Suppressive Effect of Cocoa Powder on Atherosclerosis in Kurosawa and Kusanagi-hypercholesterolemic Rabbits

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We investigated the suppressive effect of cocoa powder (cacao polyphenol content: 7.8%) on atherosclerosis in a spontaneous familial hypercholesterolemic model, Kurosawa and Kusanagi-hypercholesterolemic (KHC) rabbits. Six-month dietary administration of cocoa powder had no effects on body weight, hematology or blood chemistry parameters or a lipid profile in KHC rabbits. Antioxidative activity of low-density lipoprotein (LDL) was observed in the 2nd month and 3rd month of administration. Thiobarbituric acid reactive substances (TBARS), the marker of lipid peroxidation, in plasma were decreased in the cocoa powder treated group from the 2nd month of administration during the study period compared to that in the control group. The area of atherosclerotic lesions in the aorta was significantly smaller in the cocoa powder group (30.87%) than in the control (52.39%). Tissue cholesterol content also tended to decrease. Distensibility of the aortic wall was improved significantly in the cocoa powder treated group due to decreases in fatty streaks and intimal thickening compared to that in the control group. These results suggest that cocoa powder has suppressive effect on development of atherosclerotic lesions. We consider that antioxidative activity of polyphenols rich in cocoa powder may be a key factor for the anti-atherosclerotic effect. *J Atheroscler Thromb*, 2005; 12: 20–28.

Key words: Polyphenols, KHC rabbit, LDL oxidation, Antioxidant

Introduction

Epidemiological studies revealed that the intake of food abundant in polyphenols, antioxidative substances, reduces mortality risks from cardiovascular diseases (1–3). Cacao beans (*Theobroma cacao*) are the main ingre-

dient for chocolates and cocoa drinks. Cacao beans are rich in polyphenols including catechins and their oligomers such as procyanidins (4–6). Crude cacao polyphenol fractions have been reported so far to possess *in vitro* antioxidative activity (7, 8) and suppressive

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Abbreviations:

KHC rabbit: Kurosawa and Kusanagi-hypercholesterolemic rabbit

LDL: low-density lipoprotein

TBARS: thiobarbituric acid reactive substances

FH: familial hypercholesterolemia

V-70: 2,2'-azobis(4-methoxy)-2,4-dimethylvaleronitrile

EM_{0.5}: elastic modulus of the wall at a strain of 0.50

activity of LDL oxidation in cholesterol-fed rabbits (9). A clinical study in healthy volunteers disclosed that daily intake of cocoa powder decreased susceptibility of LDL to oxidation (10). LDL oxidation is considered to play an important role in development of atherosclerosis (11), and dietary intake of cacao polyphenol is thereby expected to suppress it. In the present study, we examined anti-atherosclerotic effect of cocoa powder in KHC rabbits, the animal model of familial hypercholesterolemia (FH) that exhibits hypercholesterolemia from birth and spontaneously develops atherosclerosis (12).

Materials and Methods

Cocoa powder

Fermented and dried cacao beans were imported from Ecuador. The beans were processed in Meiji Seika Kaisha, Ltd. (Tokyo, Japan) through roasting, grinding and compressing to prepare cocoa powder. The composition of this cocoa powder is shown in Table 1.

Animals and diets

Six male and 6 female homozygous KHC rabbits (body weight range: 1.8–2.3 kg) were purchased from Japan Laboratory Animals Inc. (Tokyo, Japan) at the age of 3 months. The animals were individually housed under controlled environment (room temperature: 21–25°C, relative humidity: 45–65%, 12-hour dark and light cycle, more than 10 times ventilation per hour). The control animals received commercially available standard diets (RC-4, Oriental Yeast, Tokyo, Japan). The animals in the treatment group received the admixture of the standard diets and cocoa powder by 10%. The mixed diets were stored at –20°C until immediately before feeding.

Table 1. General composition per 10 g of the cocoa powder.

