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Comparison of Jujube Extract with Tangerine Peel Extract in Lowering Plasma Lipids and Activities of Cholesterol Regulating Enzymes in Cholesterol-Fed Rats

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Abstract

A potential mechanism through which the jujube extract might produce a cholesterol-lowering effect was compared with that of tangerine peel extract *in vivo*. Two extracts were prepared using ethanol. Male rats were fed a high cholesterol (1%, w/w) lab chow with jujube extract (1.2%, w/w) or tangerine peel extract (6.3%, w/w) for 3 weeks. Activities of hepatic HMG-CoA reductase (289.6±12.9 and 296.7±11.6 nmole/min/mg vs. 347.9±17.5 nmole/min/mg) and ACAT (554.8±18.2 and 451.7±19.4 nmole/min/mg vs. 602.6±21.4 nmole/min/mg) were significantly lowered by both supplements compared to the control group. These two supplements also substantially reduced the concentrations of plasma cholesterol (103.3±15.9 and 101.6±19.4 mg/dL vs. 141.6±18.1 mg/dL) and triglyceride (61.3±5.5 and 55.5±3.9 mg/dL vs. 96.0±4.2 mg/dL). The inhibition of HMG-CoA reductase resulting from the supplementation of jujube or tangerine extracts could count for the reduction in plasma cholesterol. Accordingly, lipid-lowering action of both supplements appears to be similar in high-cholesterol fed rats.

Key words: high-cholesterol diet, jujube, HMG-CoA reductase, ACAT, tangerine peel extract

INTRODUCTION

A major risk factor for the development of coronary artery disease or arteriosclerosis is elevated levels of plasma cholesterol (1). Any excess cholesterol needs to be removed to such an amount consistent with maintenance of normal body functions.

The regulation of plasma cholesterol levels involves factors that influence both extracellular and intracellular cholesterol metabolism. The two key enzymes involved are 3-hydroxy-3-methylglutaryl-coenzyme A (EC 1.1.1.34) (HMG-CoA) reductase and acyl coenzyme A:cholesterol O-acyltransferase (EC 2.3.1.26) (ACAT). The rate-limiting step in cholesterol synthesis is the conversion of HMG-CoA into mevalonate by HMG-CoA reductase. Inhibition of HMG-CoA reductase results in the decrease of cholesterol synthesis and its inhibitors have been repeatedly shown to be very effective in lowering serum cholesterol in most animal species including human (2-4). The inhibitors are now widely used in hypocholesterolemic drugs (5,6). The ACAT, another key enzyme catalyzing the intracellular esterification of cholesterol, has been shown to be involved in the cholesterol absorption, the secretion of hepatic very low density lipoprotein-cholesterol and the cholesterol accumulation in the arterial wall (7). For these reasons, ACAT inhibitors have been used in test drugs as cholesterol-lowering agents as well as anti-atherosclerotic agents. Treatment of selective ACAT inhibitors has led variable degrees of reduction in plasma cholesterol in different animal species (8-10).

Some bioflavonoids have shown to be associated with a prevention of chronic diseases such as cancer and hyperlipidemia (11,12). Recently among naturally occurring flavonoids, hesperidin was pharmacologically evaluated as a potential anti-inflammatory agent (13), and a cholesterol-lowering agent by improving the cholesterol metabolism in diet-induced hypercholesterolemic rats (14). A significant decrease in plasma cholesterol was previously observed in rats fed 1% high-cholesterol diet with 0.1% hesperetin (w/w) (15), which is a glycone compound of hesperidin. However, little is known about the physiological activity of jujube. The present study was designed to compare the effects of jujube extract with a tangerine peel extract, which includes hesperidin as a major citrus bioflavonoid, on cholesterol metabolism in rats fed a high-cholesterol diet.

