Dose–Response Study of Daily Cocoa Intake on the Oxidative Susceptibility of Low-Density Lipoprotein in Healthy Human Volunteers

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A four-period crossover study was conducted to evaluate the dose-related response of daily cocoa intake on the oxidative susceptibility of low-density lipoprotein (LDL) in healthy human volunteers. Each supplementation phase consisted of a 14-day feeding period followed by a 28-day washout period. During the period, healthy male volunteers (n = 8) ingested 18, 24 or 36 g of cocoa powder per day (1.3, 1.74 or 2.61 g of polyphenols per day). During the control period, these subjects ingested sugar. LDL oxidative susceptibility was measured as the lag time of conjugated diene formation that started with the addition of a radical initiator, 2,2′-azobis (4-methoxy-2,4-dimethylvaleronitrile). Samples were analyzed at pre-, one week, and two weeks post-supplementation. In the 24 and 36 g cocoa powder ingestion period, significant lag time prolongation was observed. We conclude that the ingestion of more than 24 g of cocoa powder per day (1.74 g of polyphenols) clearly protects LDL from oxidation.

Key words —— cocoa, low-density lipoprotein, polyphenol, oxidation, dose-related

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INTRODUCTION

The oxidative modification of low-density lipoprotein (LDL) is thought to play a crucial role in the initiation of atherosclerosis.[1)] An enhanced uptake of oxidized LDL by macrophages *via* their scavenger receptors[2)] leads to the formation of lipid-laden foam cells, a hallmark of early atherosclerosis.[3)] It has been reported that oxidized LDL is a chemotactic agent for monocytes and T-lymphocytes, causes an increased production of inflammatory cytokines[4)] and growth factors,[5)] promotes procoagulant activities,[6)] and impairs arterial vasotumor responses.[7)] There have been many studies to investigate increased LDL resistance to oxidation by various antioxidants.[8–10)] Cacao beans, the seed of *Theobroma cacao*, are known to be rich in antioxidative polyphenols.[11)] The major component of cacao polyphenols are (+)-catechin, (–)-epicatechin and their oligomers that are linked at C4-C8 bonds to form B-type procyanidins.[12)] We have reported that each of these components had potent antioxidative activities in an *in vitro* LDL oxidation system.[13)] In addition, there have been several reports indicating that chocolate and cocoa ingestion decreased LDL oxidative susceptibility in healthy human volunteers.[14–17)] However, in a previous studies, the subjects ingested a large amount of cacao products daily. The aim of this study is to elucidate the dose-relative response of cocoa powder ingestion on the oxidative susceptibility of LDL in human healthy volunteers.

MATERIALS AND METHODS

Materials —— The polyphenols and other component from the test cocoa powder, which was prepared by Meiji Seika Kaisha Ltd. (Japan), are shown in Table 1. Total polyphenol concentration was determined by the Prussian blue method using epicatechin as the standard.[18)] Xanthine derivatives, such as caffeine and theobromine, catechins and procyanidins were evaluated by HPLC according to the method of Natsume *et al*.[18)] Other chemicals were commercially available products in analytical or HPLC grade.

Subject and Ethics —— We enrolled 8 healthy male Japanese subjects with a mean age of 32.5 ± 6.4 year, body weight of 62.2 ± 6.4 kg, and body mass index of 21.7 ± 2.1 kg/m^2. They were nonsmokers, normolipidemic, non-obese and unmedicated. Subjects consumed a standard Japanese diet

Table 1. Nutritional Composition of Cocoa Powder

nutrients	% (w/w)
protein	21.40
fat	12.10
sugar	7.61
fiber	36.67
ash	8.06
water	4.17
caffeine	0.28
theobromin	2.21
total polyphenol	7.25
catechin	0.20
epicatechin	0.68
procyanidin B2	0.30
procyanidin C1	0.20
cinnamtannin A2	0.32
Gal-EC-EC2	0.03
calorie (kcal/100 g)	240.00

Total polyphenols were determined by the Prussian blue method using epicatechin as the standard. Gal-EC-EC: 3-O-β-galactose-ent-(−)-epicatechin-(−)epicatechin.

during the experiment and refrained from taking fibers or antioxidant supplements starting from 4 weeks before the start of and during the experiment. The protocol for this trial was approved by the ethics committees of the National Institute of Health and Nutrition. Written informed consent was obtained from all volunteers before their participation.

Experimental Design —— The experiment was conducted as a 4-period crossover study under a free-living condition but with a strict diet regimen of cocoa powder supplementation. Each ingestion phase consisted of a 14-day feeding period followed by a 28-day washout period. During the 18 g cocoa supplement period, subjects consumed 9 g of cocoa powder and 12 g of sugar after breakfast and lunch (1.30 g per day of total polyphenol ingestion). During the 24 g cocoa supplement period, the subjects consumed 12 g of cocoa powder and 16 g of sugar after breakfast and lunch (1.74 g per day of total polyphenol ingestion). During the 36 g of cocoa supplement period, the subjects consumed 12 g of cocoa powder and 16 g of sugar after breakfast, lunch and dinner (2.61 g per day of total polyphenol ingestion). During the control period, the subjects consumed only 16 g of sugar after breakfast, lunch and dinner. Complete dietary data were obtained from each subject throughout the experimental period. These food records were analyzed with the Excell Food Frequency Questionnaire (Kenpakusha, Tokyo, Japan).

