Suppressive effects of cacao liquor polyphenols (CLP) on LDL oxidation and the development of atherosclerosis in Kurosawa and Kusanagi-hypercholesterolemic rabbits

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Received 16 July 2004; received in revised form 19 November 2004; accepted 3 December 2004
Available online 19 January 2005

Abstract

We investigated the properties of cacao liquor polyphenols (CLP), which have an antioxidative effect on low-density lipoprotein (LDL) and an anti-atherosclerotic effect in the spontaneous familial hypercholesterolemic model, the Kurosawa and Kusanagi-hypercholesterolemic (KHC) rabbit. After 6 months of dietary administration of CLP at 1% (w/w) to the KHC rabbits, a higher total cholesterol concentration was observed in the treatment group compared to the control group. However, no other effects were noted in lipid profiles in plasma or lipoproteins. The plasma concentration of thiobarbituric acid reactive substances (TBARS), which is a lipid-peroxidation index, was significantly decreased 1 month after the start of CLP administration compared to that of the control group. The antioxidative effect of CLP on LDL was observed from 2 to 4 months of administration. The area of atherosclerotic lesions in the aorta in the CLP group (32.01 ± 1.58%) was significantly smaller than that in the control group (47.05 ± 3.29%), and the tissue cholesterol and TBARS concentrations were lower in the CLP group than in the control group. The anti-atherosclerotic effect of CLP was confirmed both rheologically and histopathologically. An in vitro study using KHC rabbit-derived LDL revealed that CLP significantly prolonged the lag time of LDL oxidation that was induced by a lipophilic azo-radical initiator, 2,2′-azobis(4-methoxy)-2,4-dimethylvaleronitrile (V-70), or Cu2+ from a low concentration of 0.1 μM/mL. The antioxidative effect of CLP was superior to those of the well-known antioxidative substances, vitamin C, vitamin E and probucol. Therefore, CLP suppressed the generation of atherosclerosis, and its antioxidative effect appeared to have an important role in its anti-atherosclerotic activity.

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Keywords: Antioxidation, Atherosclerosis, Cacao liquor polyphenols, Kurosawa and Kusanagi-hypercholesterolemic (KHC) rabbit

1. Introduction

LDL oxidation is known to be one cause of atherosclerosis [1], and antioxidative substances are believed to suppress the onset and development of atherosclerosis. Many epidemiological studies have shown that the consumption of food (vegetables and fruit) or drinks that are abundant in polyphenols, which are antioxidative substances, can reduce the risks of mortality from cardiovascular diseases [2–5]. Non-clinical studies have also shown a suppressive effect of the polyphenols contained in various kinds of food.
food, as well as polyphenol extracts, in the development of atherosclerosis [6–8]. Cacao beans, which are the seeds of Theobroma cacao, are rich in polyphenols, including catechins and their oligomers [9–12]. Previously, we reported that cacao liquor is rich in polyphenols [11,12]. Raw beans are fermented, dried and ground to produce cacao liquor, which is the main ingredient of chocolate and cocoa powder. Arts et al. [13] reported that the pattern of consumption of chocolate in Germany led to a high dietary catechin intake of as much as 20%. The antioxidative effect of cacao polyphenols has been reported previously [12,14,15]. Additionally, a clinical study in healthy volunteers revealed that a daily intake of cocoa powder decreased the susceptibility of LDL to oxidation [16]. However, the suppressive effect of cacao polyphenols on the development of atherosclerosis remains to be examined. Herbal medicines containing flavonoids, which are natural antioxidative substances, have been reported to show anti-atherosclerotic activity without decreasing the plasma cholesterol concentration in the KHC rabbit [17,18]. This animal model of familial hypercholesterolemia (FH) exhibits hypercholesterolemia from birth, due to lack of LDL receptors, and spontaneously develops atherosclerosis [19]. In the present study, we aimed to determine the resistance of crude polyphenols extracted from cacao liquor to LDL oxidation, and to examine its anti-atherosclerotic effect during 6 months of administration to KHC rabbits.

