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Daily Cocoa Intake Reduces the Susceptibility of Low-Density Lipoprotein to Oxidation as Demonstrated in Healthy Human Volunteers

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Nine male volunteers were given 36 g of cocoa powder (containing 2610 mg of polyphenols) per day with sugar and 6 volunteers received an equivalent amount of sugar for 2 weeks. Conjugated diene production in LDL induced by 2–2′ azobis(4-methoxy-2, 4-dimethyl-valeronitrile) (V-70) and copper ion were evaluated. The lag time was significantly prolonged at 1 and 2 weeks in V-70 and at 2 weeks in copper ion after cocoa powder consumption. The level of excretion of epicatechin in urine was significantly higher in the cocoa group than that in the control group. In conclusion, the antioxidants in cocoa powder might be absorbed and increase the resistance of human LDL to oxidation.

Keywords: cocoa powder, polyphenols, LDL, oxidation, human volunteers, diene

INTRODUCTION

Cacao beans, the seed of *Theobroma cacao*, are known to contain various polyphenolic substances [1–3]. It has been reported that cacao liquor, one of the major ingredients of cocoa and chocolate, prepared by fermentation of dried and cracked raw beans, is rich in polyphenols. Epicatechin, catechin, and procyanidins are confirmed as major antioxidative components of cocoa and chocolate[4–6]. Long et al. reported that tea and coffee exhibited a complex mixture of anti- and pro-oxidant abilities, however, cocoa did not exhibit such pro-oxidant activity [7].

A protective role of plant polyphenols against atherosclerosis has been suggested by several studies. According to epidemiological studies, plant polyphenol consumption is associated with a reduced risk of coronary heart disease (CHD) [8–10]. Polyphenolic substances derived from tea [11] and grape seed [12] showed effectiveness in prevention of atherosclerosis in hypercholesterolemic rabbits. These studies suggested that the anti-atherosclerotic effect of these compounds is the result of inhibition of LDL oxidation. It has been reported that administration of a single high dose of cacao powder to volun-

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teers reduced the susceptibility of low-density lipoprotein to oxidation [13].

In the present study, we examined the effect of daily cocoa powder intake on the susceptibility of low-density lipoprotein to oxidation in healthy human volunteers, and also the absorption of antioxidative polyphenols from cocoa was evaluated through analyses of their plasma and urine.

MATERIALS AND METHODS

Subjects

Fifteen healthy Japanese men aged 32.5 ± 6.4 y, weight 62.2 ± 3.8 kg, body mass index 21.7 ± 2.1 kg/m^2, who were non-smokers, normolipidemic, non-obese, taking no medication, and consuming a standard Japanese diet, participated in the study. The study protocol was approved by the National Institute of Health and Nutrition Ethical Committee. All subjects signed informed consent documents.

Materials

Cocoa powder using in this study was prepared by Meiji Seika Kaisha Ltd. Daily intake of polyphenols and other nutrients from the cocoa powder is shown in Table I. Total polyphenol concentration in cacao powder was determined by the Prussian blue method using epicatechin as the standard [14]. Xanthine derivatives such as caffeine and theobromine, catechins and procyanidins were determined by HPLC [4,6]. Other chemicals were available products of analytical or HPLC grade.

Experimental procedure

The volunteers were divided into two groups, nine volunteers in the cocoa group who consumed 3 cups of cocoa drink each day, and six volunteers in the control group who consumed an equivalent amount of sugar each day. One cup of cocoa (12 g of cocoa powder and 16 g of sugar) or sugar was consumed after breakfast, lunch and dinner. Complete dietary data from food and beverage records were obtained from each subject throughout the study period. Blood samples were drawn following a 12-h fasting period after the supper meal. Blood samples were taken before cocoa intake, and after 1 and 2 weeks of cocoa consumption. Urine samples were collected for 8 hrs from 9:00 to 17:00 before cocoa intake, and after 1 and 2 weeks of cocoa consumption.

LDL oxidation

LDL oxidation was assayed by the methods of Esterbauer et al.[15]and Hirano and Kondo et al.[16]. LDL was isolated from plasma by single-spin density gradient centrifugation (417,000 × g, 40 min, 4°C) [17]. The fractions obtained were dialyzed against a 2,000-fold volume of 10 mmol/L phosphate-buffered saline at 4°C overnight. The protein concentration was determined by the bicinchonic acid method [18]. A mixture consisting of the human LDL fraction (100 μg of protein/ml) and 200 μM V-70 (2–2′-azobis (4-methoxy-2,4-dimethylvaleronitrile) or 1 μM $CuCl_2$ as the initiator of radical formation was incubated at 37°C. The kinetics of LDL oxidation were determined by monitoring the change in absorbance at 234 nm due to conjugated diene formation.

