The Inhibitory Effect of Cacao Liquor Crude Polyphenols (CLP) on Experimental Arteriosclerosis with Calcification in Rat Soft Tissue

Masako Horiuchi,[a] Naomi Osakabe,[b] Toshio Takizawa,[b] and Yoshiyuki Seyama*,[a]

[a]*Department of Clinical Chemistry, Hoshi College of Pharmacy, 2–4–41 Ebara Shinagawa-ku, Tokyo 142–8501, Japan and* [b]*Functional Food Research and Development Laboratory Meiji Seika Kaisha, Ltd., Sakadoshi, Saitama 350–0289, Japan*

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Cacao liquor crude polyphenols (CLP) contained potent anti-oxidants, such as catechin, and their oligomers. An investigation was conducted on the effects of CLP on experimental arteriosclerosis with calcification in rats, induced by giving vitamin D_2 (2.5×10^5 I.U./kg) for the first 4 consecutive days with an atherogenic diet (2% cholesterol, 1.5% cholic acid, 0.1% methylthiouracil). The diet was prepared using a vitamin E deficient diet. CLP inhibited the increase of serum lipid peroxides, the increase of calcium (Ca) in the aorta, the increase of Ca and inorganic phosphorus (P) in the aortic elastin fraction, or in the renal Ca and P of the arteriosclerotic rats. Previously, vitamin E and vitamin K_2 showed anti-calcification and radical scavenging activities under the above experimental conditions without vitamin E deficiency. Moreover, CLP reduced lipid peroxides in plasma and in tissue induced by vitamin E deficiency, without maintaining vitamin E levels. It is suggested that the inhibitory effects of CLP on the accumulation of Ca and P in the aorta or kidney were partially due to an anti-oxidant activity.

Key words —— polyphenol, anti-oxidant activity, cacao, arteriosclerosis, calcinosis

INTRODUCTION

Oxidative stress has been thought to represent one of the factors leading to chronic diseases such as atherosclerosis and diabetes mellitus; this stress is defined as an imbalance between oxidants and anti-oxidants. Vitamin E is known to be an endogenous anti-oxidant, responsible for protecting the highly unsaturated fatty acids in cell membranes from oxidation. It has been found that vitamin E prevents lipid deposition in atherosclerosis,[1] and a vitamin E derivative, *dl*-α-tocopherylnicotinate, exerted anti-arteriosclerotic effects on experimental arteriosclerosis induced by vitamin D_2 and atherogenic diet.[2] We reported that vitamin E and vitamin K_2 inhibited the increase of calcium (Ca) and inorganic phosphorus (P) in the aorta induced by vitamin D_2 with the atherogenic diet, owing to the radical scavenging activity of vitamin E and vitamin K_2.[3] Polyphenols are a large family of compounds occurring in plants, fruits, leaves, nuts and seeds. We have reported that several polyphenolic compounds were isolated from cacao liquor, which is one of the major ingredients of chocolate and cocoa.[4] It was reported that cacao liquor crude polyphenols (CLP) contained potent anti-oxidants such as epicatechin, catechin, clovamide, quercetin and their glucosides. CLP also had radical scavenging activities *in vitro*[4–6] and *in vivo*.[7] In the previous reports,[8] CLP intake resulted in a decrease in oxidative stress without maintaining vitamin E levels in the plasma and tissues. This study assessed the effect of CLP on the disorder of lipid and/or mineral metabolism, induced by vitamin D_2 with vitamin E deficiency and an atherogenic diet, in male rats.

MATERIALS AND METHODS

Materials —— CLP were prepared from cacao liquor according to a previous report.[4] Briefly, cacao liquor was defatted with n-hexane and extracted with 80% ethanol. The extract was applied to a Diaion HP2MG column (Mitsubishikasei Co., Ltd., Tokyo, Japan) and the column was washed with 20% ethanol to remove contaminants including xanthine derivative. The fraction eluted with 80% ethanol was collected as CLP and freeze-dried.

