U.S.A.). Glutathione (GSH), glutathione disulfide-reductase (GR), β-nicotinamide dinucleotide phosphate (NADPH), cumene hydroperoxide, o-phthalaldehyde (OPT), 1,1,3,3-tetraethoxypropane (TEP), trichloroacetic acid (TCA), bovine serum albumin (BSA) and thiobarbituric acid (TBA) were supplied by the Sigma Chemical Co., . (St. Louis, MO. U.S.A.). Folin-Ciocalteu phenol reagent and hydroperoxide were purchased from Merck Ltd. (Germany). AAPH was obtained from Wako Pure Chemicals Industries, Ltd., (Osaka, Japan). All other materials were commercially available reagents of

guaranteed reagent grade.

Preparation of sample Extraction conditions for isoflavone aglycones of the soybean paste, red pepper paste and soy sauce were as follows (26). Samples (1g) of solid foods were extracted with 100 ml of 70% methanol by stirring for 12hr at room temperature. In the case of soy sauce, samples (10 ml) were extracted under the same conditions after freeze-drying. Acetic acid (1%) was added to these extract solutions to prevent decarbonation reaction of the malonylglucoside isoflavones. These extract solutions were centrifuged at 10,000 rpm for 10 min. A portion of the supernatant was removed with a syringe, filtered through a 0.45 μm PVDF filter into a sample vial, and analyzed by HPLC. Ascorbate was dissolved into diluted water at 55 mg/kg B.W./ml.

HPLC analysis The standard HPLC method was used for these studies by following Wang's method (27) with a slight modification. The 20 μl filtrate was injected into the HPLC equipped with a Bondapak C_{18} column after the system had been equilibrated. The UV detector was stabilized with mobile phase (acetonitrile in 0.1% acetic acid and water in 0.1% acetic acid at a ratio of 3:7) at a flow rate of 0.8 ml/min. Quantitative data for genistein and daidzein content were calculated by comparing their peak areas of HPLC chromatogram with those of standards.

Animals, diets, and experimental design Six-week old, male, Sprague-Dawley rats weighing about 190 g were purchased from Dae Han Experimental Animal Co., Seoul, Korea. Rats were fed the following experimental diet (wt%): casein, 25; corn oil, 5; cellulose powder, 5; mineral mixture, 3.5 (AIN-76); vitamin mixture, 1 (AIN-76); sodium cholate, 0.2; and sucrose to 100. Rats were randomly allocated into five experimental groups of 6 rats/group. The five experimental groups were control, SPE (isoflavone extracted from Korean soybean paste), AAPH, SPE-AAPH (AAPH 24hr before sacrifice), and Vit. C-AAPH. The control and AAPH group were given 0.9% saline solution (5ml/kg B.W.) for 9 days. The Vit. C-AAPH group was administered orally with ascorbate solution (55 mg/kg B.W.) for 9 days. SPE and SPE-AAPH groups were administered orally (per os) with SPE (200 mg/kg B.W.) for 9 days (28). On the 10th day, the AAPH, Vit. C-AAPH and SPE-AAPH-treated groups were administered intraperitoneally with AAPH (60 mg/kg B.W.). The rats were maintained at 22 ± 2°C temperature and 60% relative humidity on a 12-h light/dark cycle with ad libitum access to feed and water.

Experimental methods Body weight and food intake were measured once a day. After 9 days, rats were deprived of food for 12h and sacrificed using diethyl ether. Blood was drawn by cardiac puncture using a syringe containing heparin. The liver was removed, weighed and stored at -75°C until analysis. Plasma samples were obtained by centrifugation of the blood at 3000 rpm for 10 min and frozen at -75°C until analysis.

Plasma analysis The plasma glutamate oxaloacetic transaminase (GOT) and glutamate pyruvate transaminase (GPT) levels were determined using a diagnostic kit by following the method of Reitman-Frankel. Plasma total cholesterol, triglyceride and phospholipids were analyzed by enzymatic colorimetric method using ready kits (Eiken Chemicals, Japan).

