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Interactive Effect of Hesperidin and Vitamin E Supplements on Cholesterol Metabolism in High Cholesterol-Fed Rats

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Abstract: Certain bioflavonoids are potent antioxidants and have pharmacologic effects similar to those of vitamin E. Accordingly, the interactive effect of hesperidin and vitamin E was studied with respect to cholesterol metabolism and the antioxidant status. Hesperidin supplement (0.1%, wt/wt) with comparable levels of vitamin E was provided with a high-cholesterol (1%, wt/wt) diet to rats for 5 weeks. The amount of vitamin E included in the hesperidin-free and hesperidin diets was either a low (low-E) or a normal (normal-E) level. The hesperidin supplement and different levels of dietary vitamin E did not significantly alter the concentrations of plasma triglycerides. However, the inclusion of hesperidin significantly lowered the concentration of plasma cholesterol in both the low-vitamin E group and the normal-vitamin E group compared to the hesperidin-free groups ($p < 0.05$). The hepatic triglyceride content was significantly lowered by the hesperidin supplement, as opposed to the plasma triglyceride content, regardless of the vitamin E level in the diet. The hepatic HMG-CoA reductase activity was significantly lowered by the hesperidin supplement with both the low-vitamin E and the normal-vitamin E compared to the hesperidin-free groups ($p < 0.05$). The hepatic HMG-CoA reductase activity was also significantly lowered with an increase in the dietary vitamin E within the hesperidin and hesperidin-free groups. The excretion of fecal neutral sterol and acidic sterols tended to be lower with the hesperidin supplement. Neither dietary hesperidin nor vitamin E significantly changed the hepatic antioxidant enzyme activity.

This data indicates that hesperidin lowers the concentration of plasma cholesterol and the hepatic triglyceride content regardless of the dietary vitamin E level. However, the concentration of plasma cholesterol in the hesperidin-free groups was dependent on the dietary vitamin E level. This information may contribute to understanding the interactive effect of hesperidin and vitamin E on cholesterol biosynthesis in high cholesterol-fed rats.

Key words: Hesperidin, vitamin E, HMG-CoA reductase, ACAT, fecal sterols, cholesterol metabolism, antioxidant enzymes

Abbreviations used: HDL, high density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; ACAT, acyl CoA:cholesterol acyltransferase.

Introduction

Flavonoids are widely recognized as naturally occurring antioxidants that inhibit lipid oxidation in a biological membrane. There has also been considerable interest in the role of the major phenolic phytochemical components of fruits and other edible plants as dietary antioxidants. Recent advances in the area of bioflavonoids clearly indicate that some of these compounds are potent antioxidants and pharmacokinetics similar to vitamin E [1]. Vitamin E is considered the first line of defense against lipid peroxidation in cell membranes. Among naturally occurring flavonoids, hesperidin has already been pharmacologically evaluated as a potential anticancer agent [2], and exhibits antiexudative activity [3] yet no mutagenic activity [4].

A high consumption of flavonoids has been reported to contribute to a decreased risk of coronary heart disease since dietary hesperidin lowers the serum cholesterol and triglycerides in rats [5, 6]. The plasma cholesterol concentration can be regulated by the biosynthesis of cholesterol, removal of cholesterol from the circulation, absorption of dietary cholesterol, and excretion of cholesterol via bile and feces. Cholesterol is essential for cell membranes, however, the unregulated accumulation of cholesterol causes atherosclerosis. Cellular cholesterol homeostasis is very important for the prevention of cardiovascular disease, and numerous studies have reported on the beneficial effects of HMG-CoA reductase and ACAT inhibitors on hypercholesteolemia and atherosclerosis [7, 8]. However, the effects of bioflavonoids on cholesterol metabolism and the antioxidative status still require clarification.

Accordingly, the present study investigated whether a hesperidin supplement can exhibit a vitamin E-like activity on cholesterol metabolism and antioxidant enzymes. The interactive effects of dietary hesperidin and vitamin E were examined in high cholesterol-fed rats.

Materials and Methods

Animals and diets: Forty male Sprague-Dawley weighing between 90 and 100 g were purchased from the Daehan Laboratory Animal Research Center Co. (Chungbuk, Korea). The animals were individually housed in stainless steel cages in a room with controlled temperature (24°C) and lighting (alternating 12-hour periods of light and dark). All the rats were fed a pellitized commercial chow diet for 6 days after arrival. Next, the rats were randomly divided into four groups (n = 10) and fed a high cholesterol diet (1%, wt/wt) either with or without a hesperidin (0.1%, wt/wt, Sigma Chemical Co.) supplement for 5 weeks. Two levels of vitamin E, a low (low-E) and a normal (normal-E) level, were included in the hesperidin and hesperidin-free diets. The composition of the experimental diet, as shown in Table I, was based on the AIN-76 semisynthetic diet [9, 10] and the low level of vitamin E used in the diet (5 IU/kg diet) included about a 20% marginal vitamin E level for growth (27 IU/kg diet) [11].