2. Materials and methods

2.1. Materials

A crude fraction of cacao liquor polyphenols (CLP) was prepared in accordance with the method reported previously [15]. Cacao liquor was defatted with n-hexane and extracted with 80% (v/v) ethanol. The extract was applied to a chromatographic column (Diatron HP 2MG; Mitsubishi Kasei, Japan) and freeze-dried. The total polyphenol content in the final product was determined as 75.3% using the Prussian blue method with epicatechin as a standard [20]. The composition of polyphenol fraction derived from cacao liquor is shown in Table 1.

Table 1

<table>
<thead>
<tr>
<th>Component</th>
<th>Content% (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total polyphenols</td>
<td>76.3</td>
</tr>
<tr>
<td>Catechin</td>
<td>0.70</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>2.78</td>
</tr>
<tr>
<td>Procyanidin B2</td>
<td>2.57</td>
</tr>
<tr>
<td>Procyanidin C1</td>
<td>3.61</td>
</tr>
<tr>
<td>Cinnamtannin A1</td>
<td>3.06</td>
</tr>
<tr>
<td>Caffeine</td>
<td>N.D.</td>
</tr>
<tr>
<td>Theobromine</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D.: not detectable.

2.2. Animals and diets

Seven males and eight females homozygous KHC rabbits (body weight range: 1.75–2.40 kg) were purchased from Japan Laboratory Animals Inc. (Tokyo, Japan) at the age of 3 months. The animals were housed individually under controlled environmental conditions (room temperature: 21–25 °C; relative humidity: 45–65%; lighting: 12-h dark/light cycle; ventilation: more than 10 cycles/h). The control animals received commercially available standard diets (RC-4; Oriental Yeast, Tokyo, Japan). The diets for the animals in the treatment group were prepared in Oriental Yeast by mixing CLP into the standard diet at 1%. The mixed diets were stored at −20 °C until immediately before feeding.

2.3. Study design

The rabbits were divided into two groups so as to avoid group differences in body weight, plasma total cholesterol and triglyceride levels: the control group contained four males and four females, and the treatment group comprised three males and four females. The animals received 100 g of the respective diets per day and were provided with tap water ad libitum. All of the rabbits were clinically observed and their food consumption was measured daily. Body weights, plasma lipid levels and LDL oxidation were measured 1 month after the initiation of the study and thereafter, at monthly intervals. At the end of the 6-month administration period the animals were euthanised by administering an overdose of pentobarbital.

2.4. Measurement of plasma lipid levels

Blood was collected from the auricular artery of each rabbit under EDTA–3K treatment (final concentration: 1 mg/mL) and centrifuged to obtain plasma. A portion of the plasma was ultracentrifuged to obtain very low-density lipoprotein (VLDL; d < 1.020 g/mL), LDL (d = 1.019–1.063 g/mL) and high-density lipoprotein (HDL; d > 1.064 g/mL). Total cholesterol, triglyceride and phospholipid levels in the plasma and in each lipoprotein fraction were measured enzymatically using COBAS FARA II (Roche, Switzerland) and commercially available kits (Wako Pure Chemical, Osaka, Japan). The concentration of TBARS in the plasma was measured as the production of thiobarbituric acid reactive substances (TBARS). The results of this analysis are shown in Table 2.

Table 2

<table>
<thead>
<tr>
<th>Component</th>
<th>Value (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total choles terol</td>
<td>160.0±20.0</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>85.0±15.0</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>40.0±5.0</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>100.0±10.0</td>
</tr>
</tbody>
</table>

N.D.: not detectable.
2.5. Evaluation of the antioxidation of LDL

LDL oxidation was measured using the method of Esterbauer et al. [23] and Hirano et al. [24]. The LDL concentration was determined in a bicinchoninic acid assay using bovine serum albumin (Pierce Laboratories, Rockford, USA) as a standard.