Determination of EC and its metabolites in plasma and urine by HPLC

Levels of EC and its metabolites in plasma and urine were determined by HPLC [19]. Glucuronide, sulfate, and glucuronide-sulfate conjugates of EC in plasma and urine were hydrolyzed to non-conjugated EC by treatment with sulfatase type H-5 (Sigma, St. Louis, MO, USA). The sam-

ple pretreated with enzymes analyzed by HPLC: column, TSKgel ODS-80Ts(5 μm 150 × 4.6 mm; TOSHO, Tokyo, Japan); elution solvent, 5.3 mol/L methanol- 0.35 mol/L acetic acid –50 mmol/L lithium acetate; detector, amperometric electrochemical detector at + 800 mV (EC-8020, TOSHO, Tokyo, Japan); flow rate: 0.9 mL/min.

Other analyses

Plasma α-tocopherol, β-carotene[20] and vitamin C [21] levels were measured by HPLC. Plasma lipoprotein was analyzed by electrophoresis using Titan gel Lipoprotein (Helena Laboratories, Saitama Japan). Plasma biochemical parameter levels were measured by conventional enzymatic methods.

TABLE I Daily intake of energy and nutrients from cocoa powder

Nutrients	*value*
Protein, g	7.4
Fat, g	5.4
Sugar, g	2.2
Fiber, g	13.2
Ash, g	2.9
Water, g	1.5
Caffeine, mg	101.0
Theobromin, g	797.0
Total polyphenols[a], mg	2610.0
Catechin, mg	71.7
Epicalechin, mg	244.1
Procyanidin B2, mg	106.2
Procyanidin C1, mg	71.9
Cinamtannin A2, mg	115.9
Ent-EC-EC-galactose, mg	9.1
Energy, kcal	86.4

a. Total polyphenols were determined by the Prussian blue method using epicatechin as the standard.

Statistical analyses

Results were expressed as mean ± standard deviation. All analyses were done using SPSS Statistical Software. Mean values were compared by Student's t-test. Values of $p < 0.05$ were considered significant.

RESULTS

LDL oxidizability

Fig 1. shows a typical pattern of V-70-induced conjugated diene production in LDL prepared from the same subjects before, and 1 and 2 weeks after intake of cocoa. Compared with the findings before cocoa intake, the lag phase was found to be prolonged. The mean and standard deviation of the lag time and the propagation rate in this study are shown in Table II. When V-70 was used, the lag time was significantly prolonged at 1 and 2 weeks compared with the control ($p<0.01$). The maximum rate of oxidation was also significantly lower at 1 week after cocoa intake ($p<0.05$). When copper ions were used, the induction period was also found to be extended significantly at 2 weeks.

Antioxidant and metabolites in plasma

The antioxidant concentrations in plasma are shown in Table III. The levels of α-tocopherol, β-carotene and ascorbic acid in plasma did not change upon supplementation of the diet with cocoa powder.

There was no significant difference in plasma glucose, total cholesterol, free cholesterol, cholesterol ester, triglyceride, phospholipid and free fatty acid levels between the control and cocoa groups. Also, cholesterol and triglyceride concentrations of isolated lipoprotein fraction by electrophoresis were not different between these two groups. Cocoa powder consumption did not influence these plasma biochemical indices (data not shown).

TABLE II Effect of cocoa powder intake on susceptibility of LDL to oxidation[1]

	Control group (no cocoa supplementation) (n=6)	*Cocoa group (36 g/day cocoa supplementation) (n=9)*
Induced by V-70		
Lag time, min		
week 0	62.9 ± 2.1	64.4 ± 3.5
week 1	67.5 ± 8.3	84.3 ± 11.2 **
week 2	67.0 ± 6.2	82.8 ± 9.1 **
Propagation rate, nmol/mg protein/min		
week 0	3.90 ± 0.67	3.26 ± 0.58
week 1	4.21 ± 0.57	3.17 ± 0.96 *
week 2	3.74 ± 0.52	3.17 ± 0.53
Induced by Cu2+		
Lag time, min		
week 0	87.7 ± 5.8	89.7 ± 6.0
week 1	95.6 ± 11.4	95.4 ± 5.2
week 2	91.3 ± 5.5	102.4 ± 8.6 *
Propagation rate, nmol/mg protein/min		
week	3.29 ± 0.51	3.14 ± 0.66
week 1	3.52 ± 0.53	3.61 ± 0.26
week 2	4.10 ± 0.79	4.25 ± 0.51

