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Absorption of intestinal free cholesterol is lowered by supplementation of Areca catechu L. extract in rats

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Abstract

Areca extracts exhibiting a strong inhibitory activity against pancreatic cholesterol esterase (pCEase) in vitro were previously found to lower the absorption of dietary cholesteryl ester. Therefore, to determine whether a combined Areca extract also affects the absorption of intestinal free cholesterol, male rats were fed a diet containing free cholesterol (1%, w/w) either with or without an Areca nut extract supplement (0.5%, w/w). The Areca extract supplement significantly lowered the plasma cholesterol concentration by 25% without any change in the plasma triglyceride concentration, when compared to the control group. The supplement also significantly lowered the small intestinal pCEase activity by 39.1% compared to that of the control group. As regards the hepatic and intestinal ACAT activities, only the intestinal enzyme activity was significantly lowered by the supplement, when compared to the control group. The absorbed cholesterol that appeared in the blood after an oral dose of [1,2(n)–^{3}H] free cholesterol was significantly lower in the rats supplemented with the Areca nut extract, compared with the control group. These results suggest that the inhibition of intestinal ACAT and possibly pCEase may facilitate the metabolic efficiency of the Areca nut extract as regards the absorption of intestinal free cholesterol. The structure and chemical properties of the active

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compound in the water-soluble Areca extract remain to be elucidated.

Keywords: Areca Nut Extract; Free Cholesterol; ACAT; Pancreatic Cholesterol Esterase

Introduction

The major form of dietary cholesterol is free cholesterol (unesterified form of cholesterol). The movement of cholesterol from the intestinal lumen into lipoprotein particles in the circulation involves many different steps, any of which can be interrupted by a cholesterol absorption inhibitor. Dietary cholesteryl esters (CE) are not absorbed per se and must be hydrolyzed into free cholesterol by pancreatic enzymes before uptake. Cholesterol initially migrates from bile acid micelles into the intestinal brush border membrane. It is then transported from the brush border to the endoplasmic reticulum by a multi-step process. At least two proteins, pancreatic cholesterol esterase (pCEase) and intestinal acyl-CoA:cholesterol acyltransferase (ACAT), are involved in the cholesterol absorption process from the small intestine to the enterocytes through the brush border [1–3]. The free cholesterol is then reesterified in the enterocytes by the action of ACAT, and the resulting cholesterol ester is packaged into chylomicrons and secreted into the lymph.

However, cholesterol absorption efficiency is a contributing factor to the plasma cholesterol level that is a risk factor for the premature development of atherosclerosis [4]. It is thus conceivable that the inhibition of pCEase or ACAT may lead to reduced cholesterol absorption and lower plasma cholesterol levels. Several potent inhibitors of pCEase [5] or ACAT [4,6,7] have been developed and found to lower plasma cholesterol levels in animal models, although the precise action mechanism of these inhibitors has not yet been determined. pCEase is generally known to catalyze both the hydrolysis and synthesis of CE [3], that is, in the lumen of the small intestine, it catalyzes the hydrolysis of dietary CE and facilitates the absorption of free cholesterol through the intestinal mucosa in rats [8], where it catalyzes the esterification of free cholesterol [9]. Accordingly, rats that are fed a high fat/high cholesterol diet exhibit an increased pCEase mRNA expression [10]. However, some studies have shown contradictory results, for example, Weng et al. [11] reported that pCEase facilitates the absorption of both free cholesterol and CE, while Howles et al. [12] demonstrated that pCEase is only responsible for the absorption of CE. ACAT, which catalyzes the intracellular esterification of cholesterol, has also been identified as being involved in cholesterol absorption, hepatic very low density lipoprotein (VLDL) secretion, and cholesterol accumulation in the vascular wall [13,14]. Recently, specific inhibitors of ACAT, such as the phenoxyl-phenyl carbamates WAY-121, 751 and WAY-121, 898, have been shown to be equally effective inhibitors of cholesterol absorption [15] and able to prevent hypercholesterolemia in cholesterol-fed animals [16]. As a result, ACAT inhibitors have been used in test drugs as both cholesterol-lowering and antiatherosclerotic agents.

