JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antiatherosclerotic Function of Kokuto, Okinawan Noncentrifugal Cane Sugar

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In the present study, we investigated the effect of phenolic compounds (PCs) and policosanol of Kokuto, Okinawan noncentrifugal cane sugar, on the development of atherosclerosis. A total of 67 male Japanese quail were divided into eight dietary groups in trial 1. The dietary groups were fed the atherosclerotic diet (AD) containing 5% corn oil, 2% cholesterol, and 30% sucrose or seven different types of Kokuto. Dietary intakes of Kokuto notably prevented the development of atherosclerosis. Furthermore, there was a significant negative correlation between the serum radical scavenging activity and the degree of atherosclerosis in the dietary groups. In trial 2, a total of 63 Japanese quail were fed AD with sucrose, Kokuto, PC extracts from Kokuto, wax extracts from sugar cane, octacosanol, vitamin C, and vitamin E. As a result, the supplementation of the diet with Kokuto and PCs significantly reduced the development of atherosclerosis as compared with the ingestion of AD with sucrose. In conclusion, these findings suggest that, among various components of Kokuto, PCs play a central role for the prevention of experimental atherosclerosis in Japanese quail.

KEYWORDS: Kokuto; phenolic compounds; wax; octacosanol; radical scavenging activity; atherosclerosis; Japanese quail

INTRODUCTION

Atherosclerosis is an underlying cause of cardiovascular and cerebrovascular diseases. Hyperlipidemia (*1, 2*) and lipids oxidation, particularly of low-density lipoprotein (LDL) cholesterol (*3, 4*), are well-known risk factors of atherosclerosis. Kokuto is a traditional noncentrifugal cane sugar produced in Okinawa Island, Japan. It has been known that Kokuto contains various nonsugar substances derived from sugar cane, such as phenolic compounds (PCs) and policosanol, because Kokuto is made by boiling sugar cane juice sapped from finely comminuted sugar cane chips.

PCs have been reported to show various physiological functions including a suppression of LDL oxidation (*5*), an improvement of endothelial dysfunction through enhanced NO production (*6, 7*), and an inhibition of vascular smooth muscle cells proliferation (*8, 9*) in vitro. Furthermore, several studies have indicated that PCs from food substances such as wine (*10*) and cacao liquor (*5*) exert an inhibitory effect on atherosclerosis in vivo. Kokuto contains various kinds of antioxidants such as glucoside and nonglucoside PCs (*11*−*13*).

The stem and reef of sugar cane have been known to contain abundant wax, the chief constituent of which is policosanol. Menéndez et al. (*14*) have reported that policosanol is a mixture of several long-chain primary alcohols composed of hexacosanol, octacosanol (OCT), triacontanol, and other minor alcohols. In this regard, several researchers have revealed that dietary intake of Okinawan sugar cane wax inhibits an elevation of serum total cholesterol (TC) concentration in rats fed a high-fat diet (*15, 16*). Furthermore, policosanol has been shown to reduce plasma TC and LDL cholesterol levels and to increase high-density lipoprotein (HDL) cholesterol level in animals (*14, 17*) and humans (*18*). A number of studies have also indicated that policosanol inhibits the development of atherosclerotic lesion in rabbits (*19, 20*) and monkeys (*21*).

We have previously reported that Kokuto exerts a preventive effect on the development of atherosclerosis in Japanese quail

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10.1021/jf802796m CCC: $40.75 © 2009 American Chemical Society
Published on Web 12/11/2008

Table 1. Contents of Nutrient, Policosanol, PCs, and Antioxidative Activity of Kokuto in Trial 1

ingredient	Kokuto[a]						
	KA	KB	KC	KD	KE	KF	KG
carbohydrates (g/100 g)[b]	89.1	90.4	90.4	90.0	91.1	91.2	88.8
protein (g/100 g)[b]	2.5	1.8	1.6	0.8	1.1	0.8	1.7
fat (g/100 g)[b]	0.0	0.1	0.0	0.2	0.1	0.1	0.0
ash (g/100 g)[b]	3.7	3.0	3.5	3.7	2.9	3.9	4.1
moisture (g/100 g)[b]	4.7	4.7	4.5	5.3	4.9	4.1	5.4
policosanol content (mg/100 g)	168.6	6.8	8.1	13.4	8.4	27.9	7.9
OCT (mg/100 g)	113.4	3.6	4.7	8.7	4.9	20.4	4.2
PCs content (mg/100 g)[c]	632.7	578.0	722.9	729.8	486.0	561.0	673.6
antioxidative activity (μmol Trolox equivalent/100 g)	937.9	900.7	1066.4	1037.6	878.6	802.8	925.7

[a] All samples of Kokuto were purchased from different sugar factories in Okinawa in 2006. KB, Kokuto B; KC, Kokuto C; KD, Kokuto D; KE, Kokuto E; KF, Kokuto F. [b] Analysis by Japan Food Research Laboratories. [c] PCs = PCs as *p*-coumaric acid equivalents.