Lipid peroxidation analysis The lipid peroxidation in rats was measured using Ohkawa's method (29). Tissue homogenates were prepared at a ratio of wet tissue 0.1g to 0.9ml of 1.15% KCl by using a Teflon Potter – Elvehjem homogenizer (U.S.A.). The reaction mixture contained 0.1ml of sample, 0.2ml of 0.8% sodium dodecyl sulfate (SDS), 1.5ml of 20% acetic acid solution (pH 3.5), 1.5 ml of 0.5 aqueous solution of TBA, and 4.0ml of distilled water.

The plasma was also measured by the method of Yagi et al (30). The reaction mixture contained 50 μl of plasma, 4.0 ml of 1/12N H_2SO_4, and 5.0 ml of 10% phosphotungstic acid. After standing at room temperature for 5 min, the mixture was centrifuged at 3000rpm for 10min. The supernatant was discarded, the sediment was mixed with 2.0 ml of 1/12 N H_2SO_4 and 0.3 ml of 10% phosphotungstic acid, and the mixture was centrifuged at 3000 rpm for 10 min. The sediment was suspended in 4.0 ml of distilled water, to which 1.0ml of TBA reagent was added. The mixture was heated at 95°C for 60min. After cooling with tap water, 5.0 ml of n-butanol was added, and the mixture was shaken vigorously. After centrifugation at 3000 rpm for 20min, the absorbance of the organic (upper) layer was measured in a spectro-fluorometer, set to 515 nm excitation and 553 nm emission. The external standard was 1,1,3,3-tetraethoxy-propane (TEP), and the level of lipid peroxides was expressed as nmol of malondialdehyde.

Measurement of glutathione (GSH) concentration The GSH concentration was measured in rats by a certain modification to Cohn-Lyle's method (31). Briefly, the reaction procedure comprised *o*-phthalaldehyde which yields a highly specific and sensitive fluorometric assay for reduced GSH.

Rat tissue was obtained by homogenizing 100mg of tissue in 1.4 ml Tris-HCl in 0.01M EDTA (pH 8.9). Proteins were removed by centrifugation at 3000 rpm for 30 min at 4°C. The supernatant fluid was diluted by adding 200 μl sample to 5.0 ml of 0.4M Tris-HCl (pH 8.9), after which 0.1 ml of OPT was added with through mixing. After about 15-20 min reaction period at room temperature, the fluorescence at 342 nm excitation and 428 nm emission was determined.

Measurement of glutathione reductase (GR) and glutathione peroxidase (GPx) activity The liver tissue was homogenized in 0.25M sucrose and the supernatant fraction was prepared by centrifugation at 105,000 × g for 1 hr at 4°C. The supernatant obtained was kept at -75°C until analysis.

The activity of GR was measured using Worthing et al's method (32). In the presence of GSSG, the oxidation of NADPH by GR was followed by a decrease in absorbance at 340 nm.The reagent mixture was added into 0.5 ml of 200 mM potassium phosphate buffer (pH 7.0), 0.1 ml of 200 mM potassium chloride, and 0.1 ml of 10 mM EDTA. The substrates used were 50 μl of 20mM GSSG and 100

μl of 2 mM NADPH. The reaction was induced by the addition of 0.1 ml of enzyme solution. The GR activity was determined by measuring the absorbance at 340 nm. A unit of enzyme activity was defined as the oxidation of 1 μmol of NADPH per min at 25°C.

Glutathione peroxidase (GPx) activity in the liver supernatant was measured by following Lawrence-Burk's method (33). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 E.U./ml GSSG-reductase, 1 mM GSH, and 1.5 mM cumene hydroperoxide or 0.25 mM H_2O_2, in a total volume of 1 ml. Enzyme source (0.1 ml) was added to 0.8ml of the above mixture and 0.1 ml of peroxide solution. Absorbance at 340 nm was recorded for 5min and the activity was calculated from the slope of the lines as NADPH (imoles) oxidized per minute.

Measurement of catalase activity Tissue homogenates were prepared in a ratio of 200mg of wet tissue to 1.8 ml of potassium phosphate buffer (pH 7.0). The catalase activity was measured by Beers - Sizer's method (34). The reaction was started by addition of 30 mM H_2O_2 solution (1.0 ml) to enzyme source (2.0 ml). The decomposition of H_2O_2 was followed directly by the decrease in extinction at 240 nm. The difference in extinction (ΔE_{240}) per unit time was used as a measure of the catalase activity.

