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Effects of Normal Diet with or without Naringin Supplement Following Ethanol Diet on Changes in Lipid Profiles and Antioxidant Enzyme Activities in Rats*

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This study was performed to investigate the effect of normal diet with or without naringin supplement on the lipid and antioxidant metabolism in ethanol-treated rats for a short term. Male Sprague-Dawley rats were divided into three groups (n=10), which were assigned to one of three dietary categories : E_8 : ethanol diet for 8 wks, E_4N_4 : ethanol diet for the first 4 wks and normal diet for the last 4 wks, E_4Nna_4 : ethanol diet for the first 4 wks and normal diet with naringin supplement for the last 4 wks. Plasma total cholesterol concentrations were significantly higher in ethanol fed rats for 8 weeks. The HDL-C/total-C ratios of the E_4N_4 and the E_4Nna_4 groups were significantly higher than that of the E_8 group, while the atherogenic index was lower in the E_4N_4 and the E_4Nna_4 groups than in the E_8 group. The E_4N_4 and E_4Nna_4 diets significantly lowered both the hepatic cholesterol and triglyceride levels compared to the E_8 group. Accumulation of hepatic lipid droplets was observed to be the highest in the E_8 group. In the current study, the naringin supplement to normal diet significantly lowered both the hepatic HMG-CoA reductase and ACAT activities in ethanol pre-treated rats for 4 weeks. Antioxidant enzyme activities were also upregulated when ethanol feeding was ceased. Naringin supplement given for 4 weeks after ethanol cessation resulted in a significant decrease in the plasma cholesterol and hepatic lipids and plasma TBARS as well as the hepatic HMG-CoA reductase and ACAT activities compared to the rats given ethanol diet for the entire 8 weeks. Replacement of normal diet following a short term ethanol feeding was effective for the recovery of ethanol-induced fatty liver and for normalizing plasma and hepatic lipid profiles and antioxidant enzyme activities, regardless of an additional phytochemical supplement, naringin. The effect of naringin could seemingly be more evident if its supplementation period had been extended longer than 4 weeks after ethanol cessation.

Key words: Naringin, Ethanol-diet, Normal diet, Cholesterol-regulating enzyme, Antioxidant enzyme

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INTRODUCTION

It has long been known that ethanol metabolism alters the intracellular redox state. Ethanol is metabolized predominantly via two well-characterized pathways. The first involves its oxidation to acetaldehyde, by cytosolic alcohol dehydrogenase, and subsequently to acetate by predominantly mitochondrial aldehyde dehydrogenase. Both steps are coupled to the reduction of NAD to NADH.[1)] Ethanol toxicity, including a decline in nutritional status, is directly due to ethanol per se and its metabolite, acetaldehyde, or is indirectly due to the metabolic sequence of ethanol oxidation such as the decreased ratio of cytoplasmic NAD^+/NADH and the involvement of reactive oxygen species.[2,3)] Liver certainly can be injured, particularly with long term alcohol consumption, and the mechanisms of liver injury may include the effects of oxygen radicals on liver cells.[4,5)]

Free radicals are highly reactive species characterized by one or more unpaired electrons in their outer orbital. Reactive oxygen species include oxygen radicals and substances closely related to oxygen radical reactions. Production of reactive oxygen species is a physiological process, but its increase and dysbalance between production of radicals and antioxidants could lead to oxidative stress with the affection of various biological functions and structural changes.[6)] Biological effects of reactive oxygen species and other radicals are controlled by antioxidant mechanisms - enzymes and substrates.

In many natural products, bioflavonoids have been

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reported to exhibit antioxidant activities[7,8] and inhibit lipid peroxidation in biological membranes.[9] The flavonoids having these antioxidant activities are known to have at least one free aromatic hydroxyl group. Previously, naringin has been demonstrated to have antiviral,[10] antiallergic[11] and anti-cancer[12] actions through the regulation of ROS. However, very little is known about the effect of naringin supplementation on ethanol feeding. Therefore, this study evaluated the recovery effect of normal diet with or without naringin supplement following 4 weeks of ethanol feeding on changes in the plasma and hepatic lipid profile and hepatic antioxidant enzyme activity.

