Ingestion of cocoa ameliorates endothelial dysfunction in mesentery arterioles induced by high fat diet in rats: An in vivo intravital microscopy study

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**ABSTRACT**

**Aims:** Numerous clinical studies have reported that ingestion of cocoa has a therapeutic effect on hypertension. However, there is only limited information on the mechanism of ingestion of cocoa on arterioles, vessels that have a major role in determining blood pressure. In this study, we used intravital video-microscopy to evaluate the effect of cocoa consumption on the mesentery microcirculation of rats fed a high fat diet.

**Main methods:** The animals were allocated to 3 groups, and fed either a control diet, a high fat diet containing 15% lard, or the HFD with 2% cocoa (HFD-C) for 6 weeks. At the end of the experimental period, the mesentery was spread in a chamber, and the vessels were treated topically with phenylephrine, acetylcholine, or papaverine. The vascular responses to phenylephrine, acetylcholine-dependent vasodilatation and endothelium-independent vasodilatation were calculated by the diameter of the mesentery artery with each treatment.

**Key findings:** Topical treatment of mesenteric arterioles with acetylcholine caused a significantly greater response in the control compared with the HFD group. In the HFD-C group, acetylcholine-dependent vasodilation was decreased marginally. Similarly, rats in the HFD group showed a significant reduction in vascular sensitivity to phenylephrine compared with the control group. However, there was no significant difference between the control and HFD-C groups. The induction of endothelial-independent arterial dilation was reduced slightly in the HFD group.

**Significance:** Our results suggest that one of the hypotensive mechanisms of cocoa is due to amelioration of endothelial dysfunction in arterioles induced by an inappropriate diet.

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Methods

Materials

Acetylcholine chloride, phenylephrine, papaverine, and Krebs–Ringer bicarbonate buffer were purchased from Sigma Chemicals (St. Louis, MO, USA). All the chemicals were dissolved in distilled water at a concentration 1,000 times higher than the final concentration and suffused to the mesenteric vascular bed in Krebs–Ringer buffer. The plasma levels of triglyceride and total cholesterol were determined using commercial available kits (Wako, Tokyo, Japan).

Animals and diets

The study was approved by the Animal Care and Use Committee of Shibaura Institute of Technology. All animals received humane care under the guidelines of this institution.

Male Wistar rats weighing 150–170 g were obtained from Saitama Experimental Animal Supply (Tokyo, Japan). The rats were kept in a room at a regulated temperature of 23–25 °C and controlled lighting (12-h light and dark cycles). The basal diet (i.e., the diet for control group) was MF obtained from the Oriental Yeast, Co. Ltd., Tokyo, Japan. The high fat diet (HFD) was MF containing 15% lard, while a HFD containing 2% cocoa (HFD-C) was prepared using cocoa provided by Meiji Co., Ltd. (Tokyo, Japan). The concentration of the cocoa powder used in this study is shown in Table 1. The concentration of polyphenols in cocoa was determined by the Prussian blue method as described previously (Osakabe et al., 1998), while the concentration of each flavan 3-ol in cocoa powder was measured by an HPLC method (Natsume et al., 2000).