Component	Content
Carbohydrate	1.6 g
Fat	1.47 g
Protein	2.11 g
Fiber	2.58 g
Mineral	0.81 g
Caffeine	34.3 mg
Theobromine	0.25 g
α -Tocopherol	18.9 μ g
γ -Tocopherol	0.3 mg
Total polyphenol	0.78 g

Study design

The rabbits were divided into 2 groups so as to have no group differences in body weight, plasma total cholesterol, or triglyceride (3 males and 3 females for each of the control and treatment groups). The control group received 100 g of the diets per day and the cocoa powder group received 110 g, and both groups were provided with drinking tap water *ad libitum*. The animals were clinically observed and food consumption was measured every day. The measurement of body weights, hematology, blood chemistry and LDL oxidation were performed 1 month after the initiation of the study and onward at 1 month intervals. The animals were euthanised by overdose of pentobarbital at the end of the 6-month administration study of cocoa powder.

Hematology and blood chemistry

Hematology [red blood cell count (RBC), hemoglobin (Hb), hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet count (Plt), white blood cell count (WBC)] was performed with a cell counter (ADVIA 120, Bayer, USA) using the blood collected from the auricular vein under EDTA-3K treatment. Blood chemistry [total protein (TP), albumin, glucose (Glu), blood urea nitrogen (BUN), creatinine (Cre), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatine phosphokinase (CPK), alkaline phosphatase (ALP), calcium] was performed with an autoanalyzer (COBAS FARA II, Roche, Switzerland) and commercially available kits (Wako Pure Chemical, Osaka, Japan) using the plasma centrifuged from the heparin-treated blood sample. Sodium, chlorine and potassium were measured with the ionic membrane electrode method using EA03 (A&T, Tokyo, Japan).

Measurement of plasma lipid levels

The plasma centrifuged from the EDTA-3K-treated blood sample was examined for total cholesterol, triglyceride, phospholipid and TBARS. The total cholesterol, triglyceride and phospholipid were measured enzymatically using COBAS FARA II (Roche) and commercially available kits (Wako Pure Chemical). TBARS was measured in accordance with the method of Yagi (13) using a fluorophotometer (Model 650-10S; Hitachi, Tokyo, Japan) and a commercially available kit (Wako Pure Chemical).

Evaluation of antioxidation in LDL

LDL oxidation was measured with the method developed by Esterbauer *et al.* (14) and Hirano *et al.* (15). The plasma was obtained from the EDTA-3K treated blood sample (final concentration: 1 mg/ml) and ultracentrifuged to obtain LDL ($d = 1.019$ – 1.063 g/ml). LDL fraction was confirmed to have a single peak in agarose gel electro-

phoresis. To the LDL fraction (final concentration of protein: 70 $\mu\text{g/ml}$), an oxidant V-70 (2,2'-azobis(4-methoxy)-2,4-dimethylvaleronitrile) was added to be 200 μM and incubated at 37°C. The formation of conjugated diene was monitored at 234 nm using Beckman Model DU-640 spectrophotometer to obtain the lag time, that is the time until the conjugated diene started increasing.

Determination of lesioned area

The aorta was excised from the origin to the iliac bifurcation. After fats and tissues adhering to the adventitia were removed, the aorta was cut open longitudinally and the luminal surface was Xerox-copied. Atherosclerotic lesions were traced carefully and the area ratio of atherosclerotic lesions to the total area was calculated by image analysis (NIH Image 1.62; National Institutes of Health, USA).

Measurement of lipid contents in the aorta

Each aorta was divided into three segments, that is aortic arch (from the origin to the 1st intercostal arteries), thoracic aorta (between the 1st to 8th intercostal arteries), and abdominal aorta (from the 8th intercostal arteries to the iliac bifurcation). Each segment of the aorta was homogenized with 1.14% KCl containing 50 mM deferoxamine. Cholesterol was extracted in a mixed solution of methanol and chloroform to determine total cholesterol using a commercially available kit (Wako Pure Chemical). To the homogenate, a mixed solution of 8.1% sodium dodecyl sulfate and 20% acetic acid, and 0.8% thiobarbituric acid were added. It was then boiled and TBARS were extracted in a mixed solution of n-butanol and pyridine to determine intensity of fluorescence.

