

# Polyphenols in *Theobroma Cacao* Ameliorate Microcirculation: *In vivo* Intravital Microscopic Observation in Rats

NAOMI OSAKABE\*

*Department of Life Science, Shibaura Institute of Technology, Saitama Japan*

## ABSTRACT

It has been reported that ingestion of cocoa or chocolate reduces blood pressure in the subjects with hypertension. To clarify the hypotensive activity of cocoa, we carried out the research on microcirculation using intravital microscopic technique. First, we estimated the influence of repeated treatment of cocoa on microcirculation. Male rats fed normal diet (ND) or 15% fat containing high fat diet (HFD) or 0.2% flavan-3-ols containing HFD for 6 weeks. Under anesthesia, the arteriole function of mesentery in rat was observed under physiological condition. Vasoconstriction induced by the phenylephrin was significantly reduced in the HFD group compared with that in ND group, there was not such change in the HFD with cocoa group. Endothelium dependent vasodilatation stimulated by acetylcholine was significantly decreased in the HFD group compared with that in ND group. On the other hand, endothelial dysfunction of arteriole induced by HFD was not shown in cocoa group. In addition, single oral administration of cocoa or its flavan 3-ols on microcirculation in skeletal muscle was estimated. Cocoa (100 mg/kg) or flavan-3-ols derived from cocoa (10 mg/kg) was administrated through a feeding tube to the animals, velocity of red blood cells in precapillary arteriole and the number of newly recruited capillary in cremaster muscle were significantly increased by the treatment of cocoa or flavon-3-ols compared with vehicle, accompany with elevation of heart rate and blood pressure. These research has revealed that the ingestion of cocoa improves endothelial dysfunction of arterioles which is major determining of blood pressure induced by the high fat diet and single oral administration of flavan 3-ols in cocoa altered microcirculation in skeletal muscle and general circulation. These acute effects might be related with the preventive action of flavan-3-ols on cardiovascular disease.

Key words: flavan 3-ols, microcirculation, intravital microscopy, endothelial dysfunction

## INTRODUCTION

Cacao beans, the seed of *Theobroma cacao*, are known to be rich in polyphenols, such as the flavan 3-ol monomers, (+)-catechin and (-)-epicatechin, and epicatechin oligomers as B-type flavan 3-ols linked by C4-C8 bonds<sup>(1-3)</sup>. Recent epidemiological evidence suggests that ingestion of flavan 3-ol monomer prevents coronary heart disease<sup>(4,5)</sup>. In addition, numerous studies support the possibility that flavan 3-ols in cacao prevent cardiovascular disease by improving blood flow<sup>(6,7)</sup> and platelet function<sup>(8,9)</sup>, and changing inflammatory responses in endothelial cells of blood vessels<sup>(10,11)</sup>. The hypotensive activity of cocoa ingestion has also been well investigated using experimental animal models and hypertension subjects. Taubert *et al.*<sup>(12)</sup> and Hooper *et al.*<sup>(13)</sup> carried out a meta-analysis of recent randomized controlled studies, they concluded that the consumption of cocoa reduce blood pressure. However, there is only limited information on the effect of cocoa or polyphenolic substances on arterioles, vessels that have a major role in determining blood pressure. In this paper, we evaluated the repeated treatment of cocoa consumption on the arteriole function of mesentery in rats using intravital video-microscopy under physiological condition. In addition, we estimated

the influence of single oral administration of cocoa or its flavan 3-ols on the microcirculation in cremaster muscle, which is a kind of skeletal muscle in rats.

## MATERIALS AND METHODS

### I. 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 suffused to the mesenteric vascular bed in Krebs-Ringer buffer. Flavan 3-ols fraction was provided by Meiji Co., Ltd (Tokyo, Japan), containing 72.4% polyphenols analyzed by prussian blue method. This polyphenol fraction included 4.56% (+)-catechin, 6.43% (-)-epicatechin, 3.54% procyanidin B2, 0.85% procyanidin B5, 2.36% procyanidin C1 and 1.45% cinnamtannin A2 analyzed by HPLC method.

### II. Animals and Diets

The study was approved by the Animal Care and Use

\* Author for correspondence. Tel: 81-48-720-6031;  
Fax: 81-48-720-6011; E-mail: nao-osa@sic.shibaura-it.ac.jp

Committee of Shibaura Institute of Technology. All animals received humane care under the guidelines of this institution. Male wistar rats weighing 150 - 170g 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).