Measurement of protein concentration The protein content in the rat liver was measured by following the method of Makwell *et. al.* (35). The bovine serum albumin was used as the standard.

Statistical analysis The results were expressed as mean ±S.D. Two-way ANOVA was used to determine the significance of the differences among the variables of the five experimental groups, followed by Duncan's multiples range test for differences between means. The level of significance was $p<0.05$.

Results and Discussion

Isoflavone contents in fermented soy foods Isoflavones, particularly genistein, have been demonstrated to exhibit antioxidative potential (36) and phytoestrogenic activity (37). Compared to soybean products, fermented soybean contained larger amounts of genisteins than unfermented soy products did. Daidzein and genistein were found in all sample extracts in the fermented soy products. Isoflavone aglycones in fermented soy products were measured by HPLC analysis. The two kinds of isoflavones in SPE this abbreviation already defined in part 4 of the M&M section above) are depicted in the HPLC chromatogram (Fig. 3). The HPLC chromatogram for daidzein and genistein in SPE is shown in Figs. 2 and 3, along with their contents. Isoflavone components, daidzein and genistein contents in soy sauce, the red pepper paste and soybean paste as measured by HPLC are shown in Figure 4. The results demonstrate that soybean paste had very high genistein content, followed by red pepper paste and soy sauce, whereas the daidzein content was slightly higher in the case of red pepper paste than soybean paste. Soy sauce had significantly higher content of both daidzein and genistein than the others.

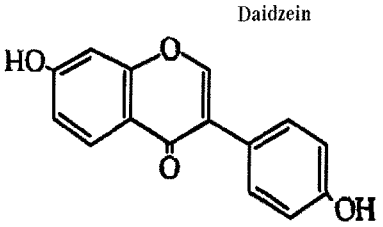

Fig. 1. Molecular structures of daidzein.

Change of body weight gain and feeding efficiency The treatment effects of five treatments on the body weight and

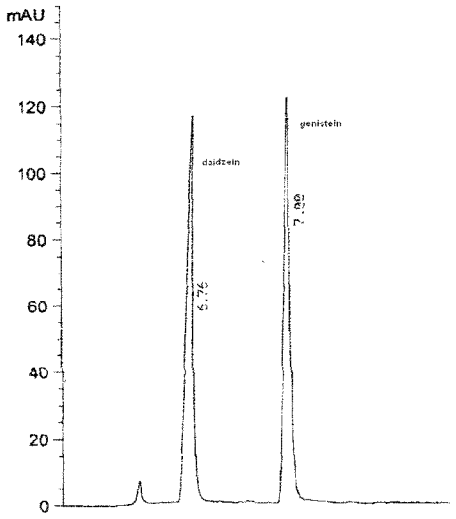

Fig. 2. Representative HPLC chromatogram of the standard for daidzein and genistein.

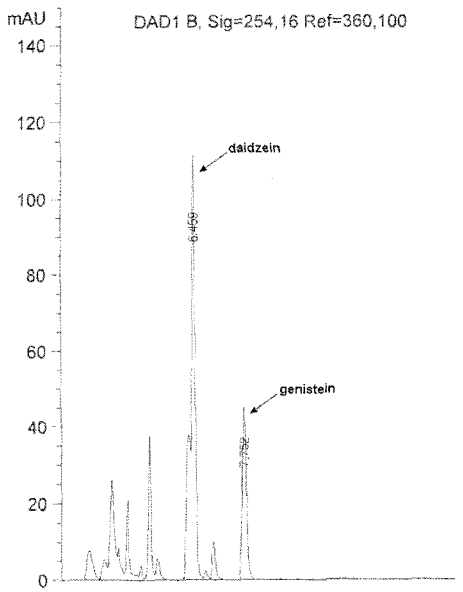

Fig. 3. Representative HPLC chromatogram of daidzein and genistein in SPE.

body weight gain during the period and the feed efficiency ratio are presented in Table 1. The study results demonstrated the significant effect of the five treatments. The body weight gain was significantly higher in the Vit. C- and AAPH-treated groups than in the control and other groups. There was no significant difference with respect to FER among the five groups.