Static rheological properties of the aorta

Circumferential wall strips of 3.0 mm in width were excised from the ascending aorta and descending portion of aortic arch (about 2 cm peripheral to the left subclavian bifurcation) where atherosclerotic lesions were relatively marked. Both ends of each strip were mounted into chucks fixed to a tensile testing instrument (TOM-30J, Minebea Co., Ltd., Tokyo, Japan), immersed into the Locke's solution at 37°C, and stretched at a speed of 4.17 mm/sec until tension reached 20–30 g as shown in the previous report (16). Immediately after the stress-strain characteristics test, the strip was relaxed to the initial length, detached from the chucks and weighed precisely. Stress value (τ) was calculated from the following formula assuming Poisson's ratio of the vascular wall as 0.5: $\tau = 1.06 \times T/W \times L_0 (1 + \Delta L/L_0)$, where 1.06, T , W , L_0 and ΔL were density of the wall, wall tension (g), sample weight (g), initial length of the strip (cm) and increment from the initial length (cm), respectively. Wall thickness (h) at the initial length was calculated from the following formula: $h = (W/1.06) / (L_0 \times Wd)$, where Wd was the width

of strip (cm). Incremental elastic modulus of the wall at a strain of 0.50 ($EM_{0.5}$) with respect to the unstressed length was determined as the mean gradient of the stress-strain curve between strains of 0.55 and 0.45, and calculated as follows: $EM_{0.5} = (\sigma_{0.55} - \sigma_{0.45}) / (\epsilon_{0.55} - \epsilon_{0.45})$, where $\sigma_{0.55}$ and $\sigma_{0.45}$ were stress at strains of 0.55 and 0.45 and $\epsilon_{0.55}$ and $\epsilon_{0.45}$ strains of 0.55 and 0.45, respectively (16). Each strip of the aorta used in the tensile test was fixed in 10% neutral buffered formalin solution. It was then embedded into paraffin, sectioned at 3 μm in thickness and stained with hematoxylin-eosin or elastica van Gieson.

Statistical analysis

All data are expressed in mean $\pm$ S.E. For determination of a significant difference from the control group, Student's t -test was used. For determination of a significant difference from the value prior to administration, paired t -test was performed. $p < 0.05$ was regarded as significant.

Results

There was no difference in all data mentioned below between males and females in each group.

General conditions

Throughout the study period, there were neither adverse effects of cocoa powder in clinical observation nor decrease in food consumption. There was no significant difference in body weight between the control and cocoa powder treated groups. The body weights were 2.1 ± 0.1 kg and 2.0 ± 0.1 kg before the start of study, and 3.3 ± 0.1 kg and 3.1 ± 0.1 kg after 6 months administration, respectively. In any test item for hematology or blood chemistry, no significant difference was noted between the control and cocoa powder groups (Table 2).

Plasma lipid levels

The level of plasma total cholesterol in the cocoa powder group was equivalent to that in the control. There was no significant difference in triglyceride or phospholipid between the control and cocoa powder groups. The level of plasma TBARS in the cocoa powder group was significantly decreased compared to the control from the 2nd month of administration (Fig. 1).

Antioxidative effect in LDL

The lag time was extended in the cocoa powder group in the 2nd month and 3rd month of administration compared to the value prior to the start of administration. When compared to the control (54.4 ± 2.2 min), the lag time in the cocoa powder group was significantly prolonged to 72.0 ± 3.1 min in the 3rd month of administration (Table 3).

Area of atherosclerotic lesions in the aorta

Photocopies of the aortic intima surfaces are shown in Fig. 2. In each location, the percent area of lesions to the total intimal area tended to decrease in the cocoa powder group. The ratios in the whole aorta and aortic arch in this group were significantly lower than those in the control (Table 4).