Experimental Procedures —— 7-week-old male Sprague-Dawley rats were obtained from Clea Japan, Inc., (Tokyo, Japan). The animals were housed in a room maintained at 26°C with 12 hr light-dark cycles and were given free access to food and distilled water. Male Sprague-Dawley rats were randomly divided into 3 groups. The rats in the normal

*To whom correspondence should be addressed: Dept. of Clinical Chemistry, Hoshi College of Pharmacy, Tokyo 142–8501, Japan. Tel.: +81-3-5498-5757; Fax: +81-3-5498-5756; E-mail: seyama@hoshi.ac.jp

Table 1. Composition of Experimental Diets

	Groups		
	N	D	CLP
Ingredients (%)			
Corn starch	41.5	36.3	36.3
Sucrose	5.0	16.5	16.5
α-potato starch	10.0	—	—
Casein	25.0	20.0	20.0
Cellulose powder	8.0	5.0	4.5
Corn oil	6.0	—	—
Vitamin E-free corn oil[a)]	—	5.0	5.0
Lard	—	10.0	10.0
Mineral mixture[b)]	3.5	3.5	3.5
Vitamin mixture[c)]	1.0	—	—
Vitamin E-free vitamin mixture[d)]	—	1.0	1.0
Cholesterol	—	2.0	2.0
Cholic acid	—	0.5	0.5
Metylthiouracil	—	0.2	0.2
CLP	—	—	0.5

a) Vitamin E-free corn oil was prepared from corn oil treated with activated charcoal and silica gel. *b*) Mineral mixture was AIN-76 mixture obtained from Oriental Yeast Co., Ltd., (Tokyo, Japan). *c*) Vitamin mixture was AIN-76 mixture including chorine bitartrate obtained from Oriental Yeast Co., Ltd., (Tokyo, Japan). *d*) Vitamin E-free vitamin mixture was the same as the AIN-76 mixture except that it was lacking vitamin E.

group (N, n = 8) were fed a CLP-free diet prepared as described in a previous report[8)] (Table 1). Experimental arteriosclerotic rats (D, n = 8) were given vitamin D_2 (Sigma Chemical Co., Ltd., St. Louis, U.S.A.) in a dose of 2.5×10^5 I.U./kg b.w./day in 1 ml olive oil via a stomach tube for the first 4 consecutive days. They were also fed an atherogenic diet (2% cholesterol, 1.5% cholic acid, 0.1% methylthiouracil) as described in a previous report[3)]; the atherogenic diet was prepared using a vitamin E-deficient diet as described in a previous report[8)] (Table 1). The CLP treatment group (CLP, n = 7) received vitamin D_2 and an atherogenic diet containing 0.5% CLP in the same way as the D group. After 6 weeks, the animals were sacrificed and serum was separated from collected blood.

Analytical Procedures —— Serum cholesterol (Ch), triglyceride (TG) and lipid peroxide levels were determined by the Ch C test, the TG CII test and LPO test, respectively (all from Wako Pure Chemical Industries, Japan). Lipid peroxides in serum were evaluated by measuring the amount of thiobarbituric acid reactive substances (TBARS), using malondialdehyde (MDA) as the standard. Serum Ca was measured with an atomic absorption spectrometer (Hitachi Z 8100 type, Tokyo, Japan) and serum P was measured by the method of Goldenberg and Fernandez.[9)] The aorta, heart and kidney were removed and freeze-dried. For determination of Ch, Ca and P, total lipids in half of the freeze-dried tissue were extracted by a chloroform-methanol solution (2 : 1), then weighed (dry defatted weight). The amount of Ch in the extracted lipid was analyzed by an enzymatic method.[10)] The defatted samples were incinerated at 600–800°C in an electric ash apparatus, then dissolved in 6 N HCl containing 1.5% $LaCl_3$. The Ca and P contents in the solution were also measured using the same methods as for the serum sample described above. For determination of elastic Ch, Ca and P, the other half of the freeze-dried aorta was fractionated by a modified version of the method described in a previous report.[11)] That is, a small piece of the aorta was suspended in water and then autoclaved. The supernatant and residue (elastin fraction) were separated by autoclaving. The elastin fraction (fr.) was freeze-dried, and the total lipids in the elastic fr. were extracted; then the elastic cholesterol was measured by the same method as the aortic Ch sample. The defatted residue (elastic fr.) was incinerated, and the Ca and P in the residues were also measured with the same methods used for the aortic ash sample. Statistical analysis results were expressed as mean ± S.D. All analyses were done using SPSS statistical soft-ware. When analysis of variance (ANOVA) revealed $p < 0.05$, data were further analyzed using Sheffe's multiple range test. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Previously, we reported that the concentration of the total polyphenols in the CLP was 49.5% as determined by the Prussian blue, using epicatechin as the standard.[4,5)] And the concentration of epicatechin, catechin and their oligomers, procyanidine B_2, procyanidine C_1, cinnamtannin A_2, in CLP were 1.83%, 0.46%, 1.69%, 2.37%, 2.01%, respectively, using HPLC.[12)] Xanthin derivatives such as caffeine and theobromine were not detected. In the previous basic experiment,[8)] the vitamin E deficient diet treatment group containing 0.5% and 1.0% CLP, showed reduction of lipid peroxides, but not the 0.25% CLP group. Therefore, the vitamin E deficient diet containing 0.5% CLP was used in this experiment. CLP did not have an adverse effect on the growth or clinical indices of the hepatic condition as previously described in our report.[3)] There-