METHODS AND MATERIALS

Animals and diets

Thirty male Sprague-Dawley rats weighing between 90 and 100 g were purchased from Bio Genomics Inc. (Seoul, Korea). The animals were individually housed in stainless steel cages in a room with controlled temperature (20~23°C) and lighting (alternating 12 hours period of light and dark) and fed a commercial chow diet for six days after arrival. They were randomly divided into three groups (n=10) and fed a 1 g/100 g high-cholesterol diet with group 1 receiving a jujube extract supplement (1.2 g/100 diet), group 2 a tangerine-peel extract supplement (6.3 g/100 g diet equivalent to 0.1 g hesperidin/100

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g diet) prepared with ethanol, and group 3 no supplement for 3 weeks. The pelleted lab chow was powdered to mix with cholesterol and two supplements. Dried jujube powder and tangerine peel weighing 6.7 kg each were extracted with 80 L of 95% ethanol for 24 h at 60°C. The extract was filtered and concentrated using a high-capacity evaporator (EYELA Rotary vacuum evaporator N-11; Tokyo Ridadidai Co., Ltd., Japan). The final weight of the jujube and tangerine-peel concentrates was 2.1 kg and 1.7 kg, respectively. Components of the tangerine-peel concentrate were: 100 g of the tangerine-peel concentrate containing 39.1 g H_2O, 2.7 g crude protein, 1.8 g crude fat, 1.0 g crude ash, 20 g fructose, 16.5 g glucose, 8.6 g sucrose, 0.6 g hesperidin, 0.03 g naringin, and 9.67 g other sugars. Components of the jujube concentrate were: 100 g of the jujube concentrate containing 30.2 g H_2O, 4.4 g crude protein, 2.5 g crude fat, 1.9 g crude ash, 22 g fructose, 15.5 g glucose, 13.1 g sucrose, and 10.4 g other sugars. The animals were given free access to food and distilled water during the entire experimental period. The food consumption and weight gains were measured every third day. At the end of the experimental period, animals were anesthetized with ketamine-HCl following a 12 h fast. Blood samples were taken from the inferior vena cava for the determination of plasma lipid. The livers were removed and rinsed with physiological saline. Plasma and livers were stored at -60°C until analyzed.

Plasma lipid analyses

Plasma cholesterol and HDL-cholesterol concentrations were determined using a commercial kit (Sigma Chemical Co., U.S.A.) based on a modification of the cholesterol oxidase method of Allain et al. (16). The HDL-fractions were separated using a Sigma kit based on the heparin-manganese precipitation procedure (17). The plasma triglyceride concentrations were measured enzymatically using a kit from Sigma Chemical Co., a modification of the lipase-glycerol phosphate oxidase method (18).

HMG-CoA reductase and ACAT activities

Microsomes were prepared according to Hulcher and Oleson (19) with a slight modification. Two grams of the liver tissues were homogenized in 4 mL of an ice-cold buffer (pH 7.0) containing 0.1 mol/L triethanolamine, 0.02 moL/L EDTA and 2 mmoL/L dithiothreitol, pH 7.0. The homogenates were centrifuged twice at both 10,000×g and 12,000×g for 10 min at 4°C. Then, the supernatants were ultracentrifuged twice at 100,000×g for 60 min at 4°C. The resulting microsomal pellets were redissolved in 1 mL of a homogenation buffer for protein determination (20) and finally analyzed for HMG-CoA reductase and ACAT activities.

The HMG-CoA reductase activities were determined as described by Shapiro et al. (21) with a slight modification of using freshly prepared hepatic microsomes. The incubation mixtures (120 μL) containing microsome (100~150 g) and 500 nmoL of NADPH (dissolved in a reaction buffer containing 0.1 moL/L triethanolamine and 10 mmoL/L EDTA) were preincubated at 37°C for 5 min. Then, 50 nmoL of [^{14}C]-HMG-CoA (specific activity; 2.1420 GB q/mmole, NEN™ Life Science Products, Inc. U.S.A.) was added and incubated for 15 min at 37°C. The reaction was terminated by the addition of 30 μL of 6 moL/L HCl and the resultant reaction mixture was further incubated at 37°C for 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 F_{254} TLC plate with a mevalonolactone standard. The plate was developed in bezene-acetone (1:1, v/v), and air-dried. Finally, the region Rf 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 Company, U.S.A.). The results were expressed as picomole mevalonate synthesized · min^{-1} · mg microsomal $protein^{-1}$.