MATERIALS AND METHODS

1. Animals and Diets

Thirty male Sprague-Dawley rats weighing between 140 and 150 g were purchased from the Bio Genomics. Inc. (Seoul, Korea). The animals were all individually housed in stainless steel cages in an air-conditioned room with controlled temperature (20~23 ℃) and automatic lighting (alternating a 12-h period of light and dark) and fed a pelletized chow diet for 1 week after arrival. Next, the animals were randomly divided into three groups (n=10), which were assigned to one of three dietary categories as shown in Fig. 1; E_8 : ethanol diet for 8 wks, E_4N_4 : ethanol diet for the first 4 wks and normal diet for the last 4 wks, E_4Nna_4 : ethanol diet for the first 4 wks and normal diet with naringin supplement for the last 4 wks. The experiment was carried out under the condition of chronic administration of ethanol after fatty liver was induced by 4 wks of ethanol liquid diet. The E_8 group consumed a liquid diet[13] shown in Table 1 containing 36% of energy as ethanol ad libitum. Ethanol was introduced into the diet gradually starting from 0% (w/v) and increased to 5% (w/v) over a 1-week period. The E_8 group was given ethanol diet (50 g/L) for next 8 weeks after the adaptation period. The rats in the E_4N_4 group received an ethanol-free liquid diet containing dextrin-maltose instead of ethanol for the last 4 weeks. The E_4Nna_4 group was given normal diet with naringin supplement (0.05 g, w/v; Arian Quimica Co., Spain) for the last 4 weeks. The dose of naringin was established by our preliminary study. The food consumption and weight gain were measured everyday and once a week, respectively. At the end of the experimental period, the rats were anesthetized with katamine-HCl following a 12-h fast. Blood was drawn from the inferior vena cava into a heparin-coated tube, and the plasma was obtained

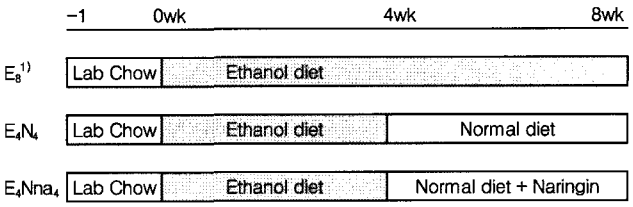

Fig. 1 Experimental schedule for feeding ethanol diet with naringin supplements.

[1)] E_8: ethanol diet for 8 wks, E_4N_4: ethanol diet for the first 4 wks and normal diet for the last 4 wks, E_4Nna_4: ethanol diet for the first 4 wks and normal diet with naringin supplement for the last 4 wks.

Table 1. Composition of Lieber-DeCarli liquid alcoholic diet[1)] and experimental diets.

(g/liter/1,000 calories)

	Ethanol diet (E)	Normal diet (N)	Normal diet + Naringin (Nna)
Casein	41.4	41.4	41.4
L-cystine	0.5	0.5	0.5
DL-Methionine	0.3	0.3	0.3
Corn oil	8.5	8.5	8.5
Olive oil	31.1	31.1	31.1
Dextrin maltose	25.6	115.2[2)]	115.2[2)]
Choline bitartrate	0.53	0.53	0.53
Fiber	10.0	10.0	10.0
Xanthan gum	3.0	3.0	3.0
Vitamin mix[3)]	2.55	2.55	2.55
Mineral mix[4)]	9.0	9.0	9.0
Ethanol	50	-	-
Naringin	-	-	0.05

[1)] The liquid diet is mixed nutritional ingredient in 1 L distilled water.
[2)] Replaced by 25.6 g of dextrin maltose and 50 g of ethanol in the ethanol formula
[3)] Vitamin mixture according to AIN-76.
[4)] Mineral mixture according to AIN-76

by centrifuging the blood at 1,000×g for 15 min at 4 ℃. The livers were then removed, rinsed with physiological saline, and weighed for enzyme analysis, lipid measurement, and morphological analysis. The plasma and livers were stored at -70 ℃ until analyzed.

2. Determination of Plasma Ethanol Concentration

Blood ethanol levels were determined using a commercial kit (Roche, Switzerland) by Cobas Integra 800 (Roche, Switzerland). This enzymatic test for ethanol utilizes the coenzyme NAD and alcohol dehydrogenase (ADH). Formation of NADH can then be measured quantitatively by the increase in the absorbance at 378 nm.[14]

3. Plasma and Hepatic Lipids

The plasma total cholesterol and high-density lipoprotein (HDL)-cholesterol concentrations were determined using

a commercial kit (Sigma) based on the modification of the cholesterol oxidase method of Allain *et al.*.[15] The HDL-fractions were separated using a Sigma kit based on the heparin-manganese precipitation procedure.[16] The plasma triglyceride concentrations were measured enzymatically using a kit from Sigma Chemical Co., a modification of the lipase-glycerol phosphate oxidase method.[17] The hepatic lipids were extracted using the procedure developed by Folch *et al.*.[18] The dried lipid residues were dissolved in 1 mL of ethanol for cholesterol and triglyceride assays. Triton X-100 and sodium cholate solution (in distilled H_2O) were added to 200 μL of the dissolved lipid solution to produce final concentrations of 5 g/L and 3 mmol/L, respectively. The hepatic cholesterol and triglycerides were analyzed with the same enzymatic kit as used in the plasma analysis.