(*22*). In the present report, the light was shed on the ingredient of Kokuto responsible for its antiatherosclerotic function by evaluating the relationships between serum lipid levels, serum radical scavenging activity (RSA), the degree of atherosclerosis, and the dietary concentrations of PCs or policosanol.

MATERIALS AND METHODS

Animal Treatments. Japanese quail (*Coturnix japonica*, male, 5 weeks old) were purchased from Tokaiyuki Co., Ltd. (Aichi, Japan). We have worked on experimental atherosclerosis using Japanese quail because it is susceptible to atherosclerosis as well as more economical than the other animal models for long-term experiments (*23*). Furthermore, our previous study demonstrated that a considerable similarity between human and Japanese quail exists in the development of atherosclerotic lesions (*23*). All experiments with Japanese quail were ethically approved under the rules and regulations of the Animal Welfare Center, University of the Ryukyus (Okinawa, Japan). Japanese quail were kept in specific pathogen-free conditions with laminar airflow and humidity. The birds were individually maintained in cages in the constant 12/12 h light/dark cycle and a temperature (24 °C)-controlled environment.

Animal Experiments. It has been shown that composition of Kokuto varies with factory (*22*), which provides us a tool to study the relationship between physiological functions and active components. In trial 1, seven samples of Kokuto [Kokuto A (KA)−Kokuto G (KG)] were purchased from the different factories on Okinawa Island, Japan, in 2006. A total of 68 Japanese quail (6 weeks old) were randomly divided into eight dietary groups. The basal commercial diet was purchased from Kyoei Co., Ltd. (Okinawa, Japan), and its compositions were 65.8% carbohydrate, 18.3% protein, 3.8% fat, 6.3% ash, 3.5% fiber, 0.7% Ca, 0.05% P, 1.25% vitamin mix, and 2842 kcal/kg. The atherosclerotic diet (AD) with Kokuto consisted of 63% basal commercial diet, 2% cholesterol, 5% corn oil, and 30% Kokuto. The control (CO) group was fed AD with 30% sucrose substituted for Kokuto. Our previous studies have demonstrated that Japanese quail did not develop atherosclerosis with the commercial diet (*24*) or with the commercial diet supplemented with 30% sucrose (data not shown). Supplementation of the diet with cholesterol was essential to induce the experimental atherosclerosis in Japanese quail. For this reason, the CO diet group of commercial diet was not included in the present study. During the experimental periods, all birds were pair-fed on the diets and served water ad libitum. At the 12th week of the feeding period, serum, liver, and the entire aorta with its branches along with the heart were collected from each quail. Serum and all tissue specimens were stored at −80 °C for lipid analysis or histological examination. In trial 2, we investigated the effect of phenolic compounds extract from Kokuto (PCE), wax extract from sugar cane (WE), and OCT (Tokyokasei, Tokyo, Japan) on the development of atherosclerosis. A total of 63 Japanese quail were randomly divided into seven dietary groups of CO, KA, WE, OCT, PCE, vitamin C (VC), and vitamin E (VE). KA was selected because KA contained the highest amount of policosanol and the moderate amount of PCs in seven types of Kokuto (shown in **Table 1**). OCT was chosen as a model substance in this study because of the main component in policosanol from Kokuto (shown in **Table 4**). Dietary compositions of CO and K groups were the same as described in trial 1. **Table 4** lists that KA contains 0.0077% policosanol, 0.0055% OCT, and 0.574% PCs. Therefore, WE, OCT, and PCE groups were each fed AD containing one of these components and sucrose at the level equivalent to those in Kokuto (0.023% policosanol, 0.017% OCT, or 0.172% PCs, respectively). With VC and VE groups, the amounts of VC and VE mixed AD with sucrose were equivalent to 0.172% PCs on the basis of antioxidative activity. All Japanese quail were pair-fed on the diets and served water ad libitum. Upon termination of feeding period of 12 weeks, we collected tissue samples from each bird the same way as in trial 1.

Preparation of PCs and Wax. Kokuto and sugar cane molasses contain a number of PCs such as phenolic acids, phenyl glycosides, phenyl propanoid glycosides, and phenyl propanoids (*12, 13, 25*). Detail analysis of polyphenols profile in Kokuto has already been reported by Takara et al. (*12, 13*). PCE was obtained from KA purchased anew in 2007 by Amberlite XAD2 (Organo, Tokyo, Japan) column chromatography as described previously (*12, 13, 25*). The extraction was carried out as follows; Kokuto solution (0.5 kg/L of pure water) was centrifuged (8000 rpm × 20 min), and then, the supernatant was filtrated by Buchner funnel with paper filter. Amberlite XAD2 resins were added to the filtrate, and the solvent was stored for 2 h. The solvent was passed through the column and eluted with 50 and 100% methanol. These two methanol extracts were combined and were dried up using rotary evaporator and freeze dryer. WE was prepared from sugar cane by Okinawa Satoukibi Kinou Kennkyuusyo (Okinawa, Japan) as described elsewhere (*26, 27*).