The ACAT activities were determined using freshly prepared hepatic microsomes according to the method developed by Erickson et al. (22) as modified by Gillies et al. (23). To prepare the cholesterol substrate, 6 mg of cholesterol and 600 mg of Tyloxapol (Triton WR-1339, Sigma Co., USA) were each dissolved in 6 mL of acetone, mixed well, and completely dried under a N_2 gas. The dried substrate was then redissolved in 20 mL of distilled water to give a final concentration of 300 μg of cholesterol/mL. Then, reaction mixture containing 20 μL of a cholesterol solution (6 g cholesterol), 20 μL of a 1 moL/L potassium-phosphate buffer (pH 7.4), 5 μL of 0.6 mM bovine serum albumin, 50~100 μg of microsomal fraction, and distilled water (up to 180 μL) was preincubated at 37°C for 30 min. The reaction was then initiated by adding 5 nmoles of [^{14}C]-Oleoyl CoA (specific activity; 2.0202 GB q/mmole, NEN™ Life Science Products, Inc., U.S.A.) to give a final volume of 200 μL; the reaction time was 30 min at 37°C. The reaction was stopped by adding 500 μL of an isopropanol-heptane mixture (4:1, v/v), 300 μL of heptane and 200 μL of 0.1 moL/L potassium phosphate (pH 7.4) and the reaction mixture was allowed to stand at room temperature for 2 min. Finally, 200 μL amount of aliquot from the supernatant was subjected to scintillation counting. The ACAT activity was expressed as picomole of cholesteryl oleate synthesized · min^{-1} · mg microsomal $protein^{-1}$.

Statistical analysis

All data were presented as the mean±SE. Significant differences among the groups were determined by one-way ANOVA using SPSS. Duncan's multiple-range test was performed if differences were identified between groups at α = 0.05.

RESULTS

There were no significant differences in the food intake, body weights, and organ weights between the control and the two experimental groups (data not shown). The supple-

mentations of jujube and tangerine-peel extract both significantly lowered the concentrations of plasma total-cholesterol and triglyceride (Table 1). However, the plasma HDL-cholesterol concentration was not significantly different among the groups. The ratios of HDL-cholesterol to total-cholesterol and atherogenic index (AI) in the tangerine-peel extract group were significantly higher than in the control group.

The HMG-CoA reductase and ACAT activities were significantly lower in the jujube and tangerine-peel extract group than in the control group (Table 2). The jujube extract apparently inhibited liver cholesterol biosynthesis and the esterification of hepatic cholesterol by 16.8% and 7.9%, respectively. In addition, tangerine-peel extract inhibited liver cholesterol biosynthesis and the esterification of hepatic cholesterol by 14.7% and 25.0%, respectively. These suggest that both supplements play important roles in cholesterol metabolism.

DISCUSSION

Few reports on the roles of jujube in cholesterol metabolism have been reported. Tangerine-peel extract includes citrus bioflavonoid. A major bioflavonoid in citrus-peel is hesperidin. The 10% hesperidin diet inhibits the pancreatic lipase and lowers the concentrations of plasma triglycerides in rats compared to control (24), and thereafter Monforte et al. (25) reported a hypolipidemic activity of hesperidin in hypercholesterolemic rats.