4. HMG-CoA (3-Hydroxy-3-Methylglutaryl-Coenzyme A) Reductase and ACAT (Acyl-CoA:Cholesterol Acyltransferase) Activities

The microsomes were prepared according to the method developed by Hulcher and Oleson[19] with a slight modification. One gram of liver tissue was homogenized in 3 mL of ice-cold buffer (pH 7.0) containing 0.1 mol/L of triethanolamine, 0.02 mol/L of EDTA, and 2 mmol/L of dithiothreitol. The homogenates were centrifuged for 15 min at 10,000×g and 12,000×g at 4 ℃. Next, the supernatants were ultracentrifuged twice at 100,000 g for 60 minutes at 4 ℃. The resulting microsomal pellets were then redissolved in 1 mL of homogenation buffer for protein determination[20] and finally analyzed for their HMG-CoA reductase and ACAT activities.

The HMG-CoA reductase activities were determined as described by Shapiro *et al.*[21] with a slight modification using freshly prepared hepatic microsomes. The incubation mixture (60 μL) containing the microsomes (100~150 μg of protein) and 500 nmol of NADPH (dissolved in the reaction buffer containing 0.1 mol/L of triethanolamine and 10 mmol/L of EDTA) was preincubated at 37 ℃ for 5 minutes. Next, 10 μL of 50 nmol of [^{14}C]HMG-CoA (specific activity, 2.1083 GBq/mmol; NEN™ Life Science Products, Boston, MA) was added, and the incubation was continued for 15 minutes at 37 ℃. The reaction was terminated by the addition of 15 μL of 10 mol/L HCl, and the resultant reaction mixture was incubated at 37 ℃ for an additional 15 minutes to convert the mevalonate into mevalonolactone. The incubation mixture was centrifuged at 10,000×g for 5 min, and the supernatant was spotted on a Silica Gel 60F_{254} TLC plate using mevalonolactone as the standard. The plate was developed in benzene-acetone (1:1, v/v), and air-dried. Finally, the region R_f 0.3~0.6 was removed by scraping with a clean razor blade and its ^{14}C radioactivity was determined using a liquid scintillation counter (Packard Tricarb 1600TR; Packard Instrument, Meriden, CT). The results were expressed as picomol of mevalonate synthesized per min per mg protein.

The ACAT activities were determined using freshly prepared hepatic microsomes, as developed by Erickson *et al.*[22] and modified by Gillies *et al.*.[23] To prepare the cholesterol substrate, 6 mg of cholesterol and 600 mg of Tyloxapol (Triton WR-1339, Sigma) were each dissolved in 6 mL of acetone, mixed well and completely dried in N_2 gas. The dried substrate was then redissolved in 20 mL of distilled water to the final concentration of 300 μg of cholesterol/mL. Next, reaction mixtures containing 20 μL of the cholesterol solution (6 μg of cholesterol), 20 μL of 1 mol/1 of potassium phosphate buffer (pH 7.4), 10 μL of 0.6 mmol/1 bovine serum albumin, 10 μg of the microsomal fraction, and distilled water (up to 180 μL) were preincubated at 37 ℃ for 30 min. The reaction was then initiated by adding 20 μL of 5.62 nmol of [^{14}C]-oleoyl CoA (specific activity; 1.9795 GBq/mmol; NEM™ Life Science Products) to a final volume of 200 μL; the reaction time was 30 min at 37 ℃. The reaction was stopped by the addition 500 μL of isopropanol: haptane (4:1, v/v), 300 μL of haptane, and 200 μL of 0.1 mol/1 potassium phosphate (pH 7.4), and the reaction mixture was allowed to stand at room temperature for 2 min. Finally, an aliquot (200 μL) of the supernatant was subjected to scintillation counting. The ACAT activities were expressed as picomol of cholesteryl oleate synthesized per min per mg protein.

5. Hepatic Morphological Comparison

Livers were removed from the rats and fixed in a buffer solution of 10% formalin. Fixed tissues were processed routinely for paraffin embedding, and 4-μm sections were prepared and dyed with hematoxylin-eosin; stained areas were viewed using an optical microscope with a magnifying power of 200×.[24,25]

6. Hepatic Antioxidant Enzyme Activities

The enzyme sources were isolated using the following procedure. Two gram of liver tissue was homogenized with 10 mL of a 0.25 M sucrose buffer, and the homogenates was then centrifuged at 600×g for 10 min to remove the nuclear fraction, and the remaining separated supernatant was recentrifuged at 10,000×g for 20 min to collect the mitochondrial fraction (pellet) for a CAT (catalase) assay. The supernatant was ultra-centrifuged at 105,000×g for 1 h to isolate the cytosolic fraction for SOD (superoxide

dismutase), GSH-Px (glutathione peroxidase), GR (glutathione reductase) and G6PD (glucose-6-phosphate dehydrogenase) assays. The activities of antioxidant enzymes were measured using the following methods that have been described very briefly.