Currently, little information is available on the inhibitory molecules involved in the process of cholesterol absorption. Therefore, the purpose of this study is to determine whether an

Areca nut extract supplement, which has recently been verified by the present authors as exerting an inhibitory effect on pCEase and the absorption of dietary CE [17], can affect the absorption of intestinal free cholesterol and ACAT activity.

Materials and methods

Preparation of Areca nut extracts and inhibition of pCEase in vitro

Six kg of Areca nuts (Herb market, Taegu, Korea) were cut into pieces, extracted 3 times using 95% ethanol under reflux, and the resulting extracts concentrated in vacuo. The partial extract was suspended in 90% aqueous methanol and then successively partitioned with chloroform, ethyl acetate, and butanol. The fractions obtained from the chloroform, chloroform/water interphase, ethylacetate, n-butanol, and aqueous layers were evaporated completely and then dissolved with either ethanol or ethanol/water. Thereafter, an aliquot from each extract was used to determine the inhibitory potential of the Areca nut fractions on pCEase in vitro.

Eight hundred and eighty g of ethanol extract was obtained from 6 kg of Areca nuts with ethanol under reflux. The ethanol extract of the Areca nut was partitioned using chloroform (81.6 g), interphase materials (213.6 g), ethylacetate (104.4 g), butanol (271.2 g), and water (162 g), successively. These solvent-soluble fractions were then measured for their inhibitory effects against pCEase activity in vitro.

Purification of bovine pCEase

A fresh bovine pancreas was obtained from a local slaughter house and the pCEase was purified according to the method developed by Lee et al. [18]. The purification procedures consisted of acid precipitation at pH 5.0, precipitation with 60% saturation $(NH_4)_2SO_4$, and a successive HPLC (high pressure liquid chromatography) on TSK gel phenyl-5PW and TSK gel heparin-5PW columns. The fractions containing cholesteryl ester hydrolyzing activity were pooled, then, after confirming their purity with SDS-PAGE the pooled fractions were used to determine the inhibitory effect of the Areca nut extracts on pCEase activity.

Measurement of pCEase inhibitory activity of Areca extracts

The cholesteryl ester-hydrolyzing activity of pCEase was measured using the method developed by Lee et al. [18], with slight modifications. Two hundred μL of the total reaction mixture included 680 Bq ^{14}C cholesteryl oleate, 10 μmol/L cholesteryl oleate, 10 μmol/L dipalmitoyl-phosphatidyl choline, 100 mmol/L cholate, 50 mmol/L Tris/HCl, at pH of 8.0, and appropriate amounts of enzyme sources, either purified pCEase or small intestinal fluid (1 μL) obtained from the following animal feeding study. To determine the inhibitory activity on pCEase, the Areca nut extracts were concentrated to a small amount and then resuspended in a solution containing cholate and Tris/HCl. Next, this suspension of substrate vesicles was

Table 1
Inhibitory activities of Areca extracts on pCEase in vitro assay

Fractions[1]	μg/100 μL reaction mixture	Inhibition (%)
Ethanol extract	5	60.5
Chloroform fraction	5	28.0
$CHCl_3/H_2O$ interphase	5	32.0
Ethyl acetate fraction	5	8.0
n-Butanol fraction	5	92.0
Aqueous fraction	5	72.0
n-butanol fr. + aqueous fr.	5	85.0

[1] Fractions were obtained from partial purification of pCEase inhibitor from Areca nut, as described in purification procedure.

added to the solution containing the enzyme. After incubation for 10 min at 37 °C, the reaction was terminated by adding 3.25 mL of methanol/chloroform/heptane (1.41 : 1.25 : 1.0) and 1.05 mL of 50 mmol/L sodium carbonate/50 mmol/L sodium borate, pH 10.0. Ten mL of the aqueous phase was removed for liquid scintillation counting. The inhibitory activities of each fraction are shown in Table 1. As the butanol and aqueous fractions exhibited stronger inhibitory activities than the most of the others, these two fractions were combined and used as the dietary supplement in the animal feeding study.