The oxidant V-70 was added to the LDL fraction (final concentration of protein: 70 μg/mL) to a final volume of 200 μM and incubated at 37 °C. The formation of conjugated dienes was monitored at 234 nm using a Beckman Model DU-640 spectrophotometer in order to obtain the lag time, that is, the time period until the amount of conjugated dienes began to increase.

2.6. Determination of the lesioned area

The aorta was excised from the origin to the iliac bifurcation. After any 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 ratio of the area of the atherosclerotic lesions to the total area was calculated using image analysis (NIH Image 1.62; National Institutes of Health, USA).

2.7. Measurement of lipid contents in the aorta

The aorta was divided into three segments: the aortic arch (from the origin to the first intercostal arteries), the thoracic aorta (from the first to the eighth intercostal arteries) and the abdominal aorta (from the eighth intercostal arteries to the iliac bifurcation). Each segment was homogenized with 1.14% NaCl and incubated at 37 °C, and stretched at a speed of 4.17 mm/s until the tension reached 20–30 g, as reported previously [25]. Immediately after the stress–strain characteristics were tested, the strip was relaxed to the initial length, detached from the chucks and weighed precisely. The stress value (τ) was calculated using the following formula (assuming Poisson’s ratio of the vascular wall to be 0.5), τ = (1.06 × T × L₀ (1 + ΔL/L₀)) / (W₂ × ΔL), where L₀ is the initial length of the strip (cm) and the increment from the initial length (cm), respectively. Wall thickness (h) at the initial length was calculated using the following formula, h = (W₀ (1.06L₀ × W₂) / W₂), where W₀ is the width of the strip (cm). The incremental elastic modulus of the wall at a strain of 0.50 (EM₀.5) 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 was calculated as follows, EM₀.5 = (σ₀.55 – σ₀.45) / (ε₀.55 – ε₀.45), where σ₀.55 and σ₀.45 are the stress at strains of 0.55 and 0.45, respectively [25].

2.9. Histopathology

Each strip of the aorta that was used in the tensile test was fixed in 10% neutral buffered formalin solution. The strips were then embedded in paraffin, sectioned (thickness: 3 μm) and stained with hematoxylin–eosin or elastica van Gieson.

2.10. In vitro susceptibility of LDL to oxidation

Plasma was obtained from the blood collected under EDTA-3K treatment (final concentration: 1 mg/mL) from three male homozygous KHC rabbits (body weight range: 2.63–2.94 kg) aged 4–5 months. The plasma was ultracentrifuged to obtain LDL (d = 1.019–1.063 g/mL). LDL to which the oxidant CuCl₂ was to be added was dialyzed with phosphate buffered saline overnight. For measurement of the LDL-oxidation lag time, the final concentrations were set at 70 μg/mL for LDL, and at 200 and 10 μM for V-70 and CuCl₂, respectively. The test substances were added to the LDL preparation (70 μg/mL) and incubated at 37 °C for 10 min before the oxidants were added. The formation of conjugated dienes was then monitored at 234 nm using a Beckman Model DU-640 spectrophotometer. Absorbance was measured at 37 °C at 3-min intervals for up to 5 h. The final concentrations of the test substances were as follows: 0.01, 0.03, 0.1, 0.3 and 1.0 μg/mL for CLP and vitamin C; 0.03, 0.1, 0.3, 1.0 and 3.0 μg/mL for progbucol; and 0.1, 0.3, 1.0, 3.0 and 10.0 g/mL for vitamin E.

2.11. Statistical analysis

All data are expressed as the mean ± standard error (S.E.). The Student’s t-test was used to determine significant differences compared with the control group. A probability (P) value of <0.05 was considered to be statistically significant.
3. Results

3.1. General conditions

Throughout the study period, no changes were observed in clinical observations or food consumption. Body weight increased in both the control and CLP animals, and there was no significant difference between the two groups; the mean body weights were 2.17 ± 0.08 and 2.19 ± 0.03 kg before the start of study, and 3.19 ± 0.06 and 3.15 ± 0.05 kg after the 6-month administration period, respectively.