The SOD activity was measured using Marklund and Marklund's[26] method with a slight modification. One hundred microliters of the cytosol supernatant was mixed with 1.5 mL of a Tris-EDTA-HCl buffer (pH 8.5), then 100 μl of 7.2 mmol/L pyrogallol was added and the reaction mixture was incubated at 25 ℃ for 10 min. The reaction was terminated by the addition of 50 μl of 1 mol/L HCl and measured at 420 nm. One unit of enzyme activity was calculated as the protein content inhibiting 50% of the auto-oxidation of pyrogallol without an enzyme source. The activity was expressed as U/mg protein. CAT activity was measured using Aebi's[27] method with a slight modification, in which the disappearance of hydrogen peroxide was monitored spectrophotometrically at 240 nm for 5 min. A molar extinction coefficient of 0.041 $mM^{-1}cm^{-1}$ was used to determine the CAT activity. The activity was defined as the amount of enzyme which oxidized H_2O_2 μmol/min/mg protein. The GSH-Px activity was measured using Paglia and Valentine's[28] method with a slight modification. The reaction mixture contained 2.6 mL of a 0.1 mol/L of Tris-HCl (pH 7.2) buffer, 100 μl of 30 mmol/L glutathione, and 100 μl of 6 mmol/L NADPH. One hundred microliters of the cytosolic supernatant was added to 2.9 mL of the reaction mixture and incubated at 25 ℃ for 5 min. The reaction was initiated by the addition of 100 μl of 7.5 mmol/L H_2O_2 and the absorbance was measured at 340 nm for 5 min. A molar extinction coefficient of 6.22×10^3 $(mmol/L)^{-1} \cdot cm^{-1}$ was used to determine the activity. One unit of GSH-Px was defined as the amount of enzyme which oxidized 1 μmol per min per mg protein. GR activity was determined with the method of Pinto and Bartley[29] by monitoring the oxidation of NADPH at 340 nm. The reaction mixture contained 1 mM EDTA and 1 mM GSSG in 0.1 M potassium phosphate buffer (pH 7.4). The activity was expressed as oxidized NADPH nmol/min/mg protein. G6PD activity was determined by the method of Pitkanen *et al.*.[30] The reaction mixture contained 55 mM Tris-HCl (pH 7.8), 3.3 mM $MgCl_2$ buffer and 6 mM G-6-P. The activity was expressed as reduced NADPH nmol/min/mg protein.

7. Plasma and Hepatic Lipid Peroxidation (TBARS assay)

The TBARS (thiobarbituric acid-reactive substances) were monitored according to the procedure previously described.[31] Briefly, 500 μL of plasma was well mixed with 3 mL of 5% trichloroacetic acid and 1 mL of freshly prepared 60 mmol/L thiobarbituric acid (TBA). After incubation at 80 ℃ for 90 min, the samples were cooled at room temperature, centrifuged at 1,000×g for 15 min at 4 ℃, and the supernatant absorbance was read at 535 nm.

The levels of hepatic lipid peroxide were determined using the method of Ohkawa *et al.*[32] with a slight modification. Tissue homogenates were prepared in the ratio of 1 g of wet tissue to 4 mL of 1.15% KCl solution using a glass or Teflon Potter-Elvehjem homogenizer. The reaction mixture containing a 0.2 mL aliquot of the homogenates, 0.2 mL of 8.1% sodium dodecyl sulfate (SDS), and 0.6 mL of distilled water was allowed to sit at room temperature for 5 min, and then mixed with 1.5 mL of 20% acetic acid (pH 3.5) and 1.5 mL of a 0.8% aqueous solution of TBA, and finally heated at 95 ℃ for 60 min. After cooling with tap water, 1 mL of distilled water and 5.0 mL of the mixture of n-butanol and pyridine (15:1, v/v) were added and the mixture was vigorously vortexed. After centrifugation at 1000×g rpm for 10 min, the absorbance of the upper layer was measured at 535 nm. A malondialdehyde (MDA) solution made freshly by the hydrolysis of 1,1,3,3-tetramethoxypropane (TMP, Sigma) was used as the standard. The results were expressed as the nmol MDA/mL plasma and nmol MDA/g liver.

8. Measurement of Glutathione (GSH) Content

GSH content was measured using the method of Ellman.[33] Five hundred microliters of the liver homogenate was mixed with 500 μL of 4% (w/v) sulfosalicylic acid and centrifuged at 600×g for 10 min. Three hundred microliters of the supermatant was added to 2.7 mL of a disulfide (5,5'-dithiobis-2-nitrobenzoic acid) reagent, and measured at 412 nm. Total GSH content was expressed as nmole/g of tissue.

9. Statistical Analysis

The parameter values were all expressed as the mean±SE. Significant differences among the groups were determined by one-way ANOVA using the SPSS package program, and the differences between the means were assessed using Duncan's multiple-range test. Statistical significance was considered at $p<0.05$.