Change of plasma biochemical parameters The effects of SPE and Vit. C on triglyceride and phospholipids plasma contents in AAPH-treated rats can be seen in Table 2. Both triglyceride and phospholipids contents were found to be significantly higher in plasma of SPE-treated rats. Very high triglyceride was also observed in Vit. C-AAPH-treated rats, although the phospholipids contents were lower in the Vit. C-AAPH group than in the SPE-AAPH and SPE-treated rats. The results demonstrated that SPE intake may adversely affect the blood plasma triglyceride and phospholipids contents.

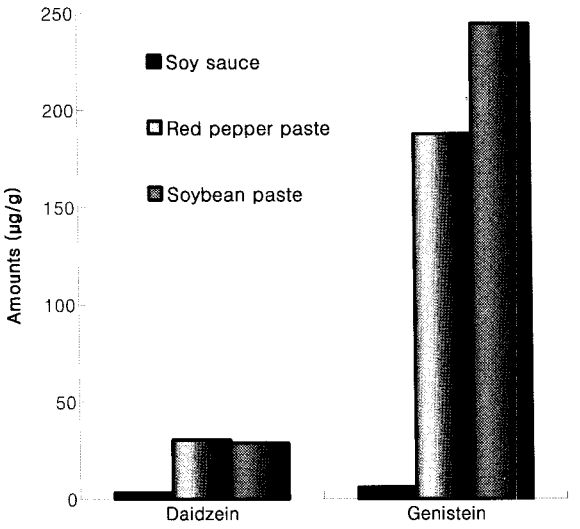

Fig. 4. Isoflavone contents of aglycone forms in fermented foods. *Each value represents the mean of four independent experiments. *Isoflavone components were analyzed by HPLC using isoflavone standard.

Table 1. Effects of SPE and Vit. C on the body weight, weight gain and FER(feeding efficiency ratio) in AAPH-treated rats

Group	Body weight (g)		Weight Gain(g)	FER
	Initial	Final		
Control	190.00±6.78	243.17±7.83	53.17±12.04^{b}	1.87±0.4^{a}
SPE	185.50±3.94	246.67±9.14	61.17±6.91^{ab}	2.09±0.2^{a}
AAPH	182.83±4.79	245.33±6.25	62.50±4.89^{ab}	2.14±0.2^{a}
SPE-AAPH	183.67±3.44	238.83±7.33	55.17±5.34^{ab}	1.79±0.1^{a}
Vit. C-AAPH	185.17±3.76	248.33±6.06	63.17±5.56^{a}	2.04±0.2^{a}

*Values represent the mean ± S.D. (n=6).
*Each value represents an average of three trials.
*Values with different letters are significantly different among experimental groups at $p< 0.05$ by Duncan's multiple range test.
Control : 0.9% saline per os 9 days before sacrifice.
SPE : 200 mg/kg B.W., extracts of soybean paste (SPE) per os 9 days.
AAPH : 60mg/kg B.W., 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH) intraperitoneal 24 hr before sacrifice.
SPE-AAPH : 200 mg/kg B.W., extracts of soybean paste (SPE) per os 9 days before AAPH administration.
Vit. C- AAPH : 55 mg/kg B.W. per os 9 days before AAPH administration.

Effects of SPE and Vit. C on serum GOT and GPT activities of AAPH-treated rats The effect of SPE and Vit. C on serum GOT and GPT activities of AAPH-treated rats is depicted in Fig. 5. The activities of GOT and GPT were significantly higher in the AAPH-treated group than in the control group. Of all the AAPH-treated groups, the SPE-AAPH group had the significantly lowest level in GOT activity.

Effects of SPE and Vit. C cholesterol parameters of AAPH-treated rats The effects of the five treatments on the rats' total blood cholesterol levels are presented in Fig. 6. The SPE-treated group showed no effect on the total cholesterol. The significant difference in plasma lipid composition between the control and SPE-treated group in this study may have resulted from the low level of isoflavone and the short experimental period. The SPE level of 200 mg/kg may have been too low to have an effect on the plasma in improving lipid composition. Choi and Lee *et al* (38) suggested that supplementation with 150 mg isoflavone demonstrated significant effects on lowering serum total cholesterol after 12 weeks of