3.2. Plasma lipid levels

The plasma total cholesterol concentration in the CLP group was initially similar to that of the control group, but was significantly higher than that of the control group after 6 months of treatment. The total cholesterol level in the lipoprotein fractions (VLDL, LDL, and HDL) did not differ significantly between the CLP and control groups (Fig. 1). Similarly, there were no significant differences in triglyceride or phospholipid concentrations between the two groups. The level of plasma TBARS in the CLP group was significantly lower than that in the control group 1 month after the administration commenced (Fig. 2).

3.3. Antioxidative effect on LDL

The lag time was prolonged significantly in the CLP group 3- and 4-month after start of administration compared to that of the control group (Table 2).

3.4. Area of atherosclerotic lesions in the aorta

At each location examined, the percent area of the lesions compared to the total intimal area tended to be lower in the CLP group. The percent areas of the lesions in the whole aorta, the thoracic aorta and the abdominal aorta were significantly lower in the CLP group than in the control group (Table 3).

3.5. Lipid contents in the aorta

In the CLP group, the total cholesterol levels in the aorta were significantly lower at all locations than those in the control group. The level of TBARS tended to be relatively low in the thoracic aorta, and a significant decrease in this parameter was observed in the abdominal aorta in the CLP group compared to the control group (Fig. 3).
3.6. Static rheological properties of the aorta

The tension–strain and stress–strain curves in the circumferential strips excised from the ascending aorta and the descending portion of the aortic arch are shown in Figs. 4 and 5, respectively. There was no significant difference between the two groups in the tension value at each strain in the ascending aorta. However, the stress value was significantly larger in the CLP group compared to that in the control group in the descending portion of the aortic arch, both the tension and stress values at each strain were significantly larger in the CLP group than those in the control group. The wall thickness of both the ascending aorta and the descending portion of the aortic arch was significantly smaller, and the value of EM0.5 was significantly greater, in the CLP group compared to the control group. In the two groups in the tension value at each strain in the ascending aorta, the stress value was significantly larger in the CLP group compared to that in the control group. In the descending portion of the aortic arch, both the tension and stress values at each strain were significantly larger in the CLP group than those in the control group. The wall thickness of both the ascending aorta and the descending portion of the aortic arch was significantly smaller, and the value of EM0.5 was significantly greater, in the CLP group compared to the control group (Table 4).

3.7. Histopathological analysis

Marked thickening of the tunica intima was observed in the wall of the aortic arch in the control group, which was mainly caused by the accumulation of foam cells; formation of a fibrous capsule and fragmentation of the internal elastic lamina were also observed. By contrast, fewer foam cells were observed in the CLP group compared to the control group, and the thickening of the tunica intima was relatively mild (Fig. 6). The effects of CLP were observed in both the ascending aorta and the descending portion of the aortic arch, and the effects were more evident in the descending portion of the aortic arch.

3.8. In vitro susceptibility of LDL to oxidation

Fig. 7 shows typical patterns of the formation of conjugated dienes with V-70 and Cu2+ after the addition of CLP to LDL. The addition of CLP prolonged the lag time of conjugated-diene formation in a dose-dependent manner. The addition of vitamin C, probucol or vitamin E had a similar effect on the lag time. However, the addition of probucol at the highest dose (3.0 µg/mL) shortened the lag time of V-70 oxidation. The lag time prolongation (%) with each antioxidative substance (the lag time with the addition of vehicle was designated as 100%). The concentrations of CLP, vitamin C, probucol and vitamin E at which the lag time of LDL oxidation was prolonged by 20% were 0.055, 0.120, 0.382 and 2.666 µM, respectively, with V-70, and 0.039, 0.117, 0.265 and 4.593 µg/mL, respectively, with Cu2+. CLP suppressed the LDL oxidation induced by either oxidant from the lowest dose.