RESULTS

1. Effect on Food Intake, Weight Gain and Liver Weights

Body weight gains and food intakes of the groups were

Table 2. Effect of normal diet with or without naringin supplementation on weight gains, food intake and organ weight in rats fed ethanol diet.[1)]

	Groups		
	E_8[2)]	E_4N_4	E_4Nna_4
Weight Gain (g/day)	2.50±0.16[a]	4.28±0.14[b]	4.20±0.15[b]
Food Intake (mL/day)	63.68±1.77[a]	83.96±2.76[b]	84.54±2.18[b]
Liver weight (g/100g B.W.)	3.70±0.10[a]	2.94±0.07[b]	3.03±0.09[b]

[1)] Mean±SE, n=10

[2)] E_8: ethanol diet for 8 wks, E_4N_4: ethanol diet for the first 4 wks and normal diet for the last 4 wks, E_4Nna_4: ethanol diet for the first 4 wks and normal diet with naringin supplement for the last 4 wks.

[ab] Means in the same row not sharing a common superscript are significantly different between groups ($p<0.05$).

significantly higher in the E_4N_4 group and the E_4Nna_4 group than those in the E_8 group (Table 2). However, both E_4N_4 and the E_4Nna_4 groups were significantly lower in the liver weight compared to the E_8 group.

2. Effect on Plasma Ethanol Level

The plasma ethanol level was significantly higher in the E_8 group than in the other groups, as shown in Fig. 2.

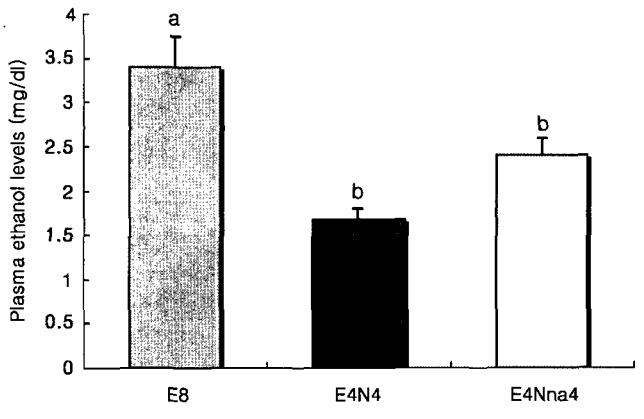

Fig. 2 Effect of normal diet with or without naringin supplementation on plasma ethanol level in rats fed ethanol diet for 4 or 8 weeks.[1)]

[1)] Mean±SE, $n=10$.

E_8: ethanol diet for 8 wks, E_4N_4: ethanol diet for the first 4 wks and normal diet for the last 4 wks, E_4Nna_4: ethanol diet for the first 4 wks and normal diet with naringin supplement for the last 4 wks.

[ab] Means not sharing a common superscript are significantly different between groups ($p<0.05$).

3. Effect on Plasma and Hepatic Lipids

The concentrations of plasma total cholesterol were significantly lower in both the E_4N_4 group and the E_4Nna_4 group than in the E_8 group (Table 3). The HDL-cholesterol concentration was not different among the groups. The HDL-C/total-C ratios of the E_4N_4 and the E_4Nna_4 groups were significantly higher than those of the E_8 group, while the atherogenic index of the E_4N_4 and the E_4Nna_4 groups was lower compared to the E_8 group. The hepatic triglyceride and cholesterol contents were significantly lower in the E_4N_4 and the E_4Nna_4 groups than those in the E_8 group (Table 3).

Table 3. Effect of normal diet with or without naringin supplementation on plasma and hepatic lipids in rat fed ethanol diet.[1)]

	Groups		
	E_8[2)]	E_4N_4	E_4Nna_4
Plasma			
Total cholesterol (mmol/L)	2.62±0.09[a]	1.96±0.02[b]	1.81±0.03[b]
HDL-cholesterol (mmol/L)	1.102±0.04	1.100±0.04	1.103±0.04
HDL-C/Total-C[3)] (%)	42.06±0.91[a]	56.12±0.91[b]	60.94±0.84[b]
Triglyceride (mmol/L)	0.51±0.03	0.57±0.02	0.57±0.03
Atherogenic index[4)]	1.38±0.04[a]	0.78±0.10[b]	0.64±0.04[b]
Liver			
Cholesterol (μmol/g)	270.5± 9.0[a]	83.7± 9.1[b]	72.4± 9.3[b]
Triglyceride (μmol/g)	86.3± 8.2[a]	46.4± 6.4[b]	31.5± 5.1[b]

[1)] Mean±SE, n=10.

[2)] E_8: ethanol diet for 8 wks, E_4N_4: ethanol diet for the first 4 wks and normal diet for the last 4 wks, E_4Nna_4: ethanol diet for the first 4 wks and normal diet with naringin supplement for the last 4 wks.

[3)] HDL-cholesterol/total cholesterol.

[4)] (Total cholesterol-HDL-cholesterol)/HDL-cholesterol.

[ab] Means in the same row not sharing a common superscript are significantly different between groups ($p<0.05$).