Policosanol Content. We determined policosanol contents in seven samples of Kokuto, WE, and PCE. The composition and content of policosanol were determined by gas chromatography and gas chromatography−mass spectrometry as previously described (*26*). Briefly, 6 g of Kokuto was placed in an thimble filter (Advantec #84) and extracted by Soxhlet method with approximately 150 mL of hexane:methanol (20:1 v/v) for 24 h. The extract was concentrated to dryness using a rotary evaporator under vacuum conditions at 40 °C and redissolved in 2 mL of toluene or chloroform for gas chromatography. WE and PCE preparations were similarly redissolved in the toluene or chloroform for analysis. A gas chromatograph used was a Shimadzu GC 17-A equipped with a flame ionization detector. The column was a fused capillary column (DB 5, 0.25 mm i.d. × 30 m) from J&W Scientific (Folsom, CA) with helium as the carrier gas. The temperature for injector and detector was 350 °C. The oven temperature was programmed to increase from the initial temperature of 150 to 320 °C in 4 °C/min increment and then to maintain 320 °C for 15 min. Samples (1 μL) were injected with split ratio of 1:10, and the standard curve for quantitation was constructed with serial dilution of authentic policosanol. Docosanol, tetraconsanol, hexacosanol, OCT, and triacontanol (Sigma Chemical, St. Louis, MO) were used as authentic standards.

PCs Content. PCs contents in seven samples of Kokuto, WE, and PCE were measured by the Folin−Denis assay as described previously (*28*). Briefly, the reaction solution was made by mixing 200 μL of Kokuto solution (10 mg/mL water), PCE solution (0.1 mg/mL water), or WE solution (1 mg/mL water) with 200 μL of Folin−Denis' reagent

(Fluka, St. Louis, MO), 400 μL of saturated sodium carbonate solution, and 3.2 mL of water. The mixture was allowed to stand for 30 min at room temperature, and its absorbance was measured at 700 nm by a spectrophotometer (UV160, Shimadzu Corp., Kyoto, Japan). The unit of total PCs was expressed as *p*-coumaric acid equivalent.

Antioxidative Activity. The antioxidative activity of PCE, WE, VC, and VE measured the decolorization of DPPH (2,2-diphenyl-1-picryl hydrazine hydrate) radical cation as described by Oki et al. (*29*). Briefly, 300 μL of Kokuto solution (50 mg/mL water), PCE solution (0.1 mg/mL water), or WE solution (1 mg/mL water), 300 μL of 20% ethanol, and 300 μL of 0.2 M morpholinoethanesulfonic acid buffer (pH 6.0) were mixed in a test tube. After the addition of 300 μL of 400 μM DPPH solution (in ethanol), the reaction mixture was left for 20 min at room temperature and its absorbance at 525 nm was measured by a spectrophotometer. The unit of DPPH RSA was expressed as Trolox (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid) equivalent.

Serum and Liver Lipids. Serum TC and triglyceride (TG) levels were measured using the TC E test kit and TG test kit (Wako Pure Chemical, Osaka, Japan). The extraction of total lipids from the liver tissue was performed by Folch's method as described previously (*30*). TC and TG levels of liver were determined by Shoenheimer-Sperry (*31*) and Fletcher's method (*32*), respectively.

Serum RSA. The measurement of serum RSA was carried out by ABTS [2,2′-azinobis(3-ethylbenzothiazolin-6-sulfonic acid)] radical cation decolorization assay as described by Re et al. (*33*). Briefly, ABTS radical solution was generated by oxidation of ABTS reagent (Fluka) with potassium persulfate. Before use, the concentration of the ABTS radical solution was diluted with PBS to an absorbance of 0.70 at 734 nm. The test reaction was made by mixing 10 μL of serum sample (diluted 10 times with PBS), Trolox (for standard), or PBS (for blank) with 200 μL of $ABTS^+$ radical solution. After incubation for 60 min at 30 °C, the absorbance of the mixtures was measured at 734 nm. Serum RSA was expressed as the inhibition rate of absorbance at 734 nm.

Histopathological Examinations. A 0.5 cm long proximal portion of ascending aorta and its large branches was excised from the heart of each quail and was fixed in 10% buffered formalin. For histopathological examination, paraffin-embedded blocks were prepared from formalin-fixed aortic tissues. Paraffin-embedded aortic tissue samples were sectioned into 4 μm thicknesses and were stained by hematoxylin eosin and elastica van Gieason. To evaluate the degree of atherosclerosis, the ratio of intimal thickening area/total original luminal area (mm^2/mm^2) was determined with two or three aortic tissues stained by elastica van Gieason method for each quail.

Statistical Analysis. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by inspection of the significant differences from mean value of CO diet group by Dunnett test. Correlation analysis was performed using Pearson product−moment correlation coefficient. All statistical analyses were performed using the SAS statistical software program (SAS Institute, Tokyo, Japan).