Experimental procedures

Twenty four animals were fed the basal diet for 4 days and then allocated to three groups, with each group being fed either the basal (n = 8), HFD (n = 8) or HFD-C (n = 8) diet for 6 weeks. At the end of the experimental period, the animals were anesthetized using urethane (1 g/kg, s.c.), followed by measurement of blood pressure using the tail-cuff method (BP-98A Softron, Tokyo Japan). Blood samples were collected from the tail vein using heparinized capillary tubes. After centrifugation, the plasma was separated and stored at −80 °C for analysis of triglyceride and total cholesterol levels. To observe the microcirculation, a mid-line abdominal incision was made and the mesentery exteriorized and spread carefully over a plastic chamber. The chamber was connected to a reservoir that allowed continuous superfusion of the mesentery with Krebs–Ringer bicarbonate buffer (pH 7.3–7.4) at 37 °C. The Krebs–Ringer solution was aerated with 95% N₂–5% CO₂ using a peristaltic pump. After a post-surgical equilibration period of 30 min, a single unbranched arteriole with a resting inner diameter of 15–30 μm was selected from the microscopic images. The vessels were pre-contracted by topical treatment with 0.3 × 10⁻⁶ mol/l phenylephrine (PE). To obtain maximal acetylcholine (Ach)-dependent responses, 10 × 10⁻⁶ mol/l of Ach was administrated topically to the microvascular field. Finally, the mesentery was treated topically with 1 × 10⁻³ mol/l papaverine (PP), an endothelium-independent vasodilator, to examine maximum vasodilatation. The concentration of all chemicals added to the tissue chamber was determined by preliminary experiments (data not shown). The microcirculation was visualized by placing the chamber on a three-way movable stage and the mesentery transilluminated with a 150-W halogen light. The microcirculation was observed using an intravital microscope (MSA, Olympus) equipped with a charge-coupled device video camera (DXC-107S, Sony, Tokyo) that we had developed previously. The images of the microcirculation were displayed on a high resolution television monitor and stored on video for off-line analysis. The inner diameters of the vessels were measured as a video image with 8-bit gray levels at 512 × 512 pixels. Each vessel was measured three times at 1 second intervals using different images of the same vessel. A scheme of the equipment is shown in Fig. 1.

Data analysis and statistical methods

The vasoactive responses were expressed as percent changes in dilator capacity according to the formula: [(DSS − DPE) - (DPP − DPE)] × 100 for phenylephrine, and [(DACH − DPE) - (DPP − DPE)] × 100 for acetylcholine. Endothelial-independent vasodilatation relative to the steady-state and maximal diameters was expressed as [DPP − (DACH × DPP)] × 100, where DSS represents between steady-state diameter, DACH the diameter 5 min after application of Ach, DPE the pre-contracted diameter with PE, and DPP the maximal diameter at the end of the experiment following administration of PP.

Nutritional composition of cocoa powder.

<table>
<thead>
<tr>
<th>Nutrients</th>
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<tbody>
<tr>
<td>Protein</td>
<td>21.40</td>
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<tr>
<td>Fat</td>
<td>12.10</td>
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<td>Sugar</td>
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<td>Water</td>
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</tr>
<tr>
<td>Caffeine</td>
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<tr>
<td>Theobromin</td>
<td>2.21</td>
</tr>
<tr>
<td>Total polyphenols</td>
<td>7.25</td>
</tr>
<tr>
<td>(+)-catechin</td>
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<tr>
<td>(−)-epicatechin</td>
<td>0.68</td>
</tr>
<tr>
<td>procyanidin B2</td>
<td>0.30</td>
</tr>
<tr>
<td>procyanidin C1</td>
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<tr>
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The data were expressed as the mean and standard deviation. The analyses were performed using the SPSS statistical software package (SPSS Inc., Chicago, IL, USA). Comparisons of the various groups were carried using one-way ANOVA, followed by Tukey’s test.

**Results**

Fig. 2 shows a video image of the microcirculation in mesentry visualized using intravital video-microscopy. The solid arrow indicates an arteriole, the dotted arrow indicates a venula.

A single unbranched arteriole with a resting inner diameter of 15–20 μm (18.5 ± 2.18 μm) was selected for analysis.

**Acetylcholine-dependent vascular dilatation**

Fig. 3 shows acetylcholine-dependent vascular dilatation, following treatment with Ach expressed as a percentage of maximal dilatation. A high level of vasodilatation was detected in the control group (89.8 ± 11.0%). The acetylcholine-dependent response was reduced significantly in the HFD group (38.5 ± 19.4%, p < 0.01), and showed a significantly different than the response in the control group. Blood pressure showed significant increases in the HFD group, however, this elevation was not observed with the ingestion of cocoa. Feeding of the experimental diets did not influence either fasting plasma triglyceride or total cholesterol levels.

**Endothelial independent vasodilatation**

Fig. 4. Endothelial-independent vasodilatation, expressed as the percentage diameter following PP treatment minus the maximum diameter following Ach is shown in Fig. 5. Endothelial-independent vasodilatation was decreased slightly in the HFD group (13.6 ± 3.8%), although this was not significantly different than the response measured in the control group (20.4 ± 7.6%). Vasodilatation was only marginally lower in the HFD-C group (18.8 ± 5.2%) compared to the controls.