LDL Oxidation —— At pre-, one week post-, and two weeks post-cocoa consumption, blood was drawn into a tube containing disodium EDTA following a 12-hr fasting period after dinner. These blood samples were used for LDL fraction isolation and general blood analyses. LDL oxidative susceptibility was measured by the method of Esterbauer[19] and Hirano *et al*.[20] LDL was isolated from plasma by single-spin density gradient centrifugation ($417000 \times g$, 40 min, 4°C). Protein concentration of the LDL fraction was determined by the bicinchonic acid method.[21] A mixture of the LDL fraction (100 μg of protein/ml) and 200 μM 2,2′-azobis (4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN), a radical initiator, was incubated at 37°C. The kinetics of LDL oxidation were determined by monitoring for 420 min the formation of conjugated dienes from the change in absorbance at 234 nm.

Blood Snalyses —— Alanine aminotransferase (ALT), aspartate aminotransferase (AST), (γ-glutamyl transpeptidase (γ-GTP), total cholesterol, high density lipoprotein (HDL)-cholesterol, triglyceride, and glucose in plasma were analyzed with Spotchem EZ SP4430 (Arkray, Saitama, Japan).

Statistical Analyses —— The results are presented as mean values with S.D. Comparisons of the initial and end values were assessed by paired *t*-test. Assessment of the effect of cocoa powder relative to the placebo was performed by using a repeated-measures general linear model. All calculations were made with SPSS for Windows, version 7.5.1 (SPSS Japan Inc., Tokyo, Japan). *p*-Values of less than 0.05 were considered statistically significant.

RESULTS

After supplementation of cocoa powder, changes in the LDL oxidative susceptibility were measured as a lag time and are shown in Fig. 1. During the period of 18 g cocoa powder ingestion per day, there were no changes in lag time among pre-, one week post-, and two weeks post-supplementation. Statistically significant lag time prolongation was observed at 2 week post-consumption compared with pre-supplementation for the 24 g per day cocoa ingestion ($p < 0.05$). For the 36 g cocoa feeding, a statistically significant lag time delay ($p < 0.01$) was observed in one week and two weeks post-ingestion compared with pre-ingestion. Table 2 shows the gen-

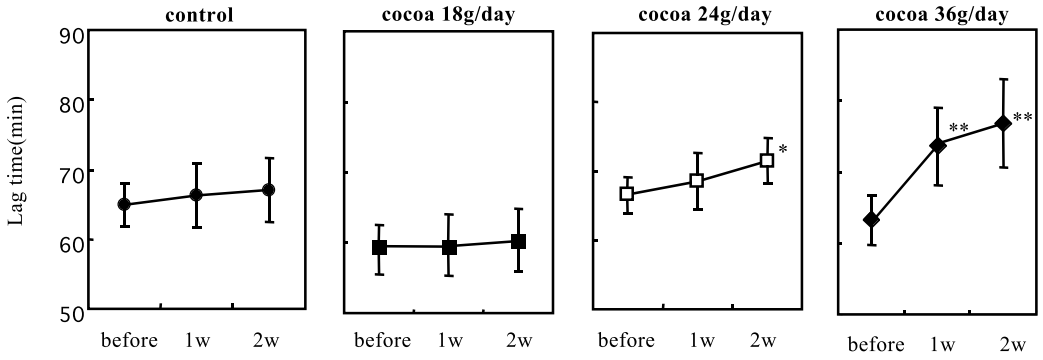

Fig. 1. Changes in LDL Oxidative Susceptibility in Subjects after Ingestion of Various Amounts of Cocoa Powder for 2 weeks
Each value represents the mean value and the standard deviation. Statistically significant differences were observed from the pre-ingestion period by paired t-test: $*p < 0.05$, $**p < 0.01$.

Table 2. Biochemical Findings in Blood before and after Various Dosages of Cocoa Supplementaion