Table I: Composition of experimental diets (%)

Groups	Hesperidin-free		Hesperidin	
	Low-E[4]	Normal-E[4]	Low-E	Normal-E
	n = 10	n = 10	n = 10	n = 10
Casein	20	20	20	20
D,L-methionine	0.3	0.3	0.3	0.3
Corn starch	15	15	15	15
Sucrose	49	49	48.9	48.9
Cellulose powder	5	5	5	5
Mineral mixture[1]	3.5	3.5	3.5	3.5
Low-E vitamin mixture[2]	1	–	1	–
Normal-E vitamin mixture[3]	–	1	–	1
Choline bitartrate	0.2	0.2	0.2	0.2
Corn oil	5	5	5	5
Cholesterol	1	1	1	1
Hesperidin	–	–	0.1	0.1
Total	100	100	100	100

[1] AIN-76 mineral mixture.
[2] Vitamin mixture with low dietary vitamin E (in g/kg mixture): thiamin HCl, 0.6; riboflavin, 0.6; pyridoxine HCl, 0.7; niacin, 3.0; D-calcium pantothenate, 1.6; folic acid, 0.2; D-biotin, 0.02; cyanocobalamin (vitamin B_{12}), 0.001; retinyl palmitate premix, 0.8; DL-α-tocopherol, 0.455; vitamin D Trituration, 0.25; menadium sodium bisulfide complex, 0.15; sucrose, finely powdered 991.624.
[3] AIN-76 vitamin mixture.
[4] Vitamin E level in low-E and normal-E diets was 5 IU and 50 IU/kg diet, respectively.

The animals were given food and distilled water ad libitum during the experimental period. The food consumption and weight gain were measured every day and every week, respectively. The feces collected during the last 3 days using metabolic cages were used for determining the fecal sterol. At the end of the experimental period, the rats were anesthetized with Ketamine following a 12-hour fast. Blood samples were taken from the inferior vena cava to determine the plasma lipid profile. The livers were removed and rinsed with physiological saline. All samples were stored at –70°C until analyzed.

Plasma and hepatic lipids: The plasma cholesterol concentration and HDL-cholesterol concentrations were determined using a commercial kit (Sigma) based on a modification of the cholesterol oxidase method of Allain *et al* [12]. The HDL-fractions were separated using a Sigma kit based on the heparin-manganese precipitation procedure [13]. The plasma triglyceride concentrations were mea-

sured enzymatically using a kit from Sigma Chemical Co., a modification of the lipase-glycerol phosphate oxidase method [14]. The hepatic lipids were extracted using the procedure developed by Folch *et al* [15]. The dried lipid residues were dissolved in 1 mL of ethanol for cholesterol and triglyceride assays. Triton X-100 and a 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.

HMG-CoA reductase and ACAT activities: Microsomes were prepared according to the method developed by Hulcher and Oleson [16] with a slight modification. Two grams of liver tissue were homogenized in 4 mL of an ice-cold buffer (pH 7.0) containing 0.1 M of triethanolamine, 0.02 M of EDTA, and 2 mM of dithiothreitol, pH 7.0. The homogenates were centrifuged for 10 min at 10,000× g and then at 12,000× g at 4°C. Next, the supernatants were ultracentrifuged twice at 100,000× g for 60 min at 4°C. The resulting microsomal pellets were then redissolved in 1 mL of a homogenation buffer for protein determination [17] and finally analyzed for HMG-CoA reductase and ACAT activities.

The HMG-CoA reductase activities were determined as described by Shapiro *et al* [18] with a slight modification using freshly prepared hepatic microsomes. An incubation mixture (120 µL) containing microsomes (100~150 µg) and 500 nmol of NADPH (dissolved in a reaction buffer containing 0.1 M of triethanolamine and 10 mM of EDTA) were preincubated at 37°C for 5 min. Next, 50 nmol of [^{14}C]-HMG-CoA (specific activity; 2.1420 GBq/mmol; NEM™ Life Science Products, Inc., Boston, MA) was added and the incubation was continued for 15 min at 37°C. The reaction was terminated by the addition 30 µL of 6 M of HCl and the resultant reaction mixture was incubated at 37°C for a further 15 min 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 60 F254 TLC plate with a mevalonolactone standard. The plate was developed in benzene-acetone (1:1, V/V), and air-dried. Finally, the Rf 0.3~0.6 region was removed by scraping with using a clean razor blade and its ^{14}C radioactivity was determined using a liquid scintillation counter (Packard Tricarb 1600TR; Packard Instrument Company, Meriden, CT). The results were expressed as picomoles mevalonate synthesized per min per mg protein.