[1]Values are means ± SD. Significant difference from control; *: $p<0.05$, **: $p<0.01$.
LDL oxidation was induced by 200 μ M V-70 or 1 μ M copper ion and conjugated diene was monitored at 234nm

Assay of EC and its metabolites in plasma and urine by HPLC

In plasma, neither free EC nor its metabolites produced through enzymatic treatment could be detected by HPLC. Total EC in the urine collected for an 8 hr period (9:00–17:00) was determined and the results are shown in Table IV. After 1 and 2 weeks, a high level of EC excretion was observed in the cocoa group compared with control group.

Dietary outcomes

Weekly energy and nutrient intake were calculated on the basis of the complete food and beverage records obtained from each subject. There was no difference in either nutrient intake or energy intake between the cocoa and control

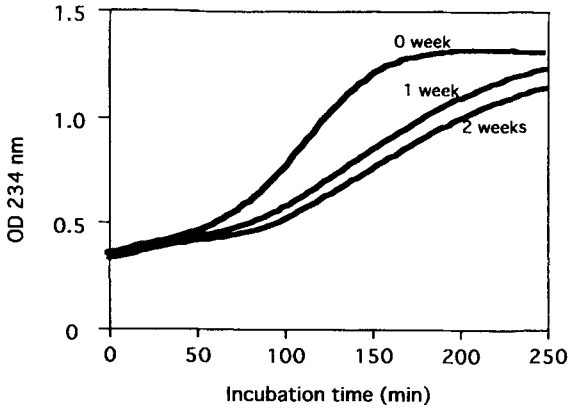

FIGURE 1 Typical kinetics of conjugated diene production induced by V-70 before (week 0), and 1 and 2 weeks after cocoa intake. A mixture consisting of the human LDL fraction (100 μg of protein/ml) and 200 μM V-70 was incubated at 37°C. Conjugated diene production in the mixture was monitored by measuring the absorbance at 234 nm

groups throughout the study period (data not shown).

DISCUSSION

Recent evidence suggests that LDL oxidation plays an important role in the pathogenesis of atherosclerosis [22–24]. Epidemiological studies on the relationship between dietary antioxidant intake and incidence of cardiovascular diseases have shown negative correlation [25,26]. A study of 805 Dutch subjects revealed that flavonoid intake was inversely associated with morbidity and mortality from coronary heart disease [7,8]. A French paradox is that low mortality from coronary heart disease was negatively correlated with the consumption of saturated fat [27]. According to recent research, there was association between this low risk of CHD incidence and red wine consumption. One of the components of red wine effective in preventing CHD was suggested to be polyphenols such as procyanidins and catechins [28,29].

TABLE III Concentration of antioxidants in plasma[1]

	Control group (no cocoa supplementation) (n=6)	*Cocoa group (36 g/day cocoa supplementation) (n=9)*
α -tocopherol, μ g/ml		
week 0	10.60 ± 0.11	10.90 ± 0.13
week 1	10.80 ± 0.19	10.70 ± 0.20
week 2	9.42 ± 0.16	9.62 ± 0.17
β carotene, μ g/ml		
week 0	1.33 ± 0.47	2.87 ± 1.45
week 1	2.32 ± 0.80	4.41 ± 3.73
week 2	1.81 ± 0.62	2.58 ± 1.37
ascorbic acid, μ g/ml		
week 0	7.9 ± 1.3	7.1 ± 0.6
week 1	6.7 ± 1.1	6.6 ± 1.1
week 2	6.4 ± 2.2	6.4 ± 1.3

[1]Values are means ± SD.

TABLE IV Epicatechin excretion in urine [1]

	Control group (no cocoa supplementation) (n=6)	*Cocoa group (36 g/day cocoa supplementation) (n=9)*
Epicatechin[2], mg/8 hrs		
week 0	0.45 ± 0.34	0.77 ± 0.37
week 1	0.86 ± 1.04	3.66 ± 1.96 *
wcok 2	0.30 ± 0.59	3.01 ± 1.83 *

[1]Values are means ± SD. Significant difference from control; *: $p<0.01$.