Table 2. Effect of Cacao Liquor Crude Polyphenols (CLP) on Cholesterol (Ch), Calcium (Ca) and Inorganic Phosphorus (P) Contents in the Serum, Aorta and Aortic Elastin Fraction Obtained from Experimental Arteriosclerosis Induced by Vitamin D_2 with Vitamin E Deficiency and an Atherogenic Diet for 6 Weeks

	N	D	CLP
n	8	8	7
Serum Glucose (mg/dl)	104 ± 23	107 ± 9	111 ± 11
Serum Ch (mg/dl)	72 ± 19	729 ± 248*	939 ± 231
Serum TG (mg/dl)	79 ± 25	83 ± 17	89 ± 12
TBARS (nmol of MDA/ml)	5.4 ± 0.7	9.1 ± 4.2*	6.1 ± 1.8#
Serum Ca (mg/dl)	12.5 ± 1.2	13.2 ± 0.5	13.4 ± 0.8
Serum P (mg/dl)	11.1 ± 1.8	7.6 ± 0.7	8.1 ± 0.9
Aortic Ch (μg/mg)	2.5 ± 0.5	4.0 ± 1.3*	3.7 ± 0.4
Aortic Ca (μg/mg)	0.3 ± 0.1	86.2 ± 18.4*	27.9 ± 33.6#
Aortic P (μg/mg)	0.4 ± 0.3	121.9 ± 23.6	146.8 ± 63.4
Elastin Ch (μg/mg)	2.5 ± 0.8	3.3 ± 1.5	2.4 ± 0.7
Elastin Ca (μg/mg)	0.2 ± 0.1	182.0 ± 42.7*	48.4 ± 49.4#
Elastin P (μg/mg)	0.9 ± 0.3	291.4 ± 59.0*	183.4 ± 77.2#
Body weight (g, 0 week)	272 ± 6	268 ± 7	274 ± 4
Body weight (g, 6 weeks)	370 ± 17	275 ± 13*	279 ± 9

Each value is the mean ± S.D. *$p < 0.05$, compared with the value for N. *$p < 0.05$, compared with the value for the D group. TBARs: thiobarbituric acid reactive substance. MDA: Malondialdehyde. The Ch content in the aorta or aortic elastin fr. was expressed as μg Ch in the aorta or aortic elastin fr./mg dry defatted weight. The Ca or P content in the aorta or aortic elastin fr./mg dry defatted weight.