The ACAT activities were determined using freshly prepared hepatic microsomes developed by Erickson *et al* [19] as modified by Gillies *et al* [20]. 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 a final concentration of 300 µg of cholesterol/mL. Next, reaction mixtures containing 20 µL of a cholesterol solution (6 µg of cholesterol), 20 µL of a 1 M of potassium-phosphate buffer (pH 7.4), 5 µL of 0.6 mM bovine serum albumin, 50~100 µg of the microsomal fraction, and distilled water (up to 180 µL) were preincubated at 37°C for 30 min. The reaction was then initiated by adding 5 nmol of [^{14}C]-Oleoyl CoA (specific activity; 2.0202 GBq/mmol; NEM™ Life Science Products, Inc.) to a final volume of 200 µL; the reaction time was 30 min at 37°C. The reaction was stopped by the addition 500 µL of isopropanol:heptane (4:1, v/v), 300 µL of heptane, and 200 µL of 0.1 M potassium phosphate (pH 7.4), then 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 activity was expressed as pmole of cholesteryl oleate synthesized per min per mg of microsomal protein.

Fecal sterols: The fecal neutral sterols were determined using a simplified micro-method developed by Czubayko *et al* [21]. Gas-liquid chromatography was carried out with a Hewlett-Packard gas chromatograph (Model 5809; Palo Alto, CA) equipped with a hydrogen flame-ionization detector and a Sac™-5 capillary column (30 m × 0.25 mm i.d., 0.25 µm film; Supelco Inc., Bellefonate, PA, USA). Helium was used as the carrier gas. The temperatures were set at 230°C for the column and 280°C for the injector/detector temperature. 5-α-cholestane (Supelco Inc.) was used as the internal standard. The daily neutral sterol excretion was calculated based on the amount of cholesterol, coprostanol, and coprostanone in each sample. The fecal bile acid was extracted with t-butanol and quantified enzymatically with 3-α-hydroxysteroid dehydrogenase [22].

Plasma vitamin E concentration: The plasma α-tocopherol was determined according to the method of Bieri *et al* [23]. Two hundred µL of plasma was added to 100 µL of tocopherol acetate (internal standard) and extracted by heptane. The collected heptane layer was then filtered using a syringe (0.5 M FH Membrane), dissolved in diethyl ether:methanol (1:3, v/v), and separated by HPLC. The chromatograph was equipped with a micro Bondapack C18 column and detected on UV 292 nm.

Antioxidant enzyme activities and TBARS concentration: The preparation of the enzyme source fraction in the liver tissue was as follows. One gram of liver tissue was homogenized in five-fold of a 0.25 M of sucrose buffer, centrifuged at 600× g for 10 min to discard any cell de-

bris, then the supernatant was centrifuged at 10,000× g for 20 min to remove the mitochondria pellet. Finally, the supernatant was further ultracentrifuged at 105,000× g for 60 min to obtain the cytosol supernatant. The amount of protein in the mitochondrial and cytosolic fractions was measured according to the method of Bradford [17] using bovine serum albumin as the standard.

The superoxide dismutase (SOD) activity was measured using Marklund & Marklund's [24] method with a slight modification. One hundred µL of the cytosol supernatant was mixed with 1.5 mL of a Tris-EDTA-HCl buffer (pH 8.5), then 100 µL of 15 mM pyrogallol was added and the reaction mixture incubated at 25°C for 10 min. The reaction was terminated by adding 50 µL of 1 N HCl, then the activity was measured at 440 nm. One unit was determined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%. The activity was expressed as units/mg protein.

The catalase (CAT) activity was measured using Aebi's [25] method with a slight modification. The mitochondria pellet was dissolved in 1.0 mL of a 0.25 M of sucrose buffer. Ten µL of the mitochondria solution was added to a cuvette containing 2.89 mL of a 50 mM potassium phosphate buffer (pH 7.4), and the reaction was initiated by adding 0.1 mL of 30 mM H_2O_2 to make a final volume of 3.0 mL at 25°C. The decomposition rate of H_2O_2 was measured at 240 nm for 5 min in a spectrophotometer. A molar extinction coefficient of 0.041 $mM^{-1}cm^{-1}$ was used to determine the CAT activity. The activity was defined as the decreased H_2O_2 µmole/min/mg protein.

The glutathione peroxidase (GSH-Px) activity was measured using Paglia & Valentine's [26] method with a slight modification. The reaction mixture contained 2.525 mL of a 0.1 M of Tris-HCl (pH 7.2) buffer, 75 µL of 30 mM glutathione, 100 µL of 6 mM NADPH, and 100 µL of glutathione reductase (0.24 unit). One hundred µL of the cytosol supernatant were added to 2.8 mL of the reaction mixture and incubated at 25°C for 5 min. The reaction was initiated by adding 100 µL of 30 mM H_2O_2 and the absorbance was measured at 340 nm for 5 min. A molar extinction coefficient of 6.22×10^3 $M^{-1}cm^{-1}$ was used to determine the activity. The activity was expressed as the oxidized NADPH µmole/min/mg protein.