RESULTS

Trial 1. *Contents of Policosanol and PCs and Antioxidative Activity of Kokuto.* **Table 1** shows the contents of nutrient, Policosanol, PCs, and antioxidative activity in seven samples of Kokuto. The composition of all Kokuto samples was roughly 90 carbohydrates, five moisture, 3−4 ash, and 1−2 protein in percentage. Policosanol in seven types of Kokuto was predominantly comprised of OCT (52.4−72.9%, **Table 1**). The policosanol content was the highest in KA, followed by KF, while there were quite lower levels for other samples than KA. No correlation was noted between policosanol contents and antioxidative activities in Kokuto. All samples of Kokuto contained PCs to variable extents (**Table 1**). Consistent with the previous report (*22*), there was a high positive correlation between PC contents and antioxidative activities of Kokuto ($r = 0.84$, $p < 0.05$).

Table 2. Growth Parameters of Japanese Quail in Trial 1

group[a]	no.	initial body weight (g)	final body weight (g)	energy intake (kcal/day)	liver weight (g/100 g body)
CO	$n = 9$	87.1 ± 1.7	101.5 ± 1.6	38.4 ± 0.4	2.73 ± 0.18
KA	$n = 8$	87.1 ± 1.5	100.0 ± 4.3	39.0 ± 0.6	2.41 ± 0.19
KB	$n = 8$	87.1 ± 1.8	91.2 ± 3.9	38.4 ± 0.6	2.85 ± 0.21
KC	$n = 9$	87.1 ± 1.4	97.5 ± 2.5	39.0 ± 0.7	2.72 ± 0.22
KD	$n = 9$	87.1 ± 1.7	96.1 ± 3.3	38.8 ± 0.4	2.78 ± 0.15
KE	$n = 9$	87.1 ± 1.6	94.1 ± 4.8	39.0 ± 0.5	2.80 ± 0.25
KF	$n = 7$	87.1 ± 1.8	97.8 ± 3.3	38.6 ± 0.5	2.64 ± 0.17
KG	$n = 8$	87.1 ± 1.6	96.8 ± 5.8	38.6 ± 0.5	3.03 ± 0.43

[a] One bird died in KF group. All data are expressed as mean values ± standard errors.

Table 3. Serum and Liver Parameters of Japanese Quail in Trial 1

	serum[a]			liver[a]	
group	TC (mg/dL)	TG (mg/dL)	relative RSA (CO = 1)	TC (mg/g liver)	TG (mg/g liver)
CO	1852 ± 242	140.8 ± 18.0	1.00 ± 0.15	28.0 ± 1.79	3.71 ± 0.27
KA	1360 ± 304	115.3 ± 29.4	1.94 ± 0.44	29.5 ± 2.49	4.42 ± 0.45
KB	1526 ± 351	109.5 ± 29.7	2.36 ± 0.44	29.3 ± 2.42	3.88 ± 0.56
KC	1666 ± 237	115.3 ± 17.2	2.14 ± 0.48	32.3 ± 1.45	3.68 ± 0.41
KD	1504 ± 198	100.9 ± 19.8	2.32 ± 0.48	31.0 ± 1.30	4.65 ± 0.79
KE	1521 ± 164	82.0 ± 12.4	1.97 ± 0.23	31.2 ± 1.07	5.51 ± 0.62
KF	1610 ± 276	133.4 ± 35.8	1.48 ± 0.17	31.5 ± 1.72	5.36 ± 0.65
KG	1552 ± 178	97.0 ± 16.3	1.87 ± 0.40	31.9 ± 1.58	4.92 ± 0.42

[a] All data are expressed as mean values ± standard errors.

Table 4. Contents of Policosanol and PCs and Antioxidative Activity of Kokuto and Its Extracts in Trial 2

ingredient	Kokuto[a]	PCE[a]	WE[a]
policosanol content (mg/g)	0.77		357.5
hexacisanol	0.12		52.5
OCT	0.55		290.0
triacontacosanol	0.09		15.0
PCs content (mg/g)[b]	5.74	725.3	4.0
antioxidative activity (μmol Trolox equivalent/g)	7.00	504.9	

[a] Kokuto, KA purchased in 2007. [b] PCs = PCs as *p*-coumaric acid equivalents.

Growth Parameters. **Table 2** summarizes the growth parameters and liver weight of Japanese quail. As a result of paired feeding, there was no significant difference in energy intake between dietary groups. Dietary intake of Kokuto had no effect on the final body weight and liver weight of Japanese quail.

Serum and Liver Parameters. **Table 3** lists serum lipid levels, serum relative RSA (relative RSA as CO = 1), and liver lipid levels of Japanese quail after the feeding period. There were no significant differences in serum and liver lipid levels of Kokuto groups as compared with CO group. However, there was a tendency for serum TG concentration of Kokuto groups to decrease in comparison with CO group. Serum RSA levels of Kokuto groups were about 1.5−2.4 fold higher than CO group but not significantly different from the CO group.