HMG-CoA reductase inhibitors are well-established drugs for the treatment of hypercholesterolemia. The cholesterol lowering effect of HMG-CoA reductase inhibitors is attributed to as increase in VLDL catabolism as well as increases in specific receptor-mediated uptake in the liver (26,27) in various animal species. When tested *in vitro*, hesperidin did not inhibit the activities of either HMG-CoA reductase or ACAT (14). As shown in the test with cholesterol-fed rats, hesperidin is a potent agent for the inhibition of HMG-CoA reductase (14,15). However, well-known HMG-CoA reductase inhibitor drugs do not have a hypocholesterolemic action in rodents (4,28), yet do in hamsters, rabbits (27) or humans (3). This might be due to differences in lipoprotein metabolism among animal species (29). Dosages of HMG-CoA reductase inhibitors must be very high to exert a hypocholesterolemic response in rats (2,30).

This study identified decreased plasma lipids in animals supplemented with jujube or tangerine-peel extracts. Cholesterol biosynthesis was concomitantly reduced by the jujube and tangerine-peel extracts as indicated by the decreased HMG-CoA reductase activities. Since the cholesterol intake was about the same for all groups, the supplementation of jujube or tangerine-peel extract seemed to promote an efficient utilization of dietary cholesterol, i.e. a possible increase of cholesterol uptake by tissues. In order for the plasma cholesterol to be unchanged or decreased with an elevated level of exogenous cholesterol, the endogenous synthesis of cholesterol would be suppressed to such a degree that suppressed synthesis can override the increased amount of cholesterol by the intestinal absorption. Thus, it seems that jujube or tangerine-peel extract exhibits a very unique mode in the regulation of cholesterol metabolism in rats fed a high-cholesterol diet.

Our results suggest these two supplements lower the cholesterol biosynthesis by an inhibition of hepatic HMG-CoA reductase, resulting in an increased absorption of dietary cholesterol, which may subsequently result in a simultaneous decrease in the fecal neutral sterols (although not measured) in the jujube or tangerine-peel extract supplemented animals. It therefore seems plausible that HMG-CoA reductase activity is inhibited first in animals fed a high-cholesterol diet with

Table 1. Effects of jujube and tangerine-peel extract supplementation on the plasma lipids in high cholesterol-fed rats

Lipids conc.	Groups	Control	Jujube extract	Tangerine-peel extract
Plasma	Total-cholesterol (mg/dL)	141.6±18.1[1)a2)]	103.3±15.9[b]	101.6±19.4[b]
	HDL-cholesterol (mg/dL)	31.1±2.4[a]	25.4±1.2[a]	29.2±1.4[a]
	HDL-C / Total-C (%)	21.9±1.1[a]	24.5±2.1[ab]	28.7±1.4[b]
	Triglyceride (mg/L)	96.0±4.2[a]	61.3±5.5[b]	55.5±3.9[b]
	AI[3)]	3.6±0.1[a]	3.1±0.2[ab]	2.5±0.2[b]

[1)]Mean±S.E., n=10
[2)]Values with different superscripts differ significantly at p=0.05.
[3)]Atherogenic index (AI): (Total cholesterol - HDL cholesterol) / HDL cholesterol

Table 2. Effect of jujube and tangerine-peel extract supplementation on hepatic HMG-CoA reductase and ACAT activities in high cholesterol-fed rats

Enzyme activities	Control	Jujube extract	Tangerine-peel extract
HMG-CoA reductase (nmole/min/mg)	347.9±17.5[1)a2)]	289.6±12.9[b]	296.7±11.6[b]
ACAT (nmole/min/mg)	602.6±21.4[a]	554.8±18.2[b]	451.7±19.4[b]

[1)]Mean±S.E., n=10
[2)]Values with different superscripts differ significantly at p=0.05.

jujube or tangerine-peel extract.

Another assumption remained to be proved is a possible increase of the hepatic cholesterol uptake by an increase in the lipoprotein receptor activities due to the inhibition of hepatic HMG-CoA reductase. The inhibition of HMG-CoA reductase was reported to induce a decrease in the rate of cholesterol biosynthesis, followed by an increase in low density lipoprotein receptors, enhancing the cellular uptake of cholesterol and lowering plasma low density lipoprotein cholesterol (26). This possibility warrants further evaluation of the functional compound of jujube extract.

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