As a marker of lipid peroxidation production, the plasma TBARS (thiobarbituric acid reactive substances) concentration was measured using the method of Tarlardgis *et al* [27]. Briefly, 500 µL of plasma were well mixed with 3 mL of 5% trichloroacetic acid and 1 mL of freshly prepared 60 mM of thiobabituric acid (TBA). After incubation at 80°C for 90 min, the samples were cooled at room temperature and centrifuged at 1,000× g for 15 min, then the absorbance of the supernatant was measured at 535 nm using tetramethoxypropane (Sigma Chemical Co.) as the standard.

Statistical analysis: All data is presented as the mean ± SE. The data was evaluated by one-way ANOVA using an SPSS program, and the differences between the means assessed using Duncan's multiple-range test. Statistical significance was considered at $p < 0.05$.

Results

Food intake, weight gains, and organ weights: There was no significant difference in the food intake, weight gain, or organ weight between the various groups (Table II). Accordingly, they were not seemingly affected by a hesperidin or vitamin E supplement.

Plasma and hepatic lipids: The supplementation of 0.1% hesperidin significantly lowered the plasma cholesterol concentration in both the low-E and normal-E groups compared to the control (hesperidin-free) groups, however, there was no change in the plasma triglyceride concentration (Table III). In contrast, the hepatic triglycerides level was significantly lowered in the hesperidin supplemented-groups compared to the hesperidin-free groups (Table III). The supplementation of hesperidin with two different levels of vitamin E produced no significant changes in the plasma HDL-C levels and HDL-C/total-C

Table II: Effect of hesperidin and vitamin E supplementation on food intake, weight gain, and organ weight in high cholesterol-fed rats*

Groups	Hesperidin-free		Hesperidin	
	Low-E	Normal-E	Low-E	Normal-E
Food intake (g/day)	19.44 ± 0.44^{NS}	20.66 ± 0.42	20.47 ± 0.31	20.17 ± 0.37
Weight Gain (g/week)	45.08 ± 1.00^{NS}	46.00 ± 1.32	45.54 ± 1.38	45.32 ± 1.29
Organ Weights (g)				
Liver	13.58 ± 0.36^{NS}	13.82 ± 0.61	14.20 ± 0.71	12.79 ± 0.51
Heart	1.16 ± 0.04^{NS}	1.11 ± 0.03	1.18 ± 0.05	1.18 ± 0.02
Kidney	2.45 ± 0.04^{NS}	2.32 ± 0.07	2.32 ± 0.08	2.25 ± 0.09

* Mean ± S.E. ^{NS}Not significantly different ($p < 0.05$) between groups.

Table III: Effect of hesperidin and vitamin E supplementation on plasma and hepatic lipids in high cholesterol-fed rats*

Groups	Hesperidin-free		Hesperidin	
	Low-E	Normal-E	Low-E	Normal-E
Plasma				
Total cholesterol (mmol/L)	3.83 ± 0.25[a]	3.40 ± 0.15[b]	2.90 ± 0.10[c]	2.62 ± 0.07[c]
HDL-cholesterol (mmol/L)	0.66 ± 0.05[NS]	0.72 ± 0.07	0.69 ± 0.06	0.67 ± 0.08
HDL-C/Total-C (%)	17.49 ± 2.29[NS]	20.78 ± 2.35	22.43 ± 2.28	25.96 ± 2.85
TG (mmol/L)	1.16 ± 0.07[NS]	1.10 ± 0.05	1.10 ± 0.18	1.01 ± 0.05
Atherogenic Index**	4.80 ± 0.62[a]	3.75 ± 0.43[b]	3.20 ± 0.30[b]	2.61 ± 0.51[b]
Liver				
Cholesterol (mmol/g)	0.31 ± 0.03[NS]	0.29 ± 0.01	0.29 ± 0.01	0.28 ± 0.01
TG (mmol/g)	0.27 ± 0.01[a]	0.25 ± 0.02[a]	0.19 ± 0.01[b]	0.18 ± 0.01[b]

* Mean ± S.E.
** Atherogenic index: (Total cholesterol-HDL-cholesterol) / HDL-cholesterol.
[abc] Means in same row not sharing a common superscript are significantly different between groups ($p < 0.05$).
[NS] Not significantly different ($p < 0.05$) between groups.

ratios compared to the hesperidin-free groups. The HDL-C/total-C ratios tended to be higher in the hesperidin-supplemented groups than in the hesperidin-free groups. The atherogenic index was significantly higher in the low-E hesperidin-free group than in all the other groups.

Hepatic HMG-CoA reductase and ACAT activities: The hepatic HMG-CoA reductase activity was significantly lowered by the hesperidin supplement in both the low-E group and the normal-E group compared to all the hesperidin-free groups (Fig. 1). This enzyme activity was also significantly lower when the dietary vitamin E was changed from low level to normal level in the hesperidin and heseridin-free groups. Accordingly, the hepatic HMG-CoA reductase inhibitory effect was significantly enhanced by an increase in the dietary vitamin E level. However, the hepatic ACAT activity (Fig. 2) was only significantly lower in the low-E hesperidin group than in the low-E hesperidin-free group.