4. Discussion

Chocolate and cocoa powder are produced from cacao beans, and are abundant in polyphenols, such as monomeric (+)-catechin and (-)-epicatechin, dimeric procyanidin B2, trimeric procyanidin C1 and tetrameric cinnamtannin A2 [10–12]. The antioxidative effect of cocoa polyphenols has been examined previously both in vitro and in vivo [11,12,14–16,26]. We found that cocoa powder enhanced LDL resistibility to oxidation and suppressed the development of atherosclerosis in KHC rabbits [27]. These effects might be attributable to polyphenols; however, cocoa powder contains many other effective substances and we were unable to determine the specific pharmacological activities of the polyphenols. Therefore, in the present study, we examined the effects of polyphenols on LDL susceptibility to

Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Aortic arch</th>
<th>Control</th>
<th>CLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wall thickness (µm) Ascending</td>
<td>157.6 ± 52.6</td>
<td>1248.1 ± 41.8</td>
<td>701.4 ± 71.4</td>
</tr>
<tr>
<td>EM0.5 (±106 dyn/cm²) Ascending</td>
<td>0.627 ± 0.054</td>
<td>0.871 ± 0.068</td>
<td>1.101 ± 0.144</td>
</tr>
</tbody>
</table>

All values are mean ± S.E. Numbers of rabbits in the groups are: n=8 (control group), n=7 (CLP group). EM0.5: elastic modulus of the wall at a strain of 0.50, significantly different from control.

* P < 0.05 (Student’s t-test).

** P < 0.01 (Student’s t-test).

Table 3

<table>
<thead>
<tr>
<th>Area of atherosclerosis (%)</th>
<th>Aortic arch</th>
<th>Control</th>
<th>CLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic arch</td>
<td>75.6 ± 4.70</td>
<td>68.03 ± 3.76</td>
<td>71.4 ± 4.45</td>
</tr>
<tr>
<td>Thoracic aorta</td>
<td>36.54 ± 3.86</td>
<td>19.68 ± 3.89</td>
<td>26.3 ± 1.32</td>
</tr>
<tr>
<td>Abdominal aorta</td>
<td>23.95 ± 3.49</td>
<td>14.32 ± 1.48</td>
<td>26.3 ± 1.22</td>
</tr>
<tr>
<td>Whole aorta</td>
<td>47.05 ± 3.29</td>
<td>32.01 ± 1.58</td>
<td>26.3 ± 1.22</td>
</tr>
</tbody>
</table>

All values are mean ± S.E. Numbers of rabbits in the groups are: n=8 (control group), n=7 (CLP group), significantly different from control.

* P < 0.05 (Student’s t-test).

** P < 0.01 (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 CLP. Data are expressed as mean ± S.E. Numbers of rabbits in the groups are: n = 8 (control group, ◦), n = 7 (CLP group, ●). Significantly different from control, *P < 0.05, **P < 0.01 (Student’s t-test). L<sub>0</sub>: Initial length, ΔL: Increment from the initial length.

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 CLP. Data are expressed as mean ± S.E. Numbers of rabbits in the groups are: n = 8 (control group, ◦), n = 7 (CLP group, ●). Significantly different from control, *P < 0.05, **P < 0.01 (Student’s t-test). L<sub>0</sub>: Initial length, ΔL: Increment from the initial length.

Fig. 6. Microphotographs of the descending portion of aortic arch in KHC rabbits with elastica van Gieson stain. Bar = 100 μm. (A) A control animal. Thickening of the tunica intima mainly due to accumulation of a large amount of foam cells is observed. Formation of the fibrous capsule and fragmentation of the internal elastic lamina are also observed in the wall of the aorta. (B) A CLP treated animal. Compared to the control animal, the CLP treated animal shows slight thickening of the tunica intima and very slight fragmentation in the internal elastic lamina.
oxidation and on atherosclerotic lesions during 6 months of dietary administration of crude polyphenols extracted from cacao liquor to KHC rabbits, from the viewpoints of biochemistry, histopathology and rheology.