Degree of Atherosclerosis. **Figure 1** presents the pathological findings of aorta in Japanese quail stained by elastica van Gieason stain. Histlogical findings of atherosclerosis-free normal aorta, lipid-containing intimal thickening lesion, and severe atherosclerotic lesion are shown in **Figure 2A−C**, respectively. The degree of atherosclerosis in each dietary group is shown in **Figure 2**. The degrees of atherosclerosis in all Kokuto groups were lower than that of CO group. Significant inhibitory effects of KB, KC, and KD on the development of atherosclerosis were noted as compared to CO group ($p < 0.05$). **Figure 3** shows the result of the correlation test between mean values of serum relative RSA and of the degree of atherosclerosis in dietary

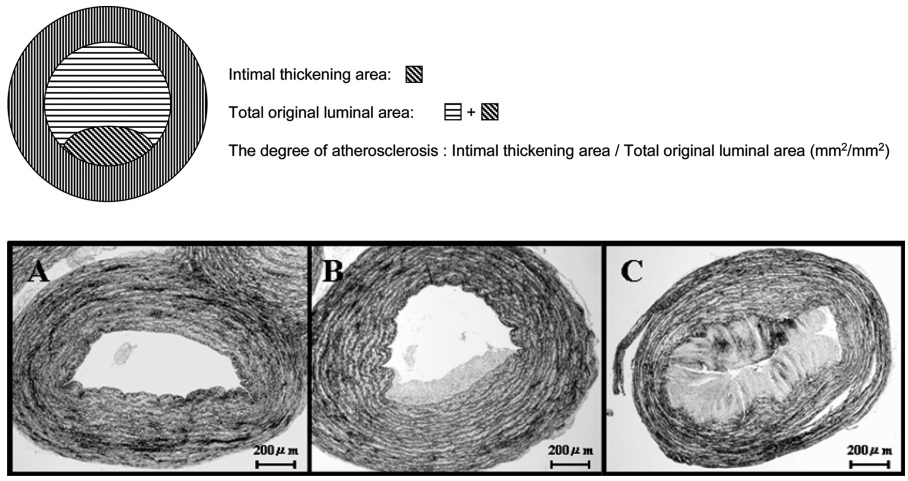

Figure 1. Quantitation of intimal thickening in aorta of Japanese quail. Japanese quail were fed AD with 30% sucrose (CO) or 30% KA−KG for 12 weeks. Pictures show an elastica van Gieason stain of normal aorta (A), a lipid-containing intimal thickening lesion (B), and a critical atheromatous lesion (C).

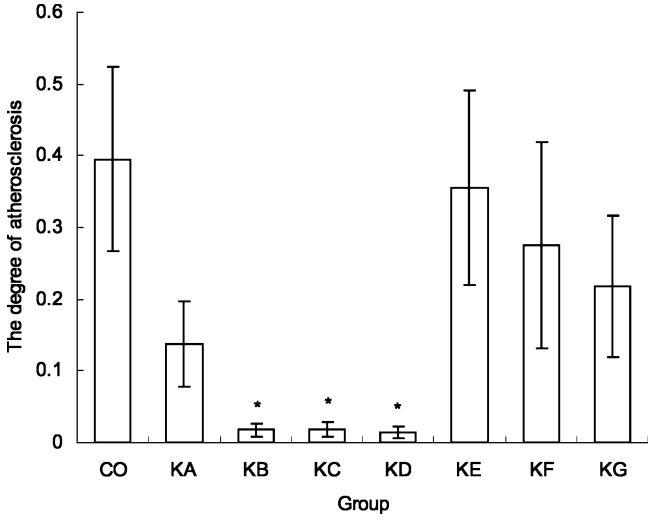

Figure 2. Degree of atherosclerosis in aorta of Japanese quail in trial 1. Japanese quail were fed AD with 30% sucrose (CO) or 30% KA−KG for 12 weeks. The longitudinal axis shows the ratio of intimal thickening area/total original luminal area (mm^2/mm^2) as the degree of atherosclerosis. The results are expressed as mean values ± standard errors for 7−9 Japanese quail. Different letters show significant difference (*$p < 0.05$).