4. Hepatic Morphological Comparisons

Accumulation of hepatic lipid droplets (indicated by the arrow) appeared to be the highest in the E_8 group, but lower in the E_4N_4 and the E_4Nna_4 groups (Fig. 3).

5. Effect on Hepatic HMG-CoA Reductase and ACAT Activities

The hepatic HMG-CoA reductase was significantly lower in the E_4Nna_4 group compared to the E_8 group (Fig. 4). The E_4N_4 and E_4Nna_4 diets resulted in lowering hepatic ACAT activity compared to the E_8 group. Especially, the ACAT activity was significantly lower in the E_4Nna_4 group than in the E_4N_4 group (Fig. 4).

6. Effects on Antioxidant Enzyme Activities, TBARS and Glutathione Contents

Regarding the hepatic antioxidant enzyme system, the hepatic SOD, GSH-Px and G6PD activities were significantly higher in both E_4N_4 and E_4Nna_4 groups compared to the E_8 group (Table 4). In contrast, the CAT and GR activities and plasma TBARS level were significantly lower in the E_4N_4 and the E_4Nna_4 groups than in the E_8 group. Hepatic TBARS content was not different among the groups. However, the hepatictotal total GSH content were significantly higher in the E_4Nna_4 group than in the E_8 group (Table 4).

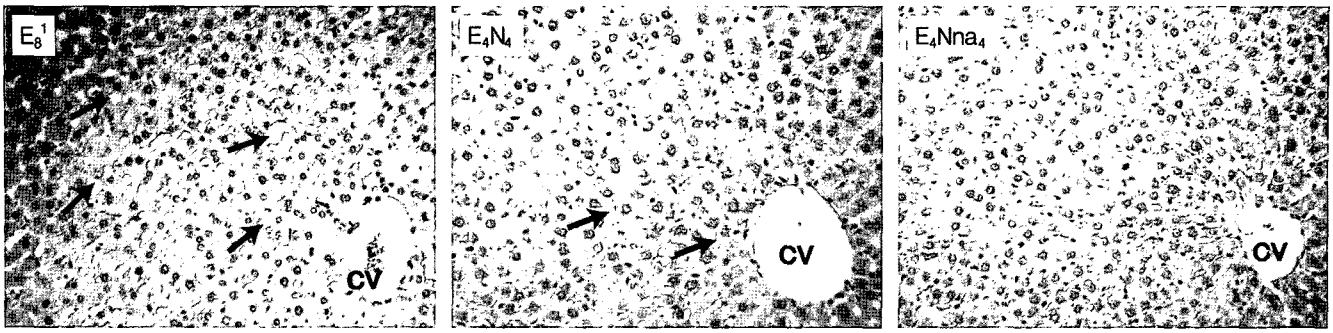

Fig. 3 Effect of normal diet with or without naringin supplementation on liver morphology in rast fed ethanol diet.[1)]

[1)] E_8: ethanol diet for 8 wks, E_4N_4: ethanol diet for the first 4 wks and normal diet for the last 4 wks, E_4Nna_4: ethanol diet for the first 4 wks and normal diet with naringin supplement for the last 4 wks. CV: central vein, Arrows indicates: lipid droplets.

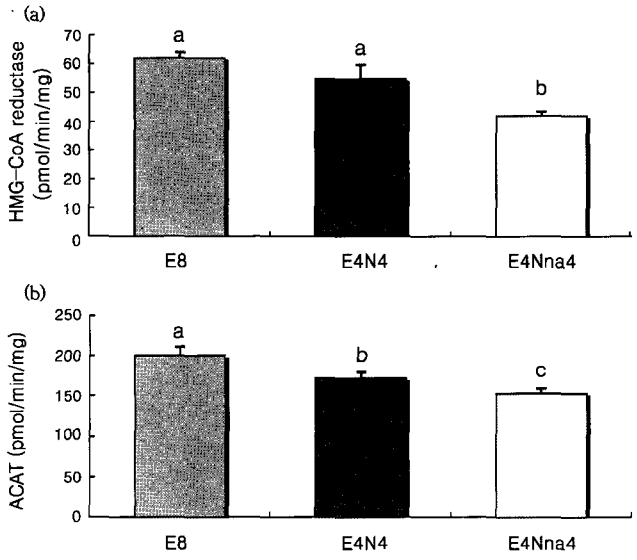

Fig. 4 Effects of normal diet with or without naringin supplementation on hepatic HMG-CoA reductase (a) and ACAT (b) activities in rats fed ethanol diet.[1,2)]

[1)] Mean±SE, n=10.

[2)] E_8: ethanol diet for 8 wks, E_4N_4: ethanol diet for the first 4 wks and normal diet for the last 4 wks, E_4Nna_4: ethanol diet for the first 4 wks and normal diet with naringin supplement for the last 4 wks.

[abc] Means not sharing a common letter are significantly different between groups ($p<0.05$).