Fecal sterols: The daily excretion of fecal sterols is shown in Table IV. The effect of the hesperidin supplementation resulted in some minor changes in the fecal neutral and acidic sterol under the cholesterol-fed conditions. The total neutral sterol was significantly lower in the two hesperidin groups compared to the low-E hesperidin-free group ($p < 0.05$). Whereas the fecal acidic sterol was significantly lower in the low-E hesperidin group than in the two hesperidin-free groups ($p < 0.05$). The total fecal sterol excretion was significantly lower in the low-E hesperidin group than in the low-E hesperidin-free group ($p < 0.05$). Accordingly, hesperidin or vitamin E supplementation would appear to decrease the excretion of neutral and acidic sterols.

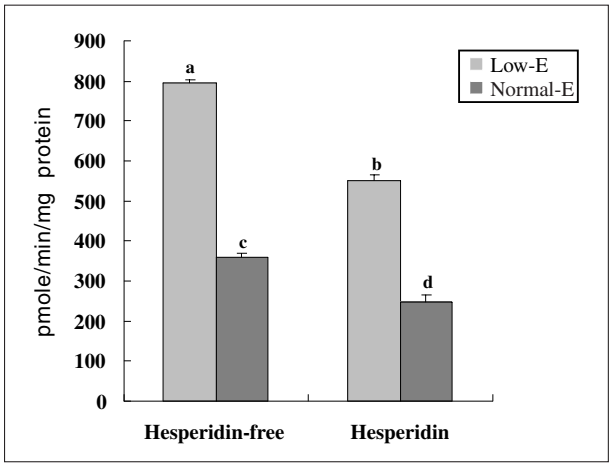

Figure 1: Effect of hesperidin and vitamin E supplementation on hepatic HMG-CoA reductase activity in high cholesterol-fed rats. Mean ± S.E. Means not sharing a common letter are significantly different between groups ($p < 0.05$).

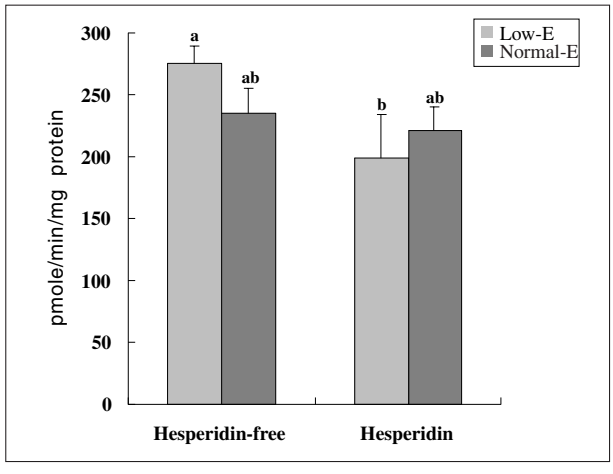

Figure 2: Effect of hesperidin and vitamin E supplementation on hepatic ACAT activity in high cholesterol-fed rats. Mean ± S.E. Means not sharing a common letter are significantly different between groups ($p < 0.05$).

Table IV: Effect of hesperidin and vitamin E supplementation on fecal sterol concentration in high cholesterol-fed rats*

Groups	Hesperidin-free		Hesperidin	
	Low-E	Normal-E	Low-E	Normal-E
Neutral sterol (mg/day)	400.30 ± 13.19[a]	334.14 ± 33.75[ab]	308.36 ± 24.88[b]	322.55 ± 15.42[b]
Acidic sterol (mg/day)	1.84 ± 0.12[a]	1.60 ± 0.14[a]	1.10 ± 0.11[b]	1.40 ± 0.12[ab]
Total fecal sterol (mg/day)	402.13 ± 13.20[a]	335.75 ± 36.49[ab]	309.91 ± 25.03[b]	323.97 ± 14.77[ab]

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

Table V: Effect of hesperidin and vitamin E supplementation on SOD, CAT , and GSH-Px activities and TBARS concentration in high cholesterol-fed rats*

Groups	Hesperidin-free		Hesperidin	
	Low-E	Normal-E	Low-E	Normal-E
SOD[1]	5.58 ± 0.18[NS]	5.55 ± 0.22	5.38 ± 0.22	5.37 ± 0.29
CAT[2]	298.88± 20.39[NS]	257.82 ± 36.26	192.87 ± 29.70	211.34 ± 50.47
GSH-Px[3]	1.38 ± 0.20[NS]	1.95 ± 0.70	1.22 ± 0.36	1.47 ± 0.17
TBARS[4]	19.19 ± 0.62[NS]	17.93 ± 0.30	20.08 ± 0.50	18.66 ± 1.09

[1] Superoxide dismutase: unit/mg protein.
[2] Catalase: decreased H_2O_2 µmole/min/mg protein.
[3] Gluthathione peroxidase: oxidized NADPH µmole /min/mg protein.
[4] Thiobarbituric acid reactive substances: nmole/mL.
* Mean ± S.E.
[NS] Not significantly different ($p < 0.05$) between groups.