Animals and diets

Twenty male Sprague-Dawley rats weighing between 90 g and 100 g were purchased from the Daehan Laboratory Animal Research Center (Chungbuk, Korea). The rats were individually housed in stainless steel cages in a room with controlled temperature (20∼23 °C) and lighting (alternating 12 h periods of light and dark) and fed a pelletized commercial chow (Samyang chow, Korea) diet for 2 weeks after arrival. The rats were then randomly divided into 2 groups (n = 10). For 6 d, both groups were fed experimental diets including 1% (w/w) free cholesterol, however, one group was also given an Areca nut extract supplement (Table 2). All rats were dosed with labeled free cholesterol before being killed. The supplementary amount of the Areca nut extract per day was established based on inhibiting the total intestinal pCEase activity in normal rats by approximately 50% in vitro. Accordingly, the amount of Areca extract included in the diet was 0.5% (w/w), respectively (Table 2). The rats were given free access to food and distilled water during the entire period. The intestinal absorption of dietary free cholesterol was measured at the end of the experimental period, on day 7. The body weights were measured every other day.

Absorption of free cholesterol

The absorption of free cholesterol was monitored by measuring the radioactivity of an oral dose of labeled free cholesterol in the intestinal contents and blood. At the end of the experimental period, on day 7, the free cholesterol-fed rats were orally dosed with a

Table 2
Composition of experimental diets (%)

Component	Control (n=10)	Areca (n=10)
Casein	20	20
D,L-Methionine	0.3	0.3
Corn starch	15	15
Sucrose	49	48.5
Cellulose powder	5	5
Mineral mixture[1]	3.5	3.5
Vitamin mixture[2]	1.0	1
Choline bitartrate	0.2	0.2
Corn oil	5	5
Free Cholesterol	1	1
Areca nut extract[3]	–	0.5
Total	100	100

[1] AIN-76 mineral mix (American Institute of Nutrition, 1977).

[2] Vitamin mixture, which furnished per kilogram of diet : thiamin HCl, 0.6; riboflavin, 0.6; pyridoxine HCl, 0.7; nicotinic acid, 0.003; D-calcium pantothenate, 0.0016; folate, 0.2; D-biotin, 0.02; cyanocobalamin (vitamin B_{12}), 0.001; retinyl palmitate premix, 0.8; DL-alpha tocopheryl acetate, premix, 20; cholecalciferol (vitamin D_3), 0.0025; menaquinone (vitamin K), 0.05; antioxidant, 0.01.

[3] n-butanol fraction plus aqueous fraction.

suspension of radiolabelled free cholesterol vesicles, prepared by sonicating 5.5×10^4 Bq [1,2(n)–^{3}H] free cholesterol (1.1 GBq/mmol) and 0.4 mg cholesterol in 1 mL of 0.5% (w/v) phosphate buffered saline, using a stomach tube after withholding food for 10 h. For the rats that received the Areca nut extract supplement, the extract was added to the free cholesterol mixture. The control group was administered a solution of phosphate buffered saline including radiolabelled free cholesterol. Blood was collected from the tail vein 5 times every hour to analyze the change in radioactivity. Seven hours after the oral administration, the rats were anesthetized with ketamine-HCl. Blood samples were taken from the inferior vena cava to determine the radioactivity and plasma lipid levels. The small and large intestines were removed and prepared according to the following procedures in order to measure the intestinal pCEase and ACAT activities and radioactive contents. All organs were washed with saline and weighed to compare any differences between the groups.