Table 4. Effect of naringin supplementation on hepatic antioxidant enzymes activities and hepatic and plasma TBARS levels in rats fed ethanol diet.[1)]

	Groups		
	E_8[2)]	E_4N_4	E_4Nna_4
SOD (units/mg)	0.60± 0.06[a]	0.97± 0.11[b]	1.05± 0.15[b]
CAT (μmol/mg/min)	0.11±0.002[a]	0.08±0.007[b]	0.10±0.003[b]
GSH-Px (nmol/mg/min)	7.89± 0.35[a]	9.98± 0.37[b]	11.18± 0.25[c]
G6PD (nmol/mg/min)	11.79± 0.76[a]	47.47± 5.85[b]	45.65± 7.68[b]
GR (nmol/mg/min)	52.97± 1.26[a]	45.68± 1.35[b]	49.68± 1.12[b]
Total GSH (μmol/g)	2.47± 0.06[a]	2.59± 0.07[ab]	2.71± 0.09[b]
Plasma TBARS (nmol/mL)	3.17± 0.10[a]	2.65± 0.10[b]	2.53± 0.05[b]
Hepatic TBARS (nmol/g)	6.66± 0.16	6.24± 0.29	6.34± 0.20

[1)] Mean±SE, n=10.

[2)] E_8: ethanol diet for 8 wks, E_4N_4: ethanol diet for the first 4 wks and normal diet for the last 4 wks, E_4NNa_4: ethanol diet for the first 4 wks and normal diet with naringin supplement for the last 4 wks.

SOD: Superoxide dismutase, CAT: Catalase, GSH-Px: Glutathione peroxidase, G6PD:Glucose-6- phosphate dehydrigenase , GR: Glutathione reductase, GSH: Glutathione, TBARS: Thiobabituric acid reactive substance.

[abc] Means in the same row not sharing a common superscript are significantly different between groups ($p<0.05$).

DISCUSSION

Alcohol-induced oxidative stress is linked to the ethanol metabolism. Each metabolic pathway [alcohol dehydrogenase, microsomal ethanol oxidation system (MEOS), catalase] produces specific metabolic and toxic disturbances.[34)]

The development of fatty liver, a characteristic early sign of alcoholic liver disease, is partially caused by the alcohol-induced suppression of lipid oxidation in the liver and by increased influx of fat from the peripheral tissues. These early changes are also associated with the typical signs of alcoholic hyperlipidemia, which include elevated serum triacylglycerol levels caused by increased hepatic secretion of very-low-density lipoproteins (VLDLs) and to the delayed removal of the VLDLs resulting from impaired lipoprotein lipase.[35)]

In the present study, total plasma cholesterol concentration was significantly elevated by ethanol feeding as shown by other's findings.[36)] The cholesterol-lowering activity of flavonoids was also identified in the preliminary study by the current authors.[37,38)] However, the plasma cholesterol concentration was not additionally lowered by the naringin supplement to normal diet after terminating ethanol feeding. The naringin supplementation period may need to be extended longer than 4 weeks to observe any additional cholesterol-lowering effect under this condition. The HDL- C/total-C ratios of the E_4N_4 and the E_4Nna_4 groups were significantly higher than those of the E_8 group,

whereas the atherogenic index was lower in the E_4N_4 and the E_4Nna_4 groups than in the E_8 group. The E_4N_4 and E_4Nna_4 diets given after terminating ethanol feeding also significantly lowered both the hepatic cholesterol and triglyceride levels than the E_8 group. These were supported by the reduced hepatic lipid droplets observed in E_4N_4 and E_4Nna_4. Chronic ethanol feeding causes the accumulation of lipids in the liver and of lipid peroxides in other tissues.[39] Accumulation of hepatic lipid droplets was the highest in the E_8 group. In the current study, although the plasma triglyceride content was not significantly different among the groups, the hepatic triglyceride content was lower in the E_4N_4 and E_4Nna_4 groups regardless of naringin supplement. In general, the naringin supplementation lowered both hepatic and plasma cholesterol in high-cholesterol fed rats as shown previously,[40] however it did not bring an additional lipid-lowering effect in spite of down-regulating the hepatic HMG-CoA reductase and ACAT activities.

The liver is the major site for the synthesis and net excretion of cholesterol and the hepatic HMG-CoA reductase activity can normally be decreased under high-cholesterol feeding conditions using a negative feedback control. The two key enzymes involved in the regulation of cholesterol metabolism are HMG-CoA reductase and ACAT.[41] ACAT is an intracellular enzyme that catalyzes the formation of cholesteryl ester from cholesterol and fatty acyl-CoA.[42] It plays key roles in the intracellular cholesterol storage, in lipoprotein assembly in the liver, and in the development of fatty streaks in arteries. Hence, the inhibition of ACAT may represent an attractive mechanism for inducing both hypolipidemic and antiatherosclerotic effects.[43-45] In the current study, the naringin supplements significantly lowered both the hepatic HMG-CoA reductase and ACAT activities. The present results can also be supported by other previous findings. For instance, Bok *et al.* [40] reported that naringin lowered cholesterol biosynthesis and the esterification of hepatic cholesterol. Kim *et al.*[46] recently reported that naringin supplementation significantly inhibited the hepatic HMG-CoA reductase activity in cholesterol diet fed LDL receptor-knockout mice.