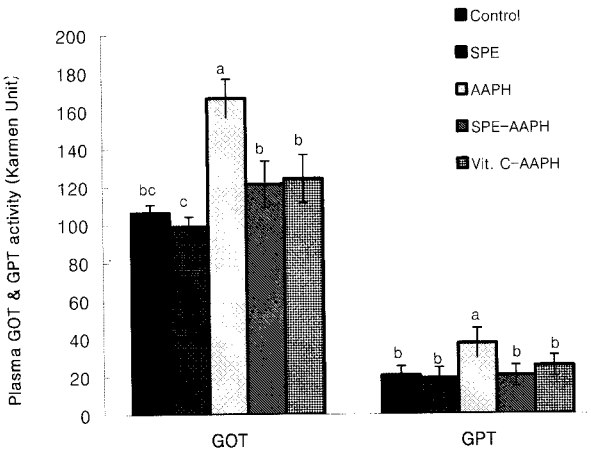

Fig. 5. Effects of SPE and Vit. C on plasma GOT and GPT activities of AAPH-treated rats. *Values represent the mean ± S.D. (n=6) *Each value represents an average of three trials. *Values with different letters are significantly different among experimental groups at $p<0.05$ by Duncan's multiple range test. For abbreviations see Table 1.

Table 2. Effects of SPE and Vit. C on the biochemical parameters of plasma in AAPH-treated rats

Group	Triglyceride	Phospholipid
Control	160.33±7.76^{ab}	97±5.85^{ab}
SPE	173.67±17.05^{ab}	111.13±8.49^{a}
AAPH	159.59±9.31^{ab}	89.75±5.61^{b}
SPE-AAPH	158.65±5.52^{b}	108.75±9.00^{a}
Vit. C-AAPH	176.63±17.86^{a}	95.63±11.75^{ab}

*Unit =mg/dl
*Values represent the mean ± S.D. (n=6)
*Each value represents an average of three trials.
*Values with different letters are significantly different among experimental groups at $p<0.05$ by Duncan's multiple range test. For abbreviations see Table 1.

isoflavone supplementation.

Change of lipid peroxidation in the plasma and liver The effect of five treatments on the change of TBARS values is presented in Figs. 7 and 8. TBARS values in the liver and plasma of the AAPH-treated rats were significantly increased compared to the control group. TBARS values in the liver of the AAPH-treated rats of the Vit. C and SPE-treated groups significantly decreased compared to the control group. Oral administration of SPE and Vit. C prior to AAPH treatment significantly suppressed the formation of malondialdehyde.

Lipid peroxidation in the plasma of the five groups of rats as expressed in terms of TBARS (nmol/ml) is depicted in Fig. 7. AAPH-induced lipid peroxidation was significantly low in the Vit. C-AAPH group of rats as compared to other treated groups. A high level of lipid peroxidation was observed in the AAPH-treated groups compared to the other treatment groups but it was not significantly different from the control. AAPH-induced lipid peroxidation in the rat livers was significantly higher in the AAPH rat groups than in the control and other treatment groups. Other rat groups did not significantly differ from the control in terms of AAPH-induced lipid peroxidation. The results proved that AAPH increased lipid peroxidation in the liver.

Change of glutathione (GSH) concentration in the liver The effect of the five treatments on the GSH content can be seen in Fig. 9.

The GSH content of the SPE-treated group was significantly higher than that of the control and AAPH group. The GSH content of the SPE-AAPH- and Vit. C-treated groups did not significantly differ from that of the AAPH-treated group. The results demonstrated that the liver GSH level is strongly influenced by SPE administration.

Change of antioxidant enzyme activities in the liver Activities of the liver GPx, GR and catalase are shown in Table 3. The GPx level was higher than that of the control group. The levels of GPx and catalase of the AAPH treatment group were significantly lower than those of the control and SPE treatment group. The GPx level of the AAPH treatment group was significantly lower than those of the control and other groups. However, the catalase levels of the SPE-AAPH and Vit. C treatment groups were significantly higher that that of the AAPH-treated group.