### III. Experimental Procedures : Experiment 1

The animals were fed the basal diet for 4 days and then divided into three groups, with each group being fed either the basal, HFD or HFD-cocoa diet for 6 weeks. At the end of the experimental period, the animals were anesthetized using urethane (1g/kg, s.c.). After receiving a midline abdominal incision, the mesentery was exteriorized and carefully spread 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% N<sub>2</sub> - 5% CO<sub>2</sub>. After a post-surgical equilibration period of 30 min, a single unbranched arteriole with a resting inner diameter of 15 - 30µm was selected from the microscopic images. The vessels were pre-contracted by topical treatment with 0.3 µM phenylephrine (PE). To obtain maximal acetylcholine (Ach)-dependent responses, 10 µM of Ach was administrated topically to the microvascular field. Finally, the mesentery was treated topically with 1 mM papaverine (PP), an endothelium-independent vasodilator, to examine maximum vasodilatation. The concentration of these chemicals was determined by preliminary examinations (data not shown).

### IV. Experiment 2

The animals were fed the basal diet for 4 days and used for experiment. A gastric tube was indwelled under anesthesia using urethane (1 g/kg, s.c.) Cremaster muscle was exteriorized and spread over a plastic chamber as described above. After a post-surgical equilibration period of 15 min, a cover slip was placed on the cremaster muscle to prevent dehydration and hypoxia. A single unbranched arteriole with a resting inner diameter of 15 - 30 µm was selected from the microscopic images. After 10 min pre-observation, 100 mg/kg body weight of cocoa, or 10 mg/kg of flavan-3ols fraction orally treated to the animals with a gastric tube. The observation period was 20 min after treatment of the chemicals. Measurement of blood pressure and heart rate after administration of the chemicals was carried out by the tail-cuff method (BP-98A Softron, Tokyo Japan).

### V. Microscopic Analysis

The microcirculation was visualized by placing the chamber on a three-way movable stage and the mesentery or cremaster trans- illuminated with a 150-W halogen light. The microcirculation was observed with an intravital microscope (M5A, Olympus) equipped with a charge-coupled device video camera (DXC-107S, Sony, Tokyo) The images were displayed on a high resolution television monitor at a final magnification of ×1450 and stored on video for off-line analysis. The inner diameters of the vessels, RBC velocity, or newly recruited of capillary was 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 Figure 1.

### VI. Data Analysis and Statistical Methods

The vascular responses to phenylephrine were expressed as percent changes of dilator capacity according to the formula:  $\frac{DSS-DPE}{Dpp-DPE} \times 100$ . The acetylcholine responses of the vessels were expressed as  $\frac{DACH-DPE}{Dpp-DPE} \times 100$ , while endothelium-independent vasodilatation was expressed as  $\frac{Dpp - DACH}{Dpp} \times 100$ , where DSS represents steady-state diameter, DACH is the diameter 5 min after application of Ach, DPE is the pre-contracted diameter with PE, and DPP is the maximal diameter determined at the end of the experiment following administration of papaverine 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 two way-ANOVA, followed by Tukey's test.

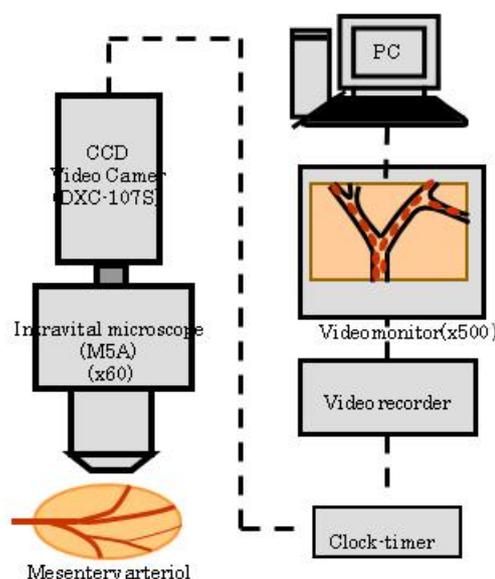


Figure 1. Scheme for intravital microscopic analysis system.