Products of alcohol metabolism include acetaldehyde formation from alcohol dehydrogenase enzymatic activity with the production of NADH. Alcohol metabolism increases the NADH to NAD ratio, which can cause the reduction of ferric to ferrous ions, which are involved in the generation of hydroxyl radical from hydrogen peroxide.[47]

All tissues have the capacity to neutralize oxygen radicals to some extent. These mechanisms include superoxide dismutase (SOD) and catalase, which convert the superoxide anion to hydrogen peroxide and hydrogen peroxide to water, respectively. In addition, the glutathione pair, GSH and GSSG, serves as an antioxidant system that relies on NADPH levels from the hexosemonophospate shunt to provide a mechanism to reduce oxidized molecules. Glutathione reductase and glutathione peroxidase (GSH-Px) provide the enzymatic components to this cycle, which is dependent upon selenium. Reduction in cellular levels of GSH and increased levels of GSSG are used as the indication of oxidative stress in a cell culture system or organ system. Vitamin E, α-tocopherol, and vitamin C, ascorbic acid, are important dietary contributions to the antioxidant capacity of the body.[4]

In the present study, the hepatic SOD activity was significantly higher in the E_4N_4 and the E_4Nna_4 groups than in the E_8 group. A study by Schlorff *et al.*[48] also reported that hepatic SOD activity was evidently decreased in ethanol-fed rats, and Chen *et al.*[49] reported that naringin had a strong scavenging activity. Chronic ethanol feeding resulted in lowering GSH-Px activity with elevated GR activity. According to others results,[50] although the increase in the GR activity can promote the recycling of glutathione for the active detoxification of xenobiotics, the decrease in GSH-Px activity may attenuate the radical scavenging function. Nonetheless, the increase in catalase activity in the livers of ethanol- fed rats may compensate, at least in part, for the curtailed radical scavenging capacity due to lowering of GSH-Px activity. This study identified an increase in catalase activity in the livers of ethanol-treated rats.

For optimal GR activity, NADPH must be supplied in adequate concentration. Thus, we have looked for alterations in the G6PD activity and found that it was decreased after chronic ethanol administration. This decrease could be explained by findings of Szweda *et al.*,[51] who described the inactivation of purified bacterial G6PD by trans-4-hydroty-2-nonenal, a toxic product of membrane lipid peroxidation. Considering the physiologic role of this enzyme in supplying NADPH, the coupling of GSH-Px and GR for glutathione recycling in the livers of rats chronically fed ethanol could be hampered by the lower G6PD activity, regardless of the changes in GR activity.[50]

GSH is one of the most important protective factors against oxidative damage[52] and GSH-dependent enzymes such as GSH-Px can act as a free radical scavenger.[53] In this study, the GSH content was concomitantly reduced in the ethanol-treated rats, which corresponds to the results reported by Shaw *et al.*.[54] The GSH content in the ethanol-treated rats was increased by E_4N_4 and E_4Nna_4, whereas the plasma TBARS level in these groups was

significantly lower than in the E_8 group. The increase in the TBARS level may be due to an increased production of oxygen free radicals that exceed the metabolizing capacity of the antioxidant enzymes.[55] Chronic ethanol feeding causes hepatic GSH depletion and lipid peroxide initiation,[56] which agrees with the results of the current study as the hepatic GSH content was significantly decreased in the ethanol-treated rats. Interestingly, the hepatic TBARS content was not altered by ethanol or naringin supplement for some reasons. Recently, naringin has been demonstrated to play an important role in regulating antioxidative capacity by increasing SOD and catalase activities and by up-regulating the gene expression of SOD, catalase and GSH-Px in rabbits fed a cholesterol-rich diet.[57] More recently, Kanno *et al.*[58] reported that naringin was a useful drug having antioxidant and anti-apoptopic activity as a natural product.

In conclusion, replacing normal diet with or without naringin supplement with ethanol diet led a decrease in the level of plasma and hepatic lipids and plasma TBARS as well as the hepatic HMG-CoA reductase and/or ACAT activities compared to the ethanol-fed rats. The change in GSH-Px activity was the highest in the E_4Nna_4 group that seems to be helpful for improving ethanol-induced fatty liver. Consequently, replacing normal diet for 4 weeks after 4 weeks of ethanol feeding seemed to be very effective for recovering fatty liver that partly could be mediated via the lipid lowering and the enhancement of antioxidant enzyme activities. Post supplementation of the naringin does not seem to be significantly effective for improving lipid profiles or enhancing antioxidant enzyme activities in ethanol fed rats when given short term period.

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