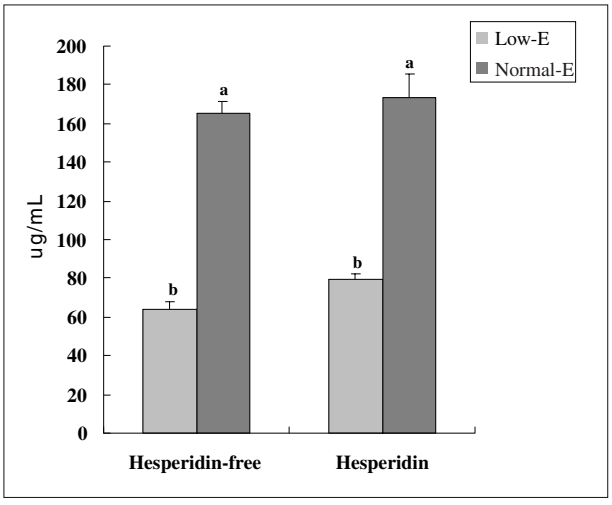

Figure 3: Effect of hesperidin and vitamin E supplementation on plasma vitamin E concentration in high cholesterol-fed rats. Mean ± S.E. Means sharing a common letter are not significantly different between groups ($p < 0.05$).

Concentrations of plasma vitamin E and TBARS, and activities of hepatic antioxidant enzymes: The plasma vitamin E concentration was significantly higher in those rats supplemented with normal-E than in the rats that received low-E ($p < 0.05$) (Fig. 3). The hesperidin supplement had no affect on the plasma vitamin E concentration.

No marked differences in the SOD, GSH-Px, and CAT activities and TBARS concentration were observed between the hesperidin-free and hesperidin-supplemented groups (Table V). Therefore, the dietary vitamin E level did not appear to have any affect on these enzyme activities.

Discussion

Hesperidin, a citrus flavonoid that is widely distributed among plants, is known to have an effect on hypolipidemia and resist coronary heart disease and cancer [28]. Kroyer [29] showed that hesperidin and naringin as well as their aglycones are responsible for the antioxidative activity of citrus peel and extracts as opposed to alpha-tocopherol *in vitro*. Accordingly, the combined effects of dietary hesperidin and vitamin E level were investigated in the present study to determine their possible interaction. The results suggest that the cholesterol-lowering effects of a hesperidin and vitamin E supplement may be different in high cholesterol-fed rats based on their cholesterol metabolism.

The liver is the major site for the synthesis and net excretion of cholesterol, either directly as free cholesterol in the bile or after conversion into bile acid. The hepatic HMG-CoA reductase activity can normally be decreased under high-cholesterol feeding conditions using a negative feedback control [30]. In this study, the plasma cholesterol concentration and hepatic HMG-CoA reductase activity were both lowered by hesperidin and vitamin E supplements, however, the biological effect of hesperidin

was more potent than that of vitamin E. Although the hepatic triglyceride content was significantly lowered in the hesperidin supplemented groups, it was unaffected by dietary vitamin E.

Dietary hesperidin is deglycosylated to hesperitine by intestinal bacteria prior to absorption [31]. The cholesterol-lowering activity of hesperidin was also identified in a preliminary study by the current authors [32]. Accordingly, part of the present result is also supported by previous observation [33], where the supplement of a citrus bioflavonoid mixture lowered the plasma cholesterol concentration and fecal neutral sterol in high cholesterol-fed rats. Recently, Kurowska *et al* [34] reported that citrus juices induce the reduction of LDL cholesterol as well as cholesterol excretion in rabbits fed a semipurified cholesterol-free diet. Furthermore, the current authors have also suggested that the citrus bioflavonoid naringenin inhibits hepatic HMG-CoA reductase during its cholesterol-lowering action [35]. Accordingly, the action of hesperidin in the present study is very similar to that of naringin in high cholesterol-fed rats. Therefore, it would appear that the lower plasma cholesterol concentration caused by the hesperidin supplement then increases the absorption of dietary cholesterol, thereby resulting in a decreased fecal sterol excretion.

The dietary hesperidin or vitamin E levels produced no effect on the antioxidant enzyme activities or plasma TBARS concentrations. The usual vitamin E requirement for most of the frequently used strains of rats is a 27 IU/kg diet when the lipid comprises less than 10% of the diet [11]. However, the low vitamin E used in this study, a 5 IU/kg diet, seemed to maintain the antioxidant function and plasma TBARS concentration at a certain level for the given experimental period in high cholesterol-fed rats. There were no significant differences in the antioxidant enzyme activities or plasma TBARS concentration between the two vitamin E levels regardless of a hesperidin supplement.