The study findings indicate that SPE and Vit. C exert a beneficial effect on the cellular antioxidant system. Pretreatment of SPE prior to AAPH administration

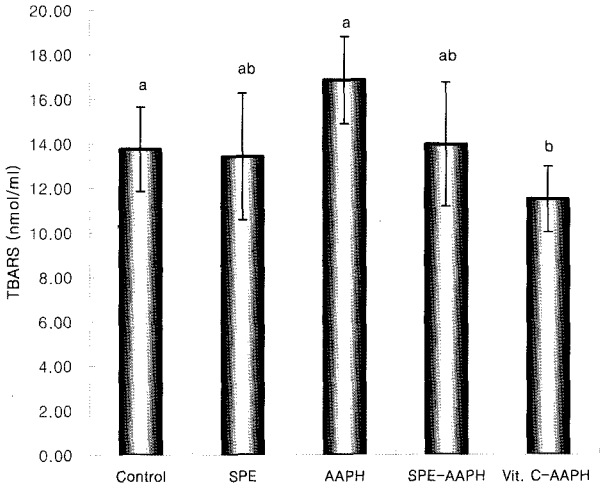

Fig. 7. Effects of SPE and Vit. C on AAPH-induced lipid peroxidation in the plasma. *Values represent the mean±S.D. (n =6) *Each value represents an average of three trials. *Values with different letters are significantly different among experimental groups at $p< 0.05$ by Duncan's multiple range test. For abbreviations see Table 1.

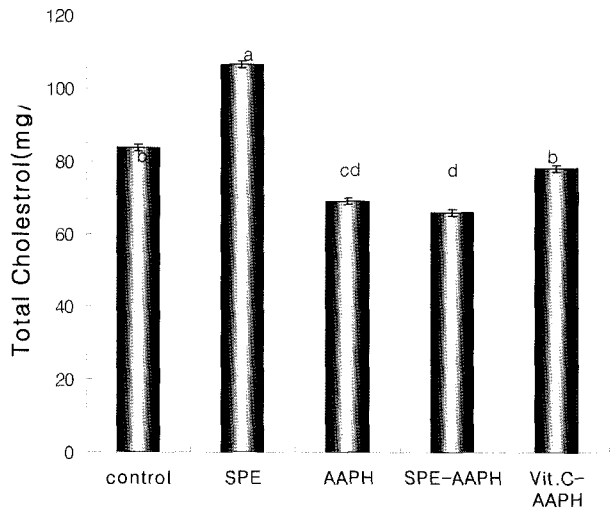

Fig. 6. Effects of SPE and Vit. C on the total cholesterol parameters of AAPH-treated rats. *Values represent the mean± S.D. (n=6) *Each value represents an average of three trials. *Values with different letters are significantly different among experimental groups at $p<0.05$ by Duncan's multiple range test. For abbreviations see Table 1.

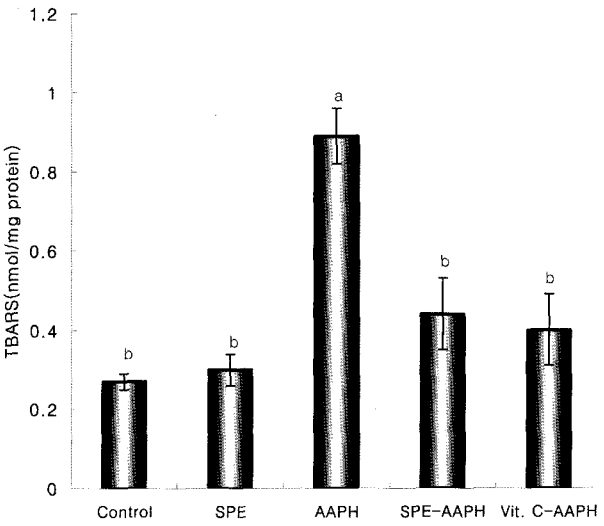

Fig. 8. Effects of SPE and Vit. C on AAPH-induced lipid peroxidation in the liver. *Values represent the mean±S.D. (n= 6) *Each value represents an average of three trials. *Values with different letters are significantly different among experimental groups at $p<0.05$ by Duncan's multiple range test. For abbreviations see Table 1.