[2]Epicatechin metabolites in urine were hydrolyzed to a non conjugated form by sulfatase type H5

In the present study, we investigated the effects of daily intake of cacao powder on indicators of atherosclerosis in healthy human volunteers. The susceptibility of LDL to oxidation was decreased one week after intake of cocoa powder as compared with the control group, and this was still evident two weeks after cocoa intake (Table II, Fig 1). Similar results were obtained when induced by copper ions. However, there was no significant change in plasma lipid levels and plasma antioxidants content between these two groups. We attempted to analyze the levels of EC and its metabolites in blood, however, these substances could not be detected. In previous study, we have evaluated EC and its metabolites in plasma and urine after 35 g of cocoa powder intake [30]. In that experiment, the EC and its metabolites (glucuronide, sulfate and sulfoglucuronide forms of non-methylated and methylated EC) showed maximum concentrations in plasma 2 hours after cocoa ingestion, and these compounds almost disappeared within 8 hours. Also it was found that 80 % of total EC and its metabolites were excreted within 8 hours in the urine. In this study, we confirmed a high level of epicatechin-related substances in the urine of the subjects in the cocoa group. This results indicated that a part of the EC or its related compound such as procyanidines in cocoa powder was absorbed, and distributed to the plasma, and finally excreted in the urine.

There have been several clinical studies examining the effect of red wine consumption on LDL oxidation. The effective polyphenols in red wine were suggested to be catechins and procyanidins [28], similar to the case of cocoa. Furman et al. [31]suggested that consumption of 400 mL of red wine per day significantly reduces the susceptibility of LDL to oxidation. Kondo et al. [29] also suggested 500 ml of red wine intake showed similar effect. Nigdikar et al. [32] have reported that 375 mL of red wine or 1 g of polyphenols derived from red wine per day is also effective to decrease LDL oxidizability. Compared with these studies, the dosage of polyphenols administered to volunteers in the present study was high (2610 mg/day). According to these findings, several possibilities were considered concerning the results of this study. First, the antioxidative polyphenols in cocoa powder were absorbed, distributed and accumulated in LDL, then LDL oxidative resistance was increased *ex vivo*. Second, antioxidative polyphenols in cocoa powder were absorbed and distributed in plasma, LDL was exposed to these polyphenols at high concentration for long time, then its oxidative resistance was altered. Third, the LDL components especially fatty acid compositions were changed by the administration of cocoa powder that contained 12 % of fat.

In conclusion, daily cocoa intake reduces the susceptibility of LDL to oxidation. Further studies are required to elucidate the mechanism of this action.

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References

[1] W.G.C. Forsyth and J.B. Roberts (1960) Cacao polyphenolic substances 5. The structure of cacao leucocianidin. *Biochemical Journal* **74**, 374–378.

[2] H. Kim and P.G. Keeney (1984) (-)-Epicatechin content in fermented and unfermented cocoa beans. *Journal of Food Science* **49**, 1090–1091.

[3] L.J Porter, Z. Ma and B.G. Chan (1991) Cacao procyanidins; Major flavonoid identification of some minor metabolites. *Phytochemistry* **30**, 1657-1663.

[4] N. Osakabe, M. Yamagishi, C. Sanbongi, M. Natsume, T. Takizawa and T. Osawa (1998) The antioxidative substances in cacao liquor. *Journal of Nutritional Science and Vitaminology* **44**, 313–321.

[5] C. Sanbongi, N. Osakabe, M. Natsume, T. Takizawa, S. Gomi and T. Osawa (1998) Antioxidative polyphenols isolated from *Theobroma cacao*. *Journal of Agricultural and Food Chemistry* **46**, 454–457.

[6] J.F. Hamerstone, S.A. Lazarus, A.E. Mitchell, R. Rucker and H.H. Schmitz (1999) Identification of procyanidins in cocoa (Theobroma cacao) and chocolate using high-performance liquid chromatography/mass. *Journal of Agricultural and Food Chemistry* **47**, 490–496.

[7] L.H. Long, A.N.B. Lan, F.T.Y. Hsuan and B. Halliwell (1999) Generation of hydrogen peroxide by "antioxi-

dant" beverages and the effect of milk addition. Is cocoa the best beverage? *Free Radical Research* 31, 67–71.

[8] M.G.L. Hertog, E.J.M. Feskens, P.C.H. Hollman, M.B. Katan and D. Kromhout (1993) Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* **342**, 1007–1011.

[9] M.G.L. Hertog, E.J.M. Feskens and D. Kromhout (1997) Antioxidant flavonols and coronary heart disease risk. *Lancet* **349**, 699.