The effect of dietary vitamin E on the antioxidant enzyme activities and serum lipid concentration appears to vary according to the species of experimental animal used. Recently, Sharma *et al* [36] reported that vitamin E is a protective factor in atherosclerosis in rhesus monkeys as a vitamin E injection significantly decreased the concentrations of lipid peroxides and serum cholesterol and triglyceride in atherogenic diet-fed monkeys. In contrast, no marked changes in the SOD, GSH-Px, and CAT activities were observed in vitamin E-deficient rats [37], whereas a vitamin E-supplemented high cholesterol diet preserved the plasma SOD activity in rabbits [38].

The present study suggests that hesperidin and vitamin E supplements were effective in lowering the plasma cholesterol concentration and hepatic HMG-CoA reductase activities. However, it is also worth mentioning that the inhibitory effect of hesperidin on hepatic HMG-CoA reductase was greater with the normal vitamin E level than with the low vitamin E level in a high-cholesterol diet. This data would appear to indicate that the impact of a hesperidin supplement is more beneficial than that of dietary vitamin E in improving the overall cholesterol metabolism.

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References

1. Chan, A. C. (1998) Vitamin E and atherosclerosis. J. Nutr. 128, 1593–1596.
2. Aboobaker, V. S., Balgi, A. D. and Bhattacharya, R. K. (1994) *In vivo* effect of dietary factors on the molecular action of aflatoxin B1: role of non-nutrient phenolic compounds on the catalytic activity of liver fraction. In vivo 8, 1095–1098.
3. Lambev, I., Krushkov, I., Zheliazkov, D. and Nikolov, N. (1980) Antiexudative effect of naringin in experimental pulmonary edema and peritonitus. Eksp. Med. Morfol. 19, 207–212.
4. Fisher, K. D. (1982) Evaluation of the health aspects of hesperidin, naringin, and citrus bioflavonoid extract as food ingredients. Prepared for Bureau of Food and Drug Administration. Department of health and human services. Washington, D.C. Contract No. FDA 223-78-2100.
5. Monforte, M. T., Trovato, A., Kirjavainen, S., Forestieri, A. M., Galati, E. M. and LoCurto, R. B. (1995) Biological effects of hesperidin, a circus flavonoid (note 2): hypolipidemic activity on experimental hypercholesterolemia in rats. Farmaco 50, 595–599.
6. Kawaguchi, K., Mizuno, T., Aida, K. and Uchino, K. (1997) Hesperidin as an inhibitor of lipase from pocrin pancreas and Pseudomonas. Bio. Biotech. Biochem. 61, 102–104.
7. Bocan, T. M., Mueller, S. B., Brown, E. Q., Lee, P., Bocan, M. J., Rea, T. and Pape, M. E. (1998) HMG-CoA reductase and ACAT inhibitors act synergistically to lower plasma cholesterol and limit atherosclerotic lesion in the cholesterol-fed rabbit. Atherosclerosis 139, 21–30.
8. Hay, J. M., Yu, W. M. and Ashraf, T. (1999) Pharmacoeconomics of lipids-lowering agents for primary and secondary prevention of coronary artery disease. Pharmacoeconomics 15, 47–74.
9. American Institute of Nutrition (1977) Report of the American Institute of Nutrition. Ad Hoc Committee on standards for nutritional studies. J. Nutr. 107, 1340–1348.
10. American Institute of Nutrition (1980) Report of Ad Hoc Committee on standards for nutritional studies. J. Nutr. 110, 1717–1726.