Our results showed that the development of atherosclerosis was suppressed without a decrease in plasma lipid concentrations in CLP-administered KHC rabbits. The total cholesterol level had increased significantly after 6 months of administration, but there was no obvious difference in the cholesterol content in the lipoprotein fractions between the CLP-treated and control groups. The increase in the total cholesterol concentration in the CLP group might have been incidental. Polyphenols have been reported to decrease plasma cholesterol levels in cholesterol-fed rats [28,29]. However, in the present study at least, CLP did not decrease the plasma cholesterol concentration of KHC rabbits.

By contrast, CLP administration increased the resistance of LDL to oxidation and significantly decreased the concentration of plasma TBARS, which is a marker for lipid peroxidation, although this was observed only during the early stage of administration. The TBARS content in the aorta was also decreased at the end of the administration period. Polyphenols must be absorbed into the bloodstream in order to exert their antioxidative activity. A recent report revealed that catechins were absorbed efficiently from the gastrointestinal tract [30]. Moreover, a correlation between the antioxidative effect of cocoa powder and the plasma concentration of epicatechin was reported in a study using cocoa powder-fed rats [26,31]. In healthy volunteers, epicatechin and its metabolites reached their concentration peaks in the plasma 1 or 2 h after the consumption of cocoa drinks or chocolate [32,33]. In the present study, the plasma polyphenol concentrations were not determined in the CLP-fed KHC rabbits. However, in vitro, the addition of CLP at a dose of 0.055 µg/mL to LDL prolonged the lag time by 20%, which was similar to the in vivo result.

We concluded that polyphenols would be absorbed at a dose showing equivalent activity to that of 0.055 µg/mL of CLP.

Previous in vitro studies reported that cacao polyphenols trapped superoxide and hydroxy radicals [11,34] and suppressed oxidation stress [12]. In the present study, the in vitro antioxidative effect of CLP on KHC rabbit-derived LDL was dose dependent for the two oxidants, V-70 and Cu²⁺, which have the different radicals. The antioxidative activity of CLP was stronger than those of the lipid-soluble antioxidants vitamin E and probucol, and was equivalent to that of the water-soluble antioxidant vitamin C. The localization of antioxidative substances depends on their properties (that is, their hy-
water-soluble antioxidants exist in the aqueous phase and lipid-soluble antioxidants exist in the lipid phase of cell membranes or lipoproteins, both of which trap radicals. Yamashiki et al. [6] showed that in vitro, the addition of grape-seed polyphenols suppressed LDL oxidation in human plasma, although they reported no significant effect on LDL that was isolated from the plasma and oxidized. This result suggests that water-soluble polyphenols are not accumulated in LDL; rather, they suppress LDL oxidation in the plasma or aqueous phase of the tissue fluid of the vascular wall. This might also be the case for CLP. Probucol shortened the lag time at a high concentration, which is consistent with the data reported by Hirano et al. [24], and it was considered that probucol acted as a pro-oxidant. CLP extended the lag time by up to 603% at the highest dose (1.0 μg/mL) and no shortening of the lag time was observed. A previous report showed an interaction between lipid- and water-soluble antioxidants in trapping radicals [24]. This activity of the lipid-soluble antioxidant was recovered by the water-soluble antioxidant. However, various aspects of the activities of polyphenols remain unclear and await future clarification.

The so-called oxidation hypothesis for the mechanism of atherosclerosis states that LDL penetrates through the vascular endothelial cell layer to the tunica intima, where it is oxidized [1]. Oxidized LDL accelerates the infiltration of monocytes into the tunica intima, which results in the stagnation of monocyte-derived macrophages. The macrophages then take up oxidized LDL and are transformed into foam cells. Accumulated foam cells develop into fatty streaks during the early stage of atherosclerosis. The oxidation of LDL in the vascular wall is also considered to be an essential step in the generation of atherosclerotic lesions in KHC rabbits. In the present study, the decrease in TBARS levels was more obvious in the arteries than in the plasma of CLP-fed KHC rabbits. Correspondingly, a marked decrease was observed in the number of foam cells in the tunica intimina, along with a decrease in the tissue cholesterol content. These results are consistent with those reported for the studies with grape-seed polyphenols [6]. In mice orally administered with labeled procyanidin, radioactivity was reported to be 5.7-fold higher in the arteries than in the blood [35]. Accordingly, CLP or its active metabolites were believed to suppress LDL oxidation in the vascular wall, thereby suppressing the transformation of macrophages into foam cells.