Lipid contents in the aorta

In the cocoa powder group, the total cholesterol level in the aorta tended to be lower in any location compared to the control, however the difference was not significant. The level of TBARS also tended to be low in any

location in the cocoa powder group, and a significant decrease was observed in the abdominal aorta (Fig. 3).

Static rheological properties of the aorta

The tension-strain curve and the stress-strain curve in the circumferential strips excised from the ascending aorta and descending portion of aortic arch are shown in

Table 2. Blood chemical and hematological findings in KHC rabbits fed with cocoa powder.

Parameter	Control		Cocoa powder	
	Baseline	6 months	Baseline	6 months
RBC ($\times 10^6/\mu\text{l}$)	6.68 $\pm$ 0.21	6.62 $\pm$ 0.20	6.48 $\pm$ 0.15	6.30 $\pm$ 0.43
Hb (g/dl)	12.9 $\pm$ 0.3	13.1 $\pm$ 0.4	12.7 $\pm$ 0.1	12.4 $\pm$ 0.2
Plt ($\times 10^3/\mu\text{l}$)	421 $\pm$ 16	402 $\pm$ 4	478 $\pm$ 51	529 $\pm$ 69
WBC ($\times 10^3/\mu\text{l}$)	7.76 $\pm$ 0.37	6.43 $\pm$ 0.68	7.92 $\pm$ 0.39	5.61 $\pm$ 0.93
TP (g/dl)	5.9 $\pm$ 0.1	6.9 $\pm$ 0.2	5.9 $\pm$ 0.0	6.5 $\pm$ 0.3
Glu (mg/dl)	129 $\pm$ 3	118 $\pm$ 2	136 $\pm$ 4	121 $\pm$ 3
BUN (mg/dl)	17.8 $\pm$ 0.8	19.2 $\pm$ 1.0	17.3 $\pm$ 1.1	19.9 $\pm$ 0.9
Cre (mg/dl)	0.62 $\pm$ 0.04	0.81 $\pm$ 0.04	0.67 $\pm$ 0.03	0.95 $\pm$ 0.07
AST (IU/l)	35 $\pm$ 2	24 $\pm$ 2	45 $\pm$ 6	20 $\pm$ 2
ALT (IU/l)	19 $\pm$ 2	21 $\pm$ 2	24 $\pm$ 3	28 $\pm$ 5
LDH (IU/l)	88 $\pm$ 11	51 $\pm$ 5	88 $\pm$ 9	44 $\pm$ 2
CPK (IU/l)	343 $\pm$ 42	372 $\pm$ 116	390 $\pm$ 30	192 $\pm$ 21
ALP (IU/l)	399 $\pm$ 34	49 $\pm$ 4	449 $\pm$ 52	44 $\pm$ 3

All values are mean $\pm$ S.E.

Numbers of rabbits in the groups are: $n = 5 - 6$ (Control group), $n = 6$ (Cocoa powder group).

No significant difference was observed by the Student's t -test.

Table 3. Effect of cocoa powder on LDL oxidation in KHC rabbits.

Group	Lag time (minutes)				
	Baseline	2 months	3 months	4 months	6 months
Control	53.5 $\pm$ 1.7	NE	54.4 $\pm$ 2.2	NE	36.1 $\pm$ 2.2
Cocoa powder	49.8 $\pm$ 2.6	56.7 $\pm$ 2.2 [§]	72.0 $\pm$ 3.1 ^{**} , ^{§§}	54.4 $\pm$ 1.9	35.9 $\pm$ 1.3

All values are mean $\pm$ S.E. ($n = 6$). NE: Not examined. Significantly different from control, ^{**} $p < 0.01$ (Student's t -test).

Significantly different in prolongation of lag time from baseline, [§] $p < 0.05$, ^{§§} $p < 0.01$ (Paired t -test).

Fig. 1. Plasma lipid levels in KHC rabbits fed with cocoa powder. Total cholesterol (A), triglyceride (B), phospholipid (C) and TBARS (D) in plasma were determined using commercial kits. Data are expressed as mean $\pm$ S.E. ($n = 6$). Control group (O); Cocoa powder group (●). Significantly different from control, ^{**} $p < 0.01$ (Student's t -test).