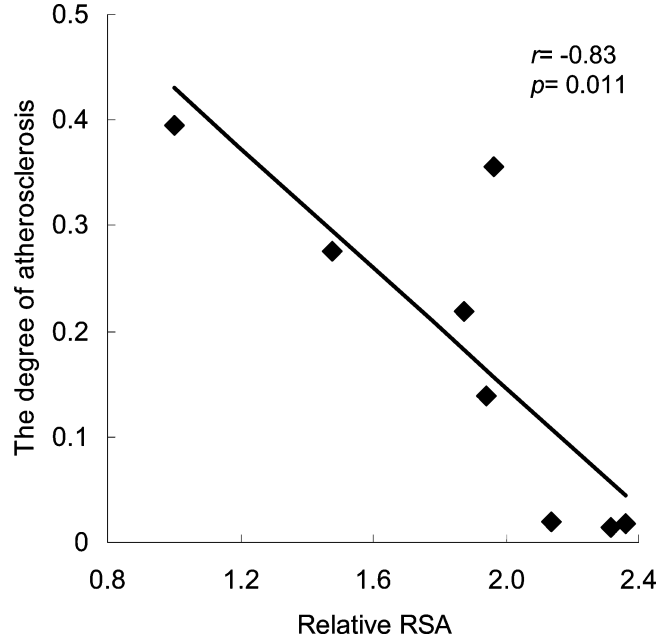

Figure 3. Correlation between the serum relative RSA and the degree of atherosclerosis in Japanese quail in trial 1. The abscissa axis shows the relative RSA. The longitudinal axis shows the degree of atherosclerosis in dietary groups. Statistical analysis was performed by the Pearson product−moment correlation test, and the ρ correlation coefficient (r) = −0.83; significant probability (p) = 0.011. Data represent the mean values of experiment.

groups. The degree of atherosclerosis was negatively correlated with serum relative RSA at the 12th week ($r = 0.83$, $p < 0.05$) in this study.

Trial 2. To ascertain which component, PCs or policosanol, is responsible for the antiatherosclerotic activity of Kokuto, each component was extracted from Kokuto, and their effects on the development of atherosclerosis were individually evaluated in trial 2.

Contents of Policosanol and PCs and Antioxidative Activity in PCE and WE. **Table 4** lists the contents of policosanol and PCs and antioxidative activity in K, PCE, and WE. We confirmed by gas chromatography that the PCE does not contain policosanol. Folin−Denis and DPPH assays found that PCE consisted of 72.5% PCs, and its antioxidant activity was 504.9 μmol of Trolox equivalent/g. Meanwhile, WE consisted of 35.8% policosanol with 5.3% hexacosanol, 29.0% OCT, and 1.5% triacontacosanol. Furthermore, WE showed reasonably low levels of PCs and no antioxidative activity.

Growth Parameters. **Table 5**summarizes the growth parameters of Japanese quail during the experimental period. Energy intakes (kcal/day) were roughly comparable between all experimental groups. The administration of active substances showed no significant effect on the final body weight and liver weight.

Table 5. Growth Parameters of Japanese Quail in Trial 2

group[a]	no.	initial body weight (g)	final body weight (g)	energy intake (kcal/day)	liver weight (g/100 g body)
CO	$n = 9$	94.1 ± 1.5	100.9 ± 1.9	38.5 ± 0.3	2.67 ± 0.17
K	$n = 9$	94.1 ± 2.1	100.0 ± 1.4	38.0 ± 0.1	3.01 ± 0.16
WE	$n = 9$	94.1 ± 1.8	100.3 ± 2.0	38.7 ± 0.3	3.02 ± 0.28
OCT	$n = 9$	94.1 ± 1.7	101.0 ± 1.5	38.5 ± 0.3	3.03 ± 0.19
PCE	$n = 9$	94.1 ± 2.1	104.2 ± 1.6	38.2 ± 0.2	2.90 ± 0.10
VC	$n = 9$	94.1 ± 2.4	102.1 ± 1.7	38.6 ± 0.2	3.03 ± 0.14
VE	$n = 9$	94.1 ± 2.0	100.8 ± 1.2	38.7 ± 0.3	2.81 ± 0.28

[a] K, Kokuto. All data are expressed as mean values ± standard errors.

Table 6. Serum and Liver Parameters of Japanese Quail in Trial 2

	serum[a]			liver[a]	
group	TC (mg/dL)	TG (mg/dL)	relative RSA (CO = 1)	TC (mg/g liver)	TG (mg/g liver)
CO	2402 ± 364	251.1 ± 48.1	1.00 ± 0.03	39.8 ± 1.13	5.78 ± 1.14
K	1879 ± 209	172.5 ± 34.6	1.38 ± 0.12	38.6 ± 0.90	4.67 ± 0.90
WE	2318 ± 429	245.7 ± 67.5	1.23 ± 0.11	39.0 ± 0.85	6.40 ± 1.51
OCT	2679 ± 339	312.9 ± 49.3	1.07 ± 0.06	39.2 ± 1.01	7.73 ± 0.78
PCE	2442 ± 286	239.0 ± 29.7	1.35 ± 0.10	40.3 ± 1.31	8.73 ± 0.50
VC	2475 ± 440	292.1 ± 78.9	1.10 ± 0.17	42.5 ± 0.42	9.01 ± 0.78
VE	2076 ± 214	342.2 ± 46.9	1.18 ± 0.08	38.9 ± 0.97	7.83 ± 0.84

[a] All data are expressed as mean values ± standard errors.