**Table 1.** Effect of repeated ingestion of cocoa on arteriol function in rat fed with HFD

	n	Acetylcholine dependent vasodilatation (%)	Vascular response to phenylephrine (%)	Endothlium independent vasodilatation
Control	8	88.3 ± 7.3	38.2 ± 16.3	21.3 ± 8.4
HFD	8	38.2 ± 12.5**	11.3 ± 8.5**	13.2 ± 5.2
2% cocoa Containing HFD	8	61.5 ± 15.3	42.4 ± 18.2	18.8 ± 6.3

**Table 2.** Influence of sigle oraladministration of coca or flavan 3-ols on microcirculation in rat cremaster muscle

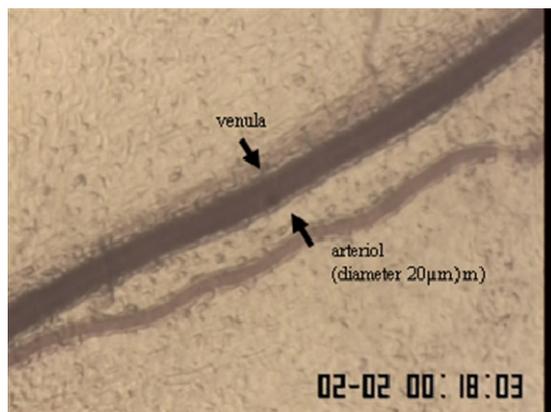
	n		Δ RBC velocity (μm/sec)	Δ newly recruited capillary (number)	Δ heart rate (beats/min)	Δ mean blood pressure (mmHg)
Vehicle	8	5 min	1.2 ± 2.6	4.3 ± 0.8	-2.1 ± 1.8	-4.6 ± 4.3
		20 min	-0.6 ± 3.1	8.6 ± 3.1	3.2 ± 8.2	2.1 ± 3.6
Cocoa	8	5 min	61.2 ± 23.3**	8.2 ± 1.3	7.3 ± 3.6	14.2 ± 9.6
		20 min	115.5 ± 26.2**	19.6 ± 5.6**	12.2 ± 4.2	30.2 ± 7.8*
Flavan	8	5 min	58.4 ± 29.7	7.6 ± 1.4	6.3 ± 2.8	13.8 ± 8.3
3-ols		20 min	98.6 ± 35.6	19.1 ± 4.2**	14.6 ± 3.1*	28.9 ± 8.8*

## RESULTS

### I. Experiment 1

#### (I) Acetylcholine- Dependent Vascular Dilatation

In Figure 2 shows the microcirculation in mesentery of rats. Table 1 shows acetylcholine-dependent vascular dilatation, expressed as percent of vasodilatation by the treatment of Ach to maximal dilatation. Approximately 90% vasodilatation was detected in the control group. The acetylcholine-dependent response was reduced significantly in the HFD group ( $p < 0.01$ ), and showed small, but insignificant decreases in the HFD-C group.

**Figure 2.** Microcirculation in mesentery of rat

#### (II) Vascular Response to Phenylephrine

The vascular responses of rat cremastar arterioles to phenylephrine, expressed as the percent of steady state vasodilatation to maximal dilatation, are shown in Table 1.

In the control group, the vascular response to phenylephrine was almost 40%. Compared to the control group, there was a significant decrease in the HFD group ( $p < 0.01$ ). This decrease was not observed in the HFD-C group.

#### (III) Endothelial Independent Vasodilatation

Endothelial-independent vasodilatation, expressed as the percent of the diameter following papaverine treatment minus the maximum diameter following Ach treatment to the maximum diameter, is shown in Table 1. Endothelial-independent vasodilatation in the HFD group was decreased slightly, but not significantly compared to the control group. This change was not observed in the HFD-C group.

### II. Experiment 2

#### (I) RBC Velocity in Arteriol

Table 2 shows RBC velocity in cremaster arteriole after oral administration of chemicals. RBC velocity was increased immediately after oral administration of cocoa or its flavan 3-ols. Significantly different between these two groups and vehicle was seen from 5 min after treatment through observation period.

#### (II) Newly Recruited Capillary

The number of newly recruited capillary in rat cremaster muscle was shown in Table 2. In increase in capillary was shown 5 min after administration of cocoa or flavan 3-ols. There were significantly different from vehicle from 10 min after treatment to the observation period.

**(III) Blood Pressure and Heart Rate**

Blood pressure and heart rate after oral administration of the chemicals were shown in Table 2. Even in 3 min after administration, elevation of blood pressure and heart rate was observed in cocoa or its flavan 3-ols group compared with vehicle, these increase was maintained at the end of observation.