Table 4. Effect of cocoa powder on the extent of atherosclerotic lesions in aortas of KHC rabbits.

	Area of atherosclerosis (%)	
	Control	Cocoa powder
Aortic arch	76.22 $\pm$ 5.05	55.52 $\pm$ 6.59 *
Thoracic aorta	48.76 $\pm$ 12.10	19.53 $\pm$ 5.11
Abdominal aorta	37.84 $\pm$ 6.58	25.85 $\pm$ 6.93
Whole aorta	52.39 $\pm$ 7.68	30.87 $\pm$ 5.61 *

All values are mean $\pm$ S.E. ($n = 6$).

Significantly different from control, * $p < 0.05$ (Student's t -test).

Fig. 4 and Fig. 5, respectively. The stress value at each strain in the descending portion of aortic arch was significantly large in the cocoa powder group compared to

Fig. 2. Xerox-copies of the aortic intima surfaces in KHC rabbits. Atherosclerotic lesions were traced with black. Control group (A); cocoa powder group (B).

Fig. 3. Effect of cocoa powder on aortic lipid contents in KHC rabbits. Total cholesterol (A) and TBARS (B) were determined in the aortic arch (Arch), thoracic aorta (Thor), abdominal aorta (Abdo) and whole aorta (Whole). Data are expressed as mean $\pm$ S.E. ($n = 6$). Control group (□); Cocoa powder group (■). Significantly different from control, * $p < 0.05$ (Student's t -test).

Fig. 4. Tension-strain curves of the circumferential strips of the aortic wall excised from the ascending aorta and descending portion of aortic arch in KHC rabbits fed with cocoa powder. Data are expressed as mean $\pm$ S.E. ($n = 6$). Control group (O); Cocoa powder group (●). No significant difference was observed by the Student's t -test. L_0 : Initial length, ΔL : Increment from the initial length.

that in the control. The wall thickness of the descending portion of aortic arch was significantly small in the cocoa powder group compared to that in the control, and the value of $EM_{0.5}$ was significantly greater in the cocoa powder group than in the control group (Table 5). In histopathology, thickening of tunica intima in the descending portion of aortic arch was mild in the cocoa powder group compared to that in the control group (Fig. 6).

Discussion

The administration of cocoa powder by 10% of the diet prevented development of atherosclerosis in KHC rabbits. The intake of cocoa powder neither affected plasma cholesterol, triglyceride or phospholipid, nor affected body weight or hematology and blood chemistry parameters. The dosage of cocoa powder in the present study was decided on the basis of a preliminary study (9), in which the short-term 1% dietary administration of crude polyphenol fraction extracted from cacao beans enhanced resistance against LDL oxidation in cholesterol-

Table 5. Thickness and elastic modulus of the wall at the ascending aorta and descending portion of aortic arch in KHC rabbits fed with cocoa powder.

Parameter	Aortic arch	Control	Cocoa powder
Wall thickness (μm)			
Ascending	1,301.4 $\pm$ 62.8	1,231.3 $\pm$ 105.9	
Descending	1,240.2 $\pm$ 50.8	739.6 $\pm$ 61.7 **	
$EM_{0.5}$ ($\times 10^6$ dyn/cm ²)			
Ascending	0.742 $\pm$ 0.101	0.858 $\pm$ 0.060	
Descending	1.058 $\pm$ 0.130	2.165 $\pm$ 0.473*	

All values are mean $\pm$ S.E. ($n = 6$). Significantly different from control, * $p < 0.05$, ** $p < 0.01$ (Student's t -test). $EM_{0.5}$: elastic modulus of the wall at a strain of 0.50.