Preparation of small and large intestines

The small intestine from the pylorus to the ileocecal junction was removed immediately after the rats were killed. The segments of the small intestine were placed in a beaker and the luminal contents drained. The lumen of each segment was then flushed twice using a small volume of cold saline to a total of 15 mL including the luminal fluid. The sample of luminal fluid was centrifuged at 600 × g for 10 min to precipitate any solid material, and the resulting supernatant was used for the pCEase analysis. The feces and drained intestinal fluid collected from the large and small intestine were used to count the radioactivity.

Hepatic and small intestinal ACAT activities

Microsomes were prepared according to the method developed by Hulcher and Oleson [19] with a slight modification. Two grams of the liver and small intestine 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 at 10,000 × g and then at 12,000 × g for 10 min each time at 4 °C. Next, the supernatants were ultracentrifuged twice at 100,000 × g for 60 min at 4 °C. Thereafter, the resulting microsomal pellets were redissolved in 1 mL of a homogenation buffer for protein determination [20] and finally analyzed for ACAT activities.

The ACAT activities were determined using freshly prepared microsomes according to the method developed by Erickson et al. [21] as modified by Gillies et al. [22]. To prepare the cholesterol substrate, 6 mg of cholesterol and 600 mg of Tyloxapol (Triton WR-1339, Sigma Chemical, St. Louis, MO, USA) were 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 mg cholesterol/L. Next, reaction mixtures containing 20 μL of a cholesterol solution (6 μg cholesterol), 20 μL of a 1 mol/L potassium-phosphate buffer (pH 7.4), 5 μL of 0.6 mmol/L bovine serum albumin, 50–100 μg of the microsomal protein, 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, NEN™ 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 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), 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 pmoles of cholesteryl oleate synthesized·min^{-1}·mg microsomal protein^{-1}.

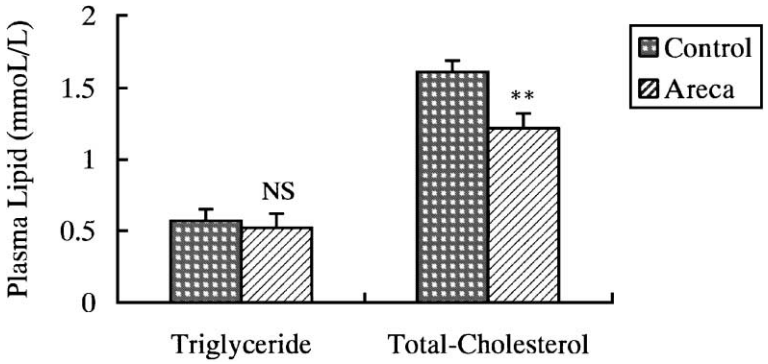

Fig. 1. Effect of Areca nut extracts on plasma triglyceride and total-cholesterol concentrations in free cholesterol-fed rats after 6 d. Mean ± SE, n=10. ** Values are significantly different ($P<0.01$) from control group.

Table 3
Effect of Areca nut extract supplement on recovery of radioactivity from oral dose of labeled free cholesterol in intestinal contents of free cholesterol-fed rats after 6 d

	Control	Areca
Small intestinal Fluid (Bq/mL)	29673 ± 1965	23047 ± 1836*
Large intestinal Content (Bq/mL)	20161 ± 1940	26487 ± 1481*

Mean ± SE, n = 10.
* Means in the same row are significantly different from control group ($P<0.05$).

Plasma lipids

The plasma cholesterol concentrations were determined using a commercial kit (Sigma Chemical, St. Louis, MO) based on a modification of the cholesterol oxidase method developed by Allain et al. [23]. The plasma triglyceride concentrations were measured enzymatically using a kit from Sigma Chemical, a modification of the lipase-glycerol phosphate oxidase method [24].

Statistical analysis

All data are presented as the mean ± SE. The differences between the groups were compared based on a students' *t*-test using standard social science statistical packages.

Results

There were no significant differences in the food intake, weight-gain, or organ weights between the two groups.