**Vascular response to phenylephrine**

The vascular responses of the arterioles to phenylephrine, expressed as a percentage of maximal dilatation, are shown in Fig. 4. In the control group, the vascular response to PE was almost 40% (38.5 ± 20.6%). Compared to the control group, the HFD group showed a significant decrease in response to PE (12.5 ± 8.3%, p < 0.01), whereas the HFD-C group did not (45.1 ± 26.4%). These findings indicate that the cocoa had normalized the response.

**Discussion**

The possibility that polyphenols can prevent cardiovascular disease has been reported (Arts et al., 2001a,b). In our previous animal studies in Kurosawa and Kusanagi-hypercholesterolemic rabbits, a model of spontaneous familial hypercholesterolemia, feeding of cocoa or its flavan-3-ol fraction for 6 months resulted in a significant reduction in the formation of atherosclerotic lesions (Kurosawa et al., 1998). Numerous clinical studies have reported that cocoa has a protective effect on cardiovascular disease.
hypotensive activity in patients with mild hypertension. Moreover, several meta-analysis confirmed the blood pressure lowering capacity of flavan 3-ols-rich cocoa products in a large set of trials than previously reported (Taubert et al., 2007; Hooper et al., 2008; Ried et al., 2010; Shrine et al., 2011). However, the experimental effect of cocoa or its flavan 3-ols on arterioles has yet to be established. Compared to observations in animals fed the basal diet, we showed that 6 weeks of a HFD caused endothelial dysfunction in mesentery arterioles associated with vascular contiguous visceral fat. According to a previous report, prolonged ingestion of a high fat diet is required to detect endothelial dysfunction in the aorta. As our study was of shorter duration than this time period, our results suggest that mesentery arterioles may be more susceptible than the aorta to the effects of adipocytokines secreted by white adipocytes, such as TNF-α, angiotensin II, and IL-6. In contrast, insignificant endothelial-dependent vasorestriction was seen in animals fed a HFD with cocoa (Fig. 3).

The reduced response to PE induced by HFD was also not observed in animals fed a HFD with cocoa (Fig. 4). These results suggested that ingestion of cocoa may prevent endothelial dysfunction induced by a HFD.

Several other reports from in vitro and clinical studies have indicated that treatment with cocoa or its flavanol 3-OH fraction prevents endothelial dysfunction. In vitro studies in isolated aortic rings from experimental animals suggested that increased vasodilatation induced by flavan 3-ols (Magos et al., 2008; Okudan et al., 2011). Clinical studies in humans have also shown that flow-mediated dilatation of the brachial artery is enhanced by ingestion of cocoa rich chocolate (Heiss et al., 2003; Loffredo et al., 2011). The aim of these in vitro and clinical studies was to examine improvement in the responses of large blood vessels. In contrast, the purpose of the present study was to evaluate the responses in small vessels such as arterioles which have a major role in determining blood pressure. In general, it is difficult to detect alterations in arteriole responses, especially endothelial dysfunction induced by vasoconstrictor and vasodilator agents under physiological conditions. To observe such changes it is necessary to use intravital microscopy, as carried out in the current study. From this point of view, the present results provide a breakthrough for clarifying the mechanism of the hypotensive effect of cocoa or rich chocolate.

Recent studies suggested that supplementation with cocoa improves several risk factors for coronary heart disease, such as insulin sensitivity (Muniyappa et al., 2008; Grassi et al., 2005), LDL oxidation (Wan et al., 2001; Baba et al., 2007), and lower plasma cholesterol levels (Tokede et al., 2011). Prevention of endothelial dysfunction in arterioles may also be a major factor in the wide range of actions associated with ingestion of cocoa.

Conclusions

Our results suggest that one of the hypotensive mechanisms of cocoa is amelioration of endothelial dysfunction in arterioles, vessels that have a major role in determining the increase in blood pressure induced by an inappropriate diet.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

Acknowledgements

We thank Meiji Co. Ltd. for donation of the cocoa powder.

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Arts ICW, Hollman PCH, Feskens EJM, Bueno de Mesquita HB, Kromhout D. Catechin intake may explain the inverse relation between tea consumption and ischemic heart disease: the Zutphen Elderly Study. Am J Clin Nutr 2001a;74:227–32.


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