**CONCLUSIONS**

In our results, the ingestion of cocoa improved endothelial dysfunction of arteriole in mesentery induced by the supplementation of high fat diet.

On the other hand, single oral administration of cocoa or its flavan 3-ols increased blood flow and number of newly recruited capillary in skeletal muscle accompany with elevation of heart rate and blood pressure. These acute effects might be related with the preventive action of flavan-3-ols on cardiovascular disease.

**ACKNOWLEDGMENTS**

The author would like to thank the Meiji Co. Ltd. for provide testing samples.

**REFERENCES**

1. Hammerstone, J. F., Lazarus, S. A., Mitchell, A. E., Rucker, R. and Schmitz, H. H. 1999. Identification of flavan 3-ol s in cocoa (*Theobroma cacao*) and chocolate using high-performance liquid chromatography/mass spectrometry. *J. Agric. Food Chem.* 47: 490-496.
2. Hatano, T., Miyatake, H., Natsume, M., Osakabe, N., Takizawa, T., Ito, H. and Yoshida, T. 2002. Proanthocyanidin glycosides and related polyphenols from cacao liquor and their antioxidant effects. *Phytochem.* 59: 749-758.
3. Sanbongi, C., Osakabe, N., Natsume, M., Takizawa, T., Gomi, S. and Osawa, T. 1998. Antioxidative Polyphenols Isolated from *Theobroma cacao*. *J. Agric. Food Chem.* 46: 454-457.
4. Arts, I. C. W., Hollman, P. C. H., Feskens, E. J. M., Bueno de Mesquita, H. B. and Kromhout, D. 2001. Catechin intake might explain the inverse relation between tea consumption and ischemic heart disease: the Zutphen Elderly Study. *Am. J. Clin. Nutr.* 74: 227-232.
5. Arts, I. C. W., Jacobs, D. R., Harnack, L. J., Gross, M. and Folsom, A. R. 2001. Dietary catechins in relation to coronary heart disease death among postmenopausal women. *Epidemiol.* 12: 668-675.
6. Heiss, C., Dejam, A., Kleinbongard, P., Schewe, T., Sies, H. and Kelm, M. 2003. Vascular effects of cocoa rich in flavan-3-ols. *JAMA* 290: 1030-1031.
7. Farouque, H. M., Leung, M., Hope, S. A., Baldi, M., Schechter, C., Cameron, J. D. and Meredith, I. T. 2006. Acute and chronic effects of flavanol-rich cocoa on vascular function in subjects with coronary artery disease: a randomized double-blind placebo-controlled study. *Clin. Sci.* 111: 71-80.
8. Keen, C. L., Holt, R. R., Oteiza, P. I., Fraga, C. G., Schmitz, H. H. 2005. Cocoa antioxidants and cardiovascular health. *Am. J. Clin. Nutr.* 81: 298S-303S.
9. Holt, R. R., Schramm, D. D., Keen, C. L., Lazarus, S. A. and Schmitz, H. H. 2002. Chocolate consumption and platelet function. *JAMA* 287: 2212-2213.
10. Schramm, D. D., Wang, J. F., Holt, R. R., Ensunsa, J. L., Gonsalves, J. L., Lazarus, S. A., Schmitz, H. H., German, J. B. and Keen, C. L. 2001. Chocolate flavan 3-ol s decrease the leuktriene-prostacyclin ratio in humans and human aortic endothelial cells. *Am. J. Clin. Nutr.* 73: 36-40.
11. Ale-Agha, N., Stahl, W. and Sies, H. 2002. (-)-Epicatechin effects in rat liver epithelial cells: stimulation of gap junctional communication and counteraction of its loss due to the tumor promoter 12-O-tetradecanoylphorbol-13-acetate. *Biochem. Pharmacol.* 63: 2145-2149.
12. Taubert, D., Roesen, R. and Schömig, E. 2007. Effect of cocoa and tea intake on blood pressure: a meta-analysis. *Arch. Intern. Med.* 167: 626-634.
13. Hooper, L., Kroon, P. A., Rimm, E. B., Cohn, J. S., Harvey, I., Le Cornu, K. A., Ryder, J. J., Hall, W. L. and Cassidy, A. 2008. Flavonoids-rich foods and cardiovascular risk: a meta-analysis of randomized controlled trials. *Am. J. Clin. Nutr.* 88: 38-50.