Fig. 5. Stress-strain curves of the circumferential strips of the aortic wall excised from the ascending aorta and descending portion of aortic arch in KHC rabbits fed with cocoa powder. Data are expressed as mean $\pm$ S.E. ($n = 6$). Control group (O); Cocoa powder group (●). Significantly different from control, * $p < 0.05$, ** $p < 0.01$ (Student's t -test). L_0 : Initial length, ΔL : Increment from the initial length.

fed rabbits. The dosage was then fixed at 10% admixture of the cocoa powder (total polyphenol content: 7.8%), so that the intake of polyphenol would be close to that in the above-mentioned study.

We confirmed that intake of cocoa powder increased resistance against oxidation of LDL, and decreased plasma TBARS in the KHC rabbits. Antioxidative effect of polyphenol that is abundant in cocoa powder was considered to be involved in this mechanism. Polyphenol is abundant in cacao beans. Especially, it is rich in processed foods such as cocoa powder and chocolates. Cacao polyphenols contain monomeric (+)-catechin and (-)-epicatechin, dimeric procyanidin B2, trimeric procyanidin C1, and tetrameric cinnamtannin A2. The crude extract fraction that contains these cacao polyphenols was shown to have antioxidative activity (7, 8). *In vitro* studies reported that these compounds trap superoxide and hydroxyl radical (6, 17), and suppress oxidation stress (18, 19). The intake of cocoa powder or crude cacao polyphenol fraction was proved to increase resistance against oxidation of plasma or LDL in rabbits (9), rats (20, 21) and humans (10). These polyphenols need to be taken up into bodies to exert their antioxidative activity, however the absorption, metabolism and distribution of them are not well known. Catechin was recently reported to be absorbed efficiently through the digestive tract compared to other flavonoids (22). In healthy volunteers, the intake of cocoa drinks or chocolates led epicatechin and its metabolite to reach their peak in plasma at 1 or 2 hours later (23, 24). Baba *et al.* reported

the correlation of antioxidative effect and plasma epicatechin concentration in rats treated with cocoa powder (20). In dietary administration of the crude polyphenol fraction to cholesterol-fed rabbits, epicatechin and its metabolite were not detected in plasma, but antioxidative activities were observed (9). It was considered that the low plasma polyphenol concentration was resulting from slow intake of the crude polyphenol fraction by rabbits due to dietary administration. In the present study, polyphenols contained in cocoa powder were administered at almost the same dosage as in the above-mentioned study. We did not determine plasma polyphenol concentrations, but a variety of polyphenols presumably existed in rabbit plasma at such concentrations as to exert antioxidative effect. It is not known exactly why enhancement of LDL resistibility against oxidation was transient in cocoa powder-fed KHC rabbits. The condition of LDL oxidation *ex vivo* may be too strong to detect antioxidative activity of cacao polyphenols in the present study. Plasma TBARS, which is both the marker of lipid peroxidation and the indirect index of oxidative stress, was, however, decreased in the cocoa powder treated group until the end of administration. We therefore assume that antioxidative effect of polyphenols has been persisted *in vivo* throughout the study period.

The KHC rabbit is a model animal of familial hypercholesterolemia (FH) that spontaneously develops atherosclerosis, exhibiting hypercholesterolemia from birth due to inheritable defect of LDL receptor, as observed in Watanabe heritable hyperlipidemic (WHHL) rabbits (12). A variety of physiological characteristics in KHC rabbits have been reported (12, 16). In the KHC rabbits, atherosclerosis in the aorta is extended to the aortic arch and major arterial bifurcations by the age of 3 months, and further descended to the peripheral regions with age (12). In the present study, marked atherosclerotic lesions were observed in the aortas of control animals at the end of the study (at the age of 9 months). In the KHC rabbits fed cocoa powder, the area of atherosclerotic lesions in the aorta was decreased significantly. Correspondingly, the cholesterol contents in the vascular walls tended to decrease. In the descending portion of aortic arch, the value of wall stress and $EM_{0.5}$ were significantly greater in the cocoa powder group than in the control group. This means the aortic wall in the control group was more viscoelastic compared to that in the cocoa powder group. Katsuda *et al.* (16) suggested in the previous report that in the process of remodeling, the aortic wall undergoes "softening" due to accumulation of foam cells in the relatively early stage of atherosclerosis and thereafter increases stiffness with progress of fibrous proliferation or calcification. As foam cells increase, the wall became viscoelastic in the relatively early stage of atherosclerosis. Similar results have been reported in cholesterol-fed cockerels (25) and cholesterol-fed rabbits (26). The co-

Fig. 6. Microphotographs of the descending portion of aortic arch in KHC rabbits with elastica van Gieson stain. Bar = 100 μ m.