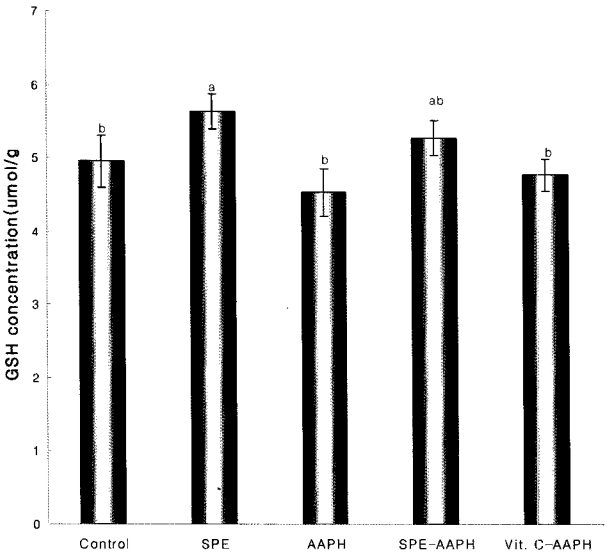

Fig. 9. Change of the liver GSH concentration. *Values represent the mean±S.D. (n=6). *Each value represents an average of three trials. *Values with different letters are significantly different among experimental groups at $p<0.05$ by Duncan's multiple range test. For abbreviations see Table 1.

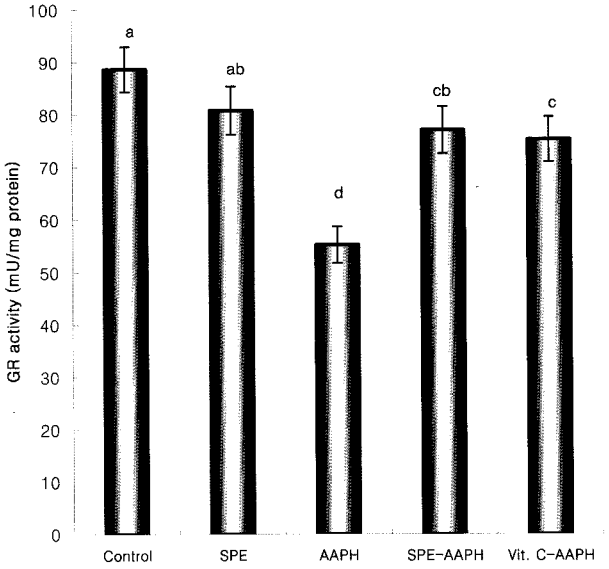

Fig. 10. Effect of AAPH on GR activity in the acute AAPH-treated rats with or without pretreatment of SPE and Vit. C. *Values represent the mean±S.D. (n=6). *Each value represents an average of three trials. *Values with different letters are significantly different among experimental groups at $p<0.05$ by Duncan's multiple range test. For abbreviations see Table 1.

Table 3. Effect on antioxidant enzyme activities in the acute AAPH-treated rats with or without pretreatment of SPE and Vit. C

Group	Catalase[1)]	Glutathione peroxidase[2)]
	(U/mg protein)	(mU/mg protein)
Control	87.40±4.69^{a}	340.8±23.43^{ab}
SPE	85.97±2.72^{ab}	395.57±20.77^{a}
AAPH	79.72±1.61^{c}	294.14±23.72^{b}
SPE-AAPH	81.43±4.26^{bc}	309.52±55.78^{b}
Vit. C-AAPH	83.00±3.54^{abc}	295.52±57.9^{b}

[1)]The unit of catalase expresses the results as μmol H_2O_2 consumed/min/mg protein.
[2)]Expressed as nmol NADPH oxidized/min/mg protein.
*Values represent the mean±S.D. (n=6)
*Each value represents an average of three trials.
*Values with different letters are significantly different among experimental groups at $p<0.05$ by Duncan's multiple range test. For abbreviations see Table 1.

inhibited the lipid oxidation in rats. These results demonstrate the protective actions of SPE against AAPH-induced free radical initiators in rats. However, the exact mechanism of SPE's antioxidative activity needs to be further investigated.

Change of GR activity in the acute AAPH-treated rats with or without SPE and Vit. C pretreatment The effect of AAPH on GR activity is presented in Fig. 10. The GR activity of the AAPH-treated groups was significantly reduced compared to the control and other groups. GR activity was lower than that of the control in all the treatment groups, and the minimum GR activity was observed in the AAPH-treated rat groups.

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