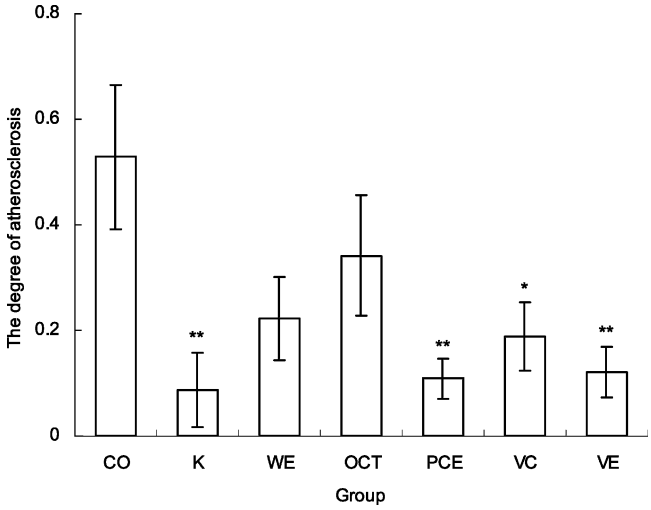

Figure 4. Degree of atherosclerosis in aorta of Japanese quail in trial 2. The abscissa axis shows dietary groups containing CO, K, WE, OCT, PCE, VC, and VE groups. The longitudinal axis shows the ratio of intimal thickening area/total original luminal area (mm^2/mm^2) as the degree of atherosclerosis. The results are expressed as mean values ± standard errors for nine Japanese quail. Different letters show significant differences (*$p < 0.05$ and **$p < 0.01$).

Serum and Liver Parameters. **Table 6** lists serum and liver lipid levels and serum relative RSA of each dietary group. No significant differences in serum and liver lipid concentrations were noted between groups. K and PCE diets increased the serum RSA level 1.3−1.4-fold over that of CO group but to a level statistically not significant.

Degree of Atherosclerosis. **Figure 4** presents the degree of atherosclerosis in each experimental group. The degrees of atherosclerosis in K, PCE, VE, and VC groups were significantly lower than that in CO group (K, PCE, and VE, $p < 0.01$; VC, $p < 0.05$). A decreasing tendency for the development of atherosclerotic lesion was noted with WE and OCT groups in comparison with the CO group.

DISCCUSION

Our present trial 1 experiment revealed a significant suppression of the development of aortic atherosclerotic lesion and a tendency toward serum TG concentration to decrease in Japanese quail fed Kokuto, as suggested by our previous report (*22*). The supplementation of grape polyphenol extract containing flavans, anthocyanins, and resveratrol has reduced plasma LDL cholesterol and TG levels in pre- and postmenopausal women (*34*). Sugiyama et al. (*35*) have suggested that oral intake of apple polyphenol extract containing oligomeric procyanidins decreases plasma TG by inhibiting pancreatic lipase activity in mice and humans. Kimura et al. (*36*) have reported that nonsugar fractions (500 or 1000 mg/day/kg body weight) eluted by methanol in crude black sugar (Kokuto) significantly decrease the serum TG level but not the TC level in rats fed a high sugar diet. We consider that the Kokuto PCE specimen in our study was almost the same in composition with the nonsugar fractions of Kokuto used by Kimura et al. (*36*) because the preparation method was largely similar. However, dietary intake of PCE (approximately 230 mg/day/kg body weight) caused no significant decrease in both serum TC and TG levels in our trial 2 experiment. Thus, the dose of PCE in our experiment was much lower than that for Kimura et al., which may largely explain the differences between two experiments.

Sho et al. (*37*) have examined the decrease of serum TC and TG levels in rat fed a high-fat diet with 2% sugar cane rind. In addition, Fukuda et al. (*16*) have reported that sugar cane wax (0.5% in diet), which is a main constituent material of sugar cane rind, reduces serum TC and TG levels in rats fed a high-fat diet. They have speculated that these effects may be associated with a change in cholesterol metabolism because the ingestion of sugar cane wax did not affect the fecal excretion of steroids. A recent study has indicated that policosanol from sugar cane rind wax has a potential to moderately control HMG-CoA reductase in liver (*38*). With respect to TC, the serum level for quail in this work was much higher than that reported for rats. The serum level of cholesterol in rats fed 1% cholesterol is usually in the range between 70 and 300 mg/dL (*15, 37*). In our previous study with Japanese quail fed a basal diet, the serum TC level was 224 mg/dL (*25*). Meanwhile, the serum level of TC in this study was approximately 1500−2500 mg/dL (**Tables 3** and **6**). Thus, it could be plausible that the serum TC level in this study might be too high to render the cholesterol-lowering effect of Kokuto clear.

Two inconsistent observations have been reported with regard to the effect of policosanol on lipid metabolism by several investigators (*14, 39−41*). Dietary intake of Cuban sugar cane-derived policosanol (50 mg/kg diet/day) has been shown to reduce plasma TC and LDL cholesterol levels but not TG level in rabbits (*14*) and healthy volunteers (*39*). Furthermore, Kato et al. (*42*) have demonstrated that the rats fed a high-fat diet with 10 g/kg of OCT significantly decrease serum TG levels but not TC levels. In contrast with previous reports, some other studies have indicated that policosanol has no effect on plasma lipid profile such as TC, LDL cholesterol, and TG in human (*40*) and hamster (*41*). In our present study, dietary intake of OCT had no significant effect on serum lipid levels. The inconsistency between these experiments might be largely ascribed to the difference in the level of doses, animals, and chemical composition of used samples.