A: A control animal. Thickening of the tunica intima mainly due to accumulation of a large amount of foam cells is observed.

B: A cocoa powder treated animal. Compared to the control animal, the cocoa powder treated animal shows slight thickening of the tunica intima.

coa powder inhibited accumulation of cholesterol-rich foam cells in the intimal layer, which was considered to be responsible for less viscoelastic properties of the aortic wall.

There is a widely accepted theory that oxidized LDL plays an important role in the onset and development of atherosclerosis (11, 27). Some epidemiological studies reported relationship between susceptibility of LDL to oxidation and atherosclerosis in humans (28, 29). Suppression of atherosclerosis by antioxidants has been proven in human and animal models. Inverse correlation between intake of antioxidants and cardiovascular diseases were shown in epidemiological studies (1–3). In FH model animals such as WHHL rabbits and KHC rabbits, natural antioxidative substances like vitamin E (30) and taurine (31), herbal medicines containing flavonoid (32, 33), and hypocholesterolemic agent possessing antioxidative activity, probucol (34, 35) were reported to suppress development of atherosclerosis. These antioxidative substances exert anti-atherosclerotic effect without decreasing plasma cholesterol. Yamakoshi *et al.* (36) reported that grape seed polyphenols significantly suppressed the susceptibility of LDL to oxidation in cholesterol-fed rabbits, and macrophage-originated foam cells filled with oxidized LDL were decreased in the thickened lesion of tunica intima without affecting plasma cholesterol level. As explained in the above, it is considered that antioxidative substances suppress LDL oxidation, which will not thereby induce foam cell formation. As is the case with these reports, LDL oxidation was suppressed and plasma lipid peroxide was decreased in the cocoa powder treated rabbits. There were no changes in plasma lipid, and atherosclerotic lesion was improved. We therefore considered that polyphenols of cocoa powder suppressed oxidation of LDL and other lipids, which prevented foam cell formation and inhibited the generation and development of atherosclerosis.

Cacao polyphenols have a good profile for healthy blood vessels, for example, inhibition of platelet aggregation (37), vasodilative activity through controlling the levels of eicosanoids and NO (38, 39), and regulation of cytokine production (40) as well as antioxidative effects on LDL. These effects might also contribute to anti-atherosclerotic effect observed in the present study. Cocoa powder contains not only polyphenols but also dietary fibers, a lot of minerals, theobromine possessing a variety of pharmacological effects, and so on (41). Dietary fibers are known to decrease serum lipid (42), however there was no change in lipids in the cocoa powder-fed KHC rabbits in our study during its 6-month study period. Minerals such as potassium, calcium and magnesium are involved in maintaining vascular functions and lowering blood pressure (43, 44). Additionally, magnesium has been reported to suppress atherosclerosis through its lipid-lowering activity and antioxidative effect (45, 46).

Recently, Azam *et al.* (47) reported xanthine derivatives such as theobromine or caffeine have possibility to act as antioxidative substances. We also assume these elements may be partially involved in suppressive effects on development of atherosclerosis in KHC rabbits. Further studies are thereby required to confirm suppressive effects of cacao polyphenols on atherosclerosis.

In conclusion, cocoa powder suppressed the development of atherosclerosis in the FH model animals, KHC rabbits. Our results suggested that the anti-atherosclerotic effect of cocoa powder was involved in the suppression of LDL oxidation. The intake of polyphenol-rich processed food of cacao beans like cocoa powder and chocolates would be beneficial to prevent the onset of atherosclerosis and cardiovascular diseases.

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