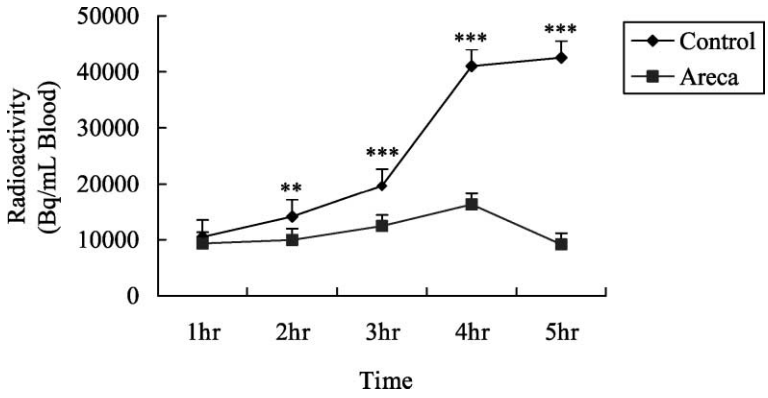

Fig. 2. Effect of Areca nut extract supplement on time-course recovery of radioactivity from oral dose of labeled free cholesterol in blood of free cholesterol-fed rats after 6 d. ** $P<0.01$, *** $P<0.001$: significantly different from those of control group in each time part.

Plasma lipids

The Areca nut extract supplement over 6 d significantly lowered the plasma cholesterol concentration when compared to the control group (1.19±0.06 vs. 1.59±0.09 mmoL/L) (Fig. 1). There was no difference in the plasma triglyceride concentration between the groups.

Recovery of radioactivity from oral dose of [1,2(n)–^{3}H] free cholesterol

The Areca nut extract supplements resulted in distinct changes in the distribution of the labeled free cholesterol in the intestinal luminal contents (Table 3). The radioactivity recovered from the small intestinal fluids was significantly lower in the group that included the Areca extract supplement than in the control group. However, the opposite was true for the large intestinal content. The absorbed cholesterol that appeared in the blood after an oral dose of [1,2(n)–^{3}H] free cholesterol was significantly lower in the rats supplemented with the Areca nut extract, compared with the control group (Fig. 2). The total radioactivity in the blood should have been very high, since the total plasma volume is assumed to be 4% of the body weight [25]. Therefore, the decreased blood radioactivity in the Areca extract supplemented group would appear to reflect a lower absorption of free cholesterol than in the control group, since the cholesterol intake levels for the two groups were similar. The highest activity of the orally dosed labeled free cholesterol was observed 4 h after oral administration.

Effect on intestinal pCEase and intestinal and hepatic ACAT

The pCEase activities of the intestinal fluids were significantly lower in the rats supplemented with cholesterol plus the Areca extract compared to the rats supplemented with only cholesterol (Table 4). The small intestinal ACAT activity was significantly lower in the rats supplemented with cholesterol plus the Areca extract compared to the rats supplemented with only free cholesterol. However, there was no difference in the hepatic ACAT activity between the two groups (Table 4).

Table 4
Effect of Areca nut extract supplement on pCEase activity in small intestinal fluid and on hepatic and small intestinal ACAT activity in free cholesterol-fed rats after 6 d

	Control	Areca
Small intestinal PCE'ase (% /μL)	46 ± 2	28 ± 2*
Hepatic ACAT (pmol/min/mg protein)	1713 ± 119^{NS}	1542 ± 73
Small Intestinal ACAT (pmol/min/mg protein)	1613 ± 78	792 ± 44*

Mean ± SE, n=10.

* Means in the same row are significantly different from control group ($P<0.05$).

^{NS} Not significantly different between groups.