variable	control	18 g cocoa/day	24 g cocoa/day	36 g cocoa/day
AST (IU/L)				
before supplementation	19.9 ± 1.6	21.5 ± 2.0	20.5 ± 2.8	19.9 ± 1.2
2 week after cocoa supplementation	20.2 ± 1.8	18.9 ± 1.8	19.3 ± 1.9	19.7 ± 1.3
ALT (IU/L)				
before supplementation	24.1 ± 1.9	29.1 ± 3.4	29.6 ± 2.4	29.3 ± 2.9
2 week after cocoa supplementation	25.1 ± 2.8	26.8 ± 4.4	28.6 ± 2.7	27.7 ± 3.8
γ-GPT (IU/L)				
before supplementation	59.0 ± 9.1	60.8 ± 10.0	61.1 ± 9.6	58.7 ± 7.5
2 week after cocoa supplementation	56.8 ± 8.5	60.1 ± 11.7	62.0 ± 11.7	60.1 ± 7.8
total cholesterol (mg/dl)				
before supplementation	160.4 ± 4.1	166.1 ± 3.8	156.8 ± 3.2	162.3 ± 4.9
2 week after cocoa supplementation	157.3 ± 4.9	157.0 ± 5.9	157.0 ± 8.9	159.5 ± 4.1
HDL-cholesterol (mg/dl)				
before supplementation	55.6 ± 2.8	56.4 ± 2.9	57.2 ± 3.1	55.8 ± 2.8
2 week after cocoa supplementation	55.4 ± 3.4	55.4 ± 3.1	53.6 ± 2.8	56.9 ± 3.0
triglyceride (mg/dl)				
before supplementation	200.2 ± 42.3	210.1 ± 51.2	214.3 ± 40.2	212.2 ± 62.5
2 week after cocoa supplementation	213.2 ± 59.3	220.0 ± 90.5	219.8 ± 76.3	221.3 ± 78.2
glucose (mg/dl)				
before supplementation	101.9 ± 4.5	106.0 ± 6.2	99.8 ± 5.2	110.1 ± 4.8
2 week after cocoa supplementation	105.6 ± 6.5	110.3 ± 9.1	102.3 ± 8.8	109.3 ± 8.8

Each value represents mean ± standard deviation.

eral biochemical analyses. There were no significant changes in these analyses between the control and cocoa groups. Weekly calorie and nutrient intake were calculated on the basis of complete food and beverage records obtained from each subject. There were no differences in either nutrient or calorie intake among all groups throughout the test period. In addition, none of the subjects discontinued their participation in this study. No adverse effects were seen in test groups throughout the test period within the limit of measurement in this study.

DISCUSSION

In this study, daily 18 g cocoa powder, containing 1.3 g of polyphenols and 0.16 g of catechins [(+)-catechin and (–)-epicatechin], did not produce an antioxidative activity against LDL oxidation. Supplementation with 24 g of cocoa powder per day, containing 1.74 g of polyphenols and 0.21 g of catechins, significantly reduced the LDL oxidative susceptibility 2 weeks after ingestion. During the consumption of 36 g of cocoa powder, marked lag time prolongation was observed in one and two weeks post-ingestion. These results confirmed our previous results.[15] Wan *et al*. reported that a 4-week feeding of 22 g of cocoa powder and 16 g of dark chocolate bar that contained 0.466 g of procyanidins and 0.111 g of catechins, significantly increased the lag time in healthy human volunteers in a cross-over trial.[16] It was also reported that LDL oxidation was significantly reduced when 37 g of chocolate bar and 30.95 g of cocoa powder were ingested for 6 weeks, *via* daily procyanidin supplementation of 650 mg.[17] According to these results, a daily consumption of 1.0 to 1.8 g cacao polyphenols, that caluculated by the ratio of catechins and procyanidins, showed protection against LDL oxidation.

There have been no epidemiological reports correlating the relationship between chocolate and cocoa consumption and decreased cardiovascular disease risk. However, several studies suggested that consumption of catechins explained its inverse relationship with incidences of coronary heart diseases.[22,23] In the Iowa Women's Health Study, the consumption of only (+)-catechin and (–)-epicatechin among catechin derivatives showed an inverse association with coronary heart disease risk. (+)-Catechin and (–)-epicatechin constitute 10% of the polyphenols in cacao. On the other hand, the ingestion of catechin conjugated with gallates was not associated with the mortalities of coronary heart disease.[23] Certainly, (+)-catechin and (–)-epicatechin appear to be high in their bioavailability. For an oral administration of cocoa, approximately 25–30% of (–)-epicatechin recovered in urine was as its metabolites.[24] In addition, the maximum level in blood of (–)-epicatechin and its metabolites reached 4.77 ± 0.94 μmol/l at 2 hr after 36 g cocoa intake. (–)-Epicatechin showed antioxidative activity on LDL oxidation more than 0.5 μmol/l.[13] These results suggested that epicatechin was contributed the antioxidative activities in the blood after ingestion of cocoa. On the other hand, we recently reported that the chemical structure of (–)-epicatechin metabolites is glucuronidated, a form thought to be distributed in blood and organ.[25] The glucuronidation of (–)-epicatechin occurs mainly at the 3′position of the B ring in human. This form seems to exhibit relatively low antioxidative activity compared with the intact form, presumably because it does not maintain the catechol structure of the B ring, the most important structure reported to have an antioxidative effect. In contrast procyanidins, constituting 90% of the polyphenols in cacao, were hardly detected in blood and only 0.5% was recovered in urine after their oral administration.[26] Recently, these procyanidins have been reported to be degraded into a more bioavailable low-molecular weight phenolic acid by microflora in the colon and then absorbed.[27,28] The contribution of these chemicals against antioxidative activity in human body remains obscure. However, previous data of antioxidative activities of cacao products or its polyphenolic fractions *in vivo* could not be explained by a monomeric catechin contribution alone. Therefore, the oligomerically derived metabolites might be acting as antioxidants in the human body.

In conclusion, a daily ingestion of more than 24 g of cocoa powder including 1.74 g polyphenols, an equivalent of two cups of cocoa, was clearly shown to protect LDL against oxidation.

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