Kurosawa et al. (*5*) have previously revealed that the addition of cacao polyphenol inhibits LDL oxidation in vitro and administration of cacao polyphenol (10 g/kg diet) suppress the development of atherosclerotic lesions in hypercholesterolemic rabbit. Fuhrman et al. (*43*) have also shown that polyphenol-rich grape powder (30 mg/day, equivalent to 150 μg of total polyphenol) elevates serum antioxidant activity and prevents aortic atherosclerosis in apolipoprotein E-deficient mice. They have suggested that the antiatherosclerotic effect of polyphenol-rich grape powder results from a decrease in macrophage-mediated LDL oxidation and cellular uptake of oxidized LDL. In our trial 1 experiment, a negative correlation ($p < 0.05$) between the degree of atherosclerosis and serum RSA was noted

as shown in **Figure 3**. This result indicates that dietary intake of PCs-rich types of Kokuto was more effective for the prevention of atherogenesis. Furthermore, in trial 2 experiment, PCE supplementation significantly lowered the degree of atherosclerosis (**Figure 4**) with an increase in serum RSA levels to statistically insignificant levels (1.35-fold over the CO level, **Table 6**). Thus, the change in the oxidation satate of serum even to a small extent could be more pronounced for the inhibition of atherosclerosis.

Several studies have reported that VC inhibits oxidative modification of LDL (*44*) and enhances resistance to oxidative stress (*45*) in human endothelial cells. Supplementary intake of VC has suppressed the blood malondialdehyde level as a marker for lipid peroxidation and prevented the hypercholesterolemia-induced atherosclerotic lesion in rabbits (*46*). Additionally, Rainwater et al. (*47*) have demonstrated that dietary supplementation of VE notably induces an up-regulation of antioxidant capacity and apolipoprotein A-I concentration and a reduction of the serum oxidized-LDL concentration in baboons. The other study has indicated that VE deficiency increases the lipid peroxidation and accelerates the development of atherosclerotic lesion in apolipoprotein E and α-tocopherol transfer protein double-knockout mice (*48*). Our results of trial 2 largely agree with these previous observations, and dietary supplementations of VC and VE decreased the degree of atherosclerotic lesion without affecting serum lipid levels.

Thus, we conclude that PCs rather than policosanol may be the effecter of antiatherogenic activity of Kokuto, probably by improving the oxidative stress in aortic lesion. It is furthermore noteworthy that the supplementation of antioxidants such as PCs and vitamins rather than the cholesterol-lowering agent could be more effective for the prevention of atherosclerosis. This view appeared to hold true, especially when the serum cholesterol level is extremely high as seen in this experiment. In this context, antioxidant has been reported to down-regulate the sensitivity of LDL to oxidation in hypercholesterolemic patients (*49*). Thus, Kokuto could be useful for medicinal purposes to prevent atherosclerosis without any side effects. However, the use of PCE rather than the use of Kokuto itself could be more practical because Kokuto consists of 90% of carbohydrates.

Noa et al. (*19*) have immunohistochemically revealed that administration of policosanol inhibits neointimal formation through suppression of proliferation of smooth muscle cells in rabbit. They also have shown with the aid of immunohistochemical localization of apoB and apoA-I that policosanol reduces the number of macrophage-derived form cells in the aortic atherosclerotic lesions of monkey (*21*). In our trial 2 experiment, the supplementation of WE appeared to inhibit the development of atherosclerotic lesion to some extent in Japanese quail. It is possible that some other activity of policosanol such as to modulate lipoprotein profile (*50*) might be involved in this antiatherosclerotic activity.

Finally, trial 1 showed that the degree of atherosclerosis did not always correlate with PCs content of Kokuto. This may suggest the presence of some other antiatherogenic component in Kokuto other than PCs and policosanol. Our ongoing study on long-chain aldehydes in sugar cane wax and Kokuto may give an answer to this question.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzothiazolin-6-sulfonic acid); AD, atherosclerotic diet; CO, control; DPPH, 2,2-diphenyl-1-picryl hydrazine hydrate; KA, Kokuto A; KB, Kokuto B; KC, Kokuto C; KD, Kokuto D; KE, Kokuto E; KF, Kokuto F; KG, Kokuto G; LDL, low-density lipoprotein; OCT, octacosanol; PCs, phenolic compounds; PCE, phenolic compounds extract from Kokuto; RSA, radical scavenging activity; TC, total cholesterol; TG, triglyceride; VC, vitamin C; VE, vitamin E; WE, wax extract from sugar cane.

ACKNOWLEDGMENT

We thank Shino Tomori, Ayako Kuniyoshi, and Masayuki Shimatani for technical assistance.

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Received for review September 9, 2008. Revised manuscript received October 24, 2008. Accepted November 10, 2008.

JF802796M