[10] P. Knekt, R. Jarvinen, A. Reunanen and J. Maatela (1996) Flavonoid intake and coronary mortality in Finland: a cohort study. *British Medical Journal* **312**, 478–481.

[11] L.B. Tijburg, S.A.Wiseman, G.W. Mejier and J.A. Westsrate (1997) Effects of green tea, black tea and dietary lipophilic antioxidants on LDL oxidizability and atherosclerosis in hypercholesterolemic rabbits. *Atherosclerosis* **135**, 37–47.

[12] J. Yamakoshi, S. Kataoka, T. Koga and T. Ariga (1999) Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Atherosclerosis* **142**, 139–149.

[13] K. Kondo, R. Hirano, A. Matsumoto, O. Igarashi and H. Itakura (1996) Inhibition of LDL oxidation by cocoa. *Lancet* **348**, 1514.

[14] M.L. Price and L.G. Butler (1977) Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. *Journal of Agricultural Food Chemistry* **25**, 1268–1273.

[15] H. Esterbauer, G. Striegl, H. Puhl and M. Rotheneder (1989) Continuous monitoring *in vitro* oxidation of human low density lipoprotein, *Free Radical Research Communications* **6**, 67–75.

[16] R. Hirano, K. Kondo, T. Iwamoto, O. Igarashi and H. Itakura (1997) Effect of antioxidants on oxidative susceptibility of low-density lipoprotein. *Journal of Nutritional Science and Vitaminology* **43**, 435–444.

[17] B.H. Chung, J.P. Segrest, M.J. Ray, J.D. Brunzell, J.E. Hokanson, R.M. Krauss, K. Beaudrie and J.T. Cone (1986) Single vertical spin gradient ultracentrifugation. *Methods in Enzymology* **128**, 181–209.

[18] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson and D.C. Klenk (1985) Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* **150**, 76–85.

[19] M. Piskura and J. Terao (1998) Accumulation of (-) epicatechin metabolites in rat plasma after oral administration and distribution of conjugation enzymes in rat tissues. *Journal of Nutrition* **128**, 1172–1178.

[20] S.B. Lotito and C.G. Fraga (1998) (+)-Catechin prevents human plasma oxidation *Free Radical Biology and Medicine* **24**, 435-441.

[21] A.J. Speek, J. Schrijver and W.H.P. Schreurs (1984) Fluorometric determination of total vitamin C in whole blood by high-performance liquid chromatography with pre-column derivatization. *Journal of Chromatography* **305**, 53–60.

[22] D. Steinberg, S. Parthasarathy, T.E. Carew and J.L. Witztim (1989) Beyond cholesterol; Modification of low-density lipoprotein that increase its atherogenesity. *New England Journal of Medicine* **320**, 915–924.

[23] B. Halliwell (1995) Oxidation of low-density lipoproteins; Question of initiation, propagation, and the effect of antioxidants. *American Journal of Clinical Nutrition* **61**, 670–677.

[24] J.L. Witztum (1994) The oxidation hypothesis of atherosclerosis. *Lancet* **344**, 393–795.

[25] R.M. Hoffman and H.S. Garewal (1995) Antioxidants and prevention of coronary heart disease. *Archives of Internal Medicine* **155**, 241–246.

[26] P.B. Duell (1995) The role of dietary antioxidants in prevention of atherosclerosis. *Endocrinologist* **5**, 347–356.

[27] S. Renoud and M. de Lorgeril (1992) Wine alcohol, platelets, and French paradox for coronary heart disease. *Lancet* **339**, 1523–1526.

[28] E.N. Frankel, J. Kanner, J.B. German, E. Parks and J.E. Kineslla (1993) Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* **341**, 454–457.

[29] K. Kondo, A. Matsumoto, H. Kurata, H. Tanahashi, H. Koda, T. Amachi and H. Itakura (1994) Inhibition of oxidation of low-density lipoprotein with red wine. *Lancet* **344**, 1152.

[30] S. Baba, N. Osakabe, A. Yasuda, M. Natsume, T. Takizawa, T. Nakamura, and J. Terao Bioavailability of (-)-epicatechin upon intake of chocolate and cocoa in human volunteers, *Free Radical Research* in press.

[31] B. Furman, A. Lavy and M. Aviram (1995) Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. *American Journal of Clinical Nutrition* **61**, 549–554.

[32] S.V. Nigdikar, N.R. Williams, B.A. Griffin and A.N. Howard (1998) Consumption of red wine polyphenols reduces the susceptibility of low-density lipoproteins to oxidation in vivo. *American Journal of Clinical Nutrition* **68**, 258–265.