11. National Research Council (1995) Nutrient requirements of laboratory animals. Fourth revised edition. National academy press, Washington, D.C. pp.11–79.
12. Allain, C. C., Poon, L. S. and Chan, C. S. G. (1974) Enzymatic determination of total serum cholesterol. Clin. Chem. 20, 470–475.
13. Waenic, R. G. and Albers, J. J. (1978) A comprehensive evaluation of the heparin-manganease precipitation procedure for estimating a high density lipoprotein cholesterol. J. Lipid Res. 19, 65–76.
14. McGowan, M. W., Artiss, J. D., Strandbergh, D. R. and Zak, B. (1983) A peroxidase-coupled method for the colorimetric determination of serum triglycerides. Clin. Chem. 29, 538–542.
15. Folch, J., Lees, M. and Sloan-Stanley, G. H. (1957) A simple method for isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509.
16. Hulcher, F. H. and Oleson, W. H. (1973) Simplified spectrophotometric assay for microsomal 3-hydroxy-3-methylglutaryl CoA reductase by measurement of coenzyme A. J. Lipid Res. 14, 625–631.
17. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
18. Shapiro, D. J., Nordstrom, J. L., Mitschelen, J. J., Rodwell, V. W. and Schimke, R. T. (1974) Micro assay for 3-hydroxy-3-methylglutaryl-CoA reductase in rat liver and in L-cell fibroblasts. Biochim. Biophy. Act. 370, 369–377.
19. Erickson, S. K., Schrewsbery, M. A., Brooks, C. and Meyer, D. J. (1980) Rat liver acyl-coenzyme A:cholesterol acyltransferase: its regulation *in vivo* and some of properties in vitro. J. Lipid Res. 21, 930–941.
20. Gillies, P. J., Rathgeb, K. A. and Robinson, C.S. (1986) Regulation of Acyl-CoA:Cholesterol Acyltransferase activity in normal and atherosclerotic rabbit aortas: Role of a cholesterol substrate pool. Exp. Mol. Path. 44, 320–339.
21. Czubayko, F., Beumers, B., Lutjohann, D. and von Bergmann, K. (1992) A simplified micro-method for quantification of fecal excretion of neutral and acidic sterols for outpatient studies in humans. J. Lipid Res. 32, 1861–1867.
22. Michael, J. C. and Ian, A. M. (1980) Enzymic Determination of 3α-, 7α-, and 12α-Hydroxyl Groups of Fecal Bile salts. Clin. Chem. 26, 1298–1300.
23. Bieri, G., Tolliver, J. J. and Catignani, G. L. (1979) Simultaneous determination of alpha-tocopherol and retinol in plasma or red blood cells by high pressure liquid chromatograph. Am. J. Clin. Nutr. 32, 2143–2149.
24. Marklund, S. and Marklund, G. (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J. Biochem. 47, 469–474.
25. Aebi, H. (1974) Catalase in "Method of Enzymatic Analysis". Academic Press, New York, vol. 2, 673–684.
26. Paglia, E. D. and Valentine, W. N. (1967) Studies on the quantitative and qualitative characterization of erythrocytes glutathione peroxidase. J. Lab. Clin. Med. 70, 158–169.
27. Tarladgis, B. G., Pearson, A. M. and Dugan, L. R. (1964) Chemistry of the 2-thiobarbituric acid test for determination of oxidative rancidity in foods. J. Sci. Food Agri. 15, 602–607.
28. Isabelle, M., Gerarad, L., Pirre, C. and Josiane, C. (1994) Role of flavonoids and iron chelation in antioxidant action. Meth. Enzymol. 234, 437–443.
29. Kroyer, G. (1986) The antioxidant activity of citrus fruit peels. Z Ernährungswiss. 25, 63–69.
30. Bjorkhem, I. (1985) Mechanism of bile acid biosynthesis in mammalian liver. In: New comprehensive Biochemistry (Danielsson, H. and Sjovall, J. eds.), pp. 231–278, Elsevier Scientific publishing Co., Amsterdam:
31. Ammer, B., Weintraub, R. A., Johnson, J. V., Yost, R. A. and Rouseff, R. L. (1996) Flavanone absorption after naringin, hesperidin, and citrus administration. Clin. Pharmacol. Ther. 60, 34–40.
32. Lee, S. H., Jeong, T. S., Park, Y. B., Kwon, Y. K., Choi, M. S. and Bok, S. H. (1999) Hypocholesterolemic effect of hesperidin mediated by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase and acyl coenzyme A: cholesterol acyltransferase in rats fed high-cholesterol diet. Nutr. Res. 19, 1245–1258.
33. Bok, S. H., Lee, S. H., Park, Y. B., Bae, K. H., Son, K. H., Jeong, T. S. and Choi, M. S. (1999) Plasma and hepatic cholesterol and hepatic activities of 3-hydroxy-3-methyl-glutaryl-CoA reductase and CoA: cholesterol acyltransferase are lower in rats fed citrus peel extract or a mixture of citrus bioflavonoids. J. Nutr. 129, 1182–1185.
34. Kurowska, E. M., Borradaile, N. M., Spence, J. D. and Carroll, K. K. (2000) Hypocholesterolemic effects of dietary citrus juices in rabbits. Nutr. Res. 20, 121–129.
35. Lee, S. H., Park, Y. B., Bae, K. H., Bok, S. H., Kwon, Y. K., Lee, E. S. and Choi, M. S. (1999) Cholesterol-lowering activity of naringenin via inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase and coenzyme A: cholesterol acyltransferase in rats. Ann. Nutr. Metab. 43, 173–180.
36. Sharma, N., Desigan, B., Ghash, S., Sanyal, S. N., Ganguly, N. K. and Majumdar, S. (1999) Effect of antioxidant vitamin E as a protective factor in experimental atherosclerosis in rhesus monkeys. Ann. Nutr. Metab. 43, 181–190.
37. Masugi, F. and Nakamura, T. (1976) Effect of vitamin E deficiency on the level of superoxide dismutase, glutathione peroxidase, catalase and lipid peroxide in rat live. Int. J. Vitam. Nutr. Res. 46, 187–191.
38. Chen, L., Haught, W. H., Yang, B., Saldeen, T. G., Parathasarathy, S. and Mehta, J. L. (1997) Preservation of endogenous antioxidant activity and inhibition of lipid peroxidation as common mechanisms of antiatherosclerotic effects of vitamin E, lovastatin and amlodipine. J. AM. Coll. Cardiol. 30, 569–575.

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