Discussion

The human intestine is responsible for absorbing approximately 200∼600 mg/day of dietary cholesterol and, under normal conditions, about only 40∼60% of dietary cholesterol is absorbed in average and the remainder is excreted as fecal sterol. Lowering plasma cholesterol by dietary and/or pharmacological manipulation has been shown to decrease the incidence of death by coronary artery disease as well as total morbidity [26,27]. The inhibition of cholesterol absorption causes a significant drain on sterol pools and lowers plasma cholesterol levels by up to 15% [4]. Consequently, a significant amount of work has been devoted to discover agents that can block cholesterol absorption.

However, there have been few reports on the effects of Areca nut compounds on cholesterol metabolism. The Areca nut includes both antiviral and antifungal properties and contains a large quantity of tannin, along with gallic acid, a fixed oil gum, a small amount of volatile oil, lignin, and various saline substances [28]. Four alkaloids have also been found in the Areca nut – Arecoline, Arecain, Guracine, and a fourth existing in very small quantities. The compounds included in the Areca nut extract used in the present study have not yet been identified. The reason an Areca nut extract was used in the current in vivo test was because pCEase inhibitory activities were previously identified in several Areca nut-extract fractions while screening for a pCEase inhibitor from various oriental herb extracts. This study identified decreases in the free cholesterol absorption that may include biliary cholesterol in addition to dietary cholesterol in small intestine, plasma cholesterol concentration, pCEase activity, and intestinal ACAT activity in rats supplemented with free cholesterol and a combined Areca extract.

When generally accepted concepts on cholesterol homeostasis are integrated with the findings in the current study, it would appear that the inhibition of intestinal ACAT and possibly pCEase facilitates the metabolic efficiency of the Areca nut extract. As such, aqueous Areca nut extracts included in a 1% free cholesterol and 5% fat diet decrease free cholesterol absorption through the inhibition of intestinal ACAT and possibly pCEase, thereby resulting in a lower plasma cholesterol concentration and simultaneous increase in fecal cholesterol excretion. A high-cholesterol diet produces an increase in the enzymatic activities of pCEase [10] as well as hepatic ACAT and cholesterol-7α-hydroxylase [29,30]. Whereas, in the present study, a reduction in ACAT and possibly pCEase activity eventually led to less free cholesterol being available for intestinal absorption and chylomicron packing. Two ACAT variants recently reported are ACAT1 and ACAT2. ACAT2 is predominant in the intestine and liver for the synthesis of CE or lipoprotein assembly whereas ACAT1 accounts for most ACAT activity in liver and macrophages [31]. There is possibly a selective inhibition of ACAT2 by the Areca extract with no effect on ACAT1 based on the data that intestinal ACAT activity was decreased whereas hepatic ACAT activity was unchanged.

The action of the Areca extract in the absorption of intestinal free cholesterol was similar to that previously reported in the absorption of dietary CE [17]. However, this study established that the Areca nut extract was able to inhibit intestinal ACAT as well as pCEase during the absorption of intestinal free cholesterol. Krause et al. [5] suggested that WAY-121, 898 acts as an inhibitor of pCEase as well as ACAT. In another report, a cholesterol absorption inhibitor

was found to block the movement of ^{3}H-cholesterol from the intestinal lumen into the brush borders of the enterocytes, which would seem to indicate the existence of a transporter that can hasten the transit of cholesterol from the lumen to the plasma membrane of intestinal cells [32]. Additional studies are needed, with various animal models, to explain the inhibitory activities of pCEase and ACAT in vivo along with their hypocholesterolemic effects. The structure and chemical properties of the active compounds in the Areca nut extract need to be elucidated to further define the nature of its cholesterol-lowering action.

The Areca nut extract would appear to be a potent agent for inhibiting ACAT and possibly pCEase, and thus beneficial for lowering the plasma cholesterol concentration. Therefore, the inhibition of pCEase or the ACAT enzyme may be a potential therapeutic approach for decreasing the plasma cholesterol concentration. The cholesterol metabolism of rats is different from that of human, nevertheless rats are still useful animal model in testing cholesterol absorption.

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