fore, it seems likely that there was no growth retardation or general health disturbance caused by CLP. The effect of vitamin D_2 (2.5×10^5 I.U./kg b.w.) with or without CLP on various parameters is illustrated in Table 2. The serum Ch was significantly higher for 6 weeks in the D group than in the N group (Table 2). The serum TBARS levels were significantly higher in the D group than in the N group (Table 2). The aortic and aortic elastin Ch were higher or tended to increase in the D group compared to the N group, which is similar to that phenomena previously described in our reports[2,3,11] (Table 2). The serum Ca concentrations of the three groups were similar. However, the Ca and P contents of the aorta or the aortic elastin fr. were significantly higher in the D group than in the N group (Table 2). The increases of Ca and P in the aorta or the increases of Ca and P in the aortic elastin fr. reflected the experimental arteriosclerosis induced by vitamin D_2 and the atherogenic diet prepared by a vitamin E deficient diet. The lipid and mineral depositions in the aorta and aortic elastin fr. were similar to those in human atherosclerosis as described by Kramsch *et al.*[13] Recently, Bennani-Kabchi *et al.*[14] reported that vitamin D_2 and a high Ch diet administration induced advanced atherosclerotic lesions in the arterial wall with elevated oxidized LDL and calcification. These authors estimated that deposition of Ch, Ca and P in the aorta was affected by increases in lipid peroxides produced by vitamin D_2, a vitamin E deficiency, and the atherogenic diet. The Ca content in the aorta and the Ca and P contents in the aortic elastin fr. of the CLP group were significantly lower than those of the D group (Table 2). From these observations, it would appear that CLP inhibited the accumulation of Ca in the aorta. CLP also inhibited the increase of Ca and P in the aortic elastin fr. induced by vitamin D_2 with a vitamin E deficiency and an atherogenic diet. In addition, the Ch contents in the liver and heart were significantly higher in the D group than in the N group. The Ca and P contents in the kidney were also significantly higher in the D group than in the N group (Table 3). The increase in the Ch or Ca and P in the soft tissues reflects the experimental arteriosclerosis induced by vitamin D_2 with a vitamin E deficiency and an atherogenic diet. The Ch contents in the heart of the CLP group were significantly lower than those of the D group. The Ca in the kidney of the CLP group was significantly lower than those of the D group. From these observations, it would appear CLP also inhibited the deposition of Ch in the heart or Ca in the kidney, induced by vitamin D_2 with a vitamin E deficiency and an atherogenic diet (Table 3). In an *in vitro* experiment, vitamin E, vitamin K_1 and vitamin K_2 also showed lipid radical scavenging activity as stated in our previous report.[3] The lipid radical scavenging activity of CLP was similar to that of vita-

Table 3. Effect of Cacao Liquor Crude Polyphenols (CLP) on Cholesterol (Ch), Calcium (Ca) and Inorganic Phosphorus (P) Contents in the Heart, Kidney and Liver Obtained from Experimental Arteriosclerosis Induced by Vitamin D_2 with Vitamin E Deficiency and Atherogenic Diet for 6 Weeks

	N	D	CLP
n	8	8	7
Heart Ch (μg/mg)	4.4 ± 2.5	7.6 ± 2.8*	4.7 ± 0.9$^{\#}$
Heart Ca (μg/mg)	0.1 ± 0.1	0.8 ± 0.6	1.1 ± 1.3
Heart P (μg/mg)	1.5 ± 1.3	3.1 ± 2.7	2.8 ± 1.5
Kidney Ch (μg/mg)	19.5 ± 3.5	18.9 ± 5.0	19.0 ± 3.9
Kidney Ca (μg/mg)	0.1 ± 0.1	4.3 ± 3.5*	1.9 ± 1.2$^{\#}$
Kidney P (μg/mg)	1.5 ± 0.2	13.7 ± 4.6*	14.1 ± 6.9
Liver Ch (μg/mg)	11.8 ± 1.5	313.7 ± 123.8*	252.5 ± 92.5
Liver Ca (μg/mg)	0.06 ± 0.02	0.07 ± 0.02	0.07 ± 0.02
Liver P (μg/mg)	2.6 ± 0.6	1.8 ± 0.4	1.7 ± 0.3

Legends for Table 3 refer to the legends in Table 2. Each value is mean ± S.D. *$p < 0.05$, compared with the value for N. $^{\#}p < 0.05$, compared with the value for the D group.

min E in our previous report.[4–8] In our previous report,[8] the increase of lipid peroxide in the liver, kidney, heart, brain and plasma were inhibited in a dose dependent manner as a results of supplementation of the vitamin E-deficient diet with 0.25, 0.5, or 1.0% CLP. Additionally, CLP also inhibited increased serum lipid peroxides in the D group (Table 2). These phenomena suggested that the inhibitory effects of CLP on the accumulation of Ca or P in the aorta and kidney, induced by the vitamin D_2 and an atherogenic diet, were partially due to an anti-oxidant activity. Oxidative stress has been thought to represent one of the mechanisms or etiological factors leading to chronic disease such as arteriosclerosis, calcinosis and diabetes mellitus.

Therefore, anti-oxidant nutrients against free radical reactions such as CLP, vitamin E and vitamin K_1 suggested a possible protective role against arteriosclerosis with calcification.

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