Substantial atherosclerotic lesions were observed in the aorta of control KHC rabbits. These lesions resulted from intimal thickening, which was mainly induced by foam cells, and comprised 47.05% of the total area of the aorta. In the CLP-fed KHC rabbits, the area of the atherosclerotic lesions was significantly reduced to 32.01% (P < 0.01) of the total area of the aorta (Table 3). The anti-atherosclerotic effect of CLP was clearly observed in the thoracic and abdominal aortas compared to that in the aortic arch. Correspondingly, the cholesterol content was low in the distal part of the vascular wall. It was reported previously that atherosclerosis in the aorta of KHC rabbits was generated in the aortic arch and the main arterial bifurcation, and subsequently extended to the peripheral parts with age [19]. We suggest that CLP consumption suppressed the development of atherosclerosis and a stronger effect was noted in the peripheral part in which lesions developed during the later phase. Regarding the static rheological properties of the aorta, the stress and EM0.5 values of the vascular wall were significantly larger, and the thickness of the wall was significantly smaller, in both the ascending aorta and the descending portion of the aortic arch, in the CLP group compared to the control group. The difference in these parameters between the two groups was relatively large in the peripheral part. This implies that the aortic wall was more viscoelastic in the control group compared to the CLP-fed group. Katsuda et al. [25] suggested that during the process of remodeling, the aortic wall underwent ‘softening’ due to the accumulation of foam cells at a relatively early stage of atherosclerosis, and thereafter the stiffness increased with the progression of fibrous proliferation or calcification. As the number of foam cells increased, the wall became viscoelastic at a relatively early stage of atherosclerosis. Similar results have been reported in cholesterol-fed cockerels [36] and rabbits [37]. CLP inhibited the accumulation of cholesterol-rich foam cells in the intimal layer, which was thought to lead to the less viscoelastic properties of the aortic wall.

Research into preventive and therapeutic measures for atherosclerosis has been performed in various animal models. Several studies using the WHHL rabbit, which is an FH animal model, reported that hypocholesterolemic agents possessing antioxidative activity, such as probucol [38,39] and fluvastatin (HMG-CoA inhibitor) [40], showed anti-atherosclerotic effects at high doses owing to their action in lowering plasma cholesterol levels, whereas they suppressed the development of atherosclerotic lesions at low doses owing to their antioxidative effects on LDL. Natural antioxidative substances, such as vitamin E [41] and taurine [42], and herbal medicines containing flavonoids [17,18] have been reported to exert anti-atherosclerotic effects by suppressing LDL susceptibility to oxidation without decreasing plasma cholesterol levels. Similarly, CLP suppressed lipid oxidation in the plasma and arteries without lowering the cholesterol level in the plasma of KHC rabbits, and decreased the number of foam cells in the thickened tunica intimina. The anti-oxidative effect of CLP is therefore considered to play an important role in the anti-atherosclerotic activity of CLP.

Cacao polyphenols have properties that are well suited to the maintenance of healthy blood vessels; for example, inhibition of platelet aggregation [43], vasodilative activity through controlling the levels of eicosanoids and NO [44,45], and regulation of cytokine production [46], as well as the antioxidative effect on LDL. These effects might also contribute to the anti-atherosclerotic effect observed in the present study.
In conclusion, CLP suppressed the development of atherosclerosis in the KHC rabbit, which is an FH model. The consumption of food or drinks produced from polyphenol-rich cacao beans, such as chocolate and cocoa powder, might therefore be beneficial in preventing the onset of atherosclerosis and cardiovascular diseases.

References


