Original Research

Flavonoid-Rich Dark Chocolate Improves Endothelial Function and Increases Plasma Epicatechin Concentrations in Healthy Adults

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Background: Dark chocolate derived from the plant (*Theobroma cacao*) is a rich source of flavonoids. Cardioprotective effects including antioxidant properties, inhibition of platelet activity, and activation of endothelial nitric oxide synthase have been ascribed to the cocoa flavonoids.

Objective: To investigate the effects of flavonoid-rich dark chocolate on endothelial function, measures of oxidative stress, blood lipids, and blood pressure in healthy adult subjects.

Design: The study was a randomized, double-blind, placebo-controlled design conducted over a 2 week period in 21 healthy adult subjects. Subjects were randomly assigned to daily intake of high-flavonoid (213 mg procyanidins, 46 mg epicatechin) or low-flavonoid dark chocolate bars (46 g, 1.6 oz).

Results: High-flavonoid chocolate consumption improved endothelium-dependent flow-mediated dilation (FMD) of the brachial artery (mean change = 1.3 ± 0.7%) as compared to low-flavonoid chocolate consumption (mean change = −0.96 ± 0.5%) ($p = 0.024$). No significant differences were noted in the resistance to LDL oxidation, total antioxidant capacity, 8-isoprostanes, blood pressure, lipid parameters, body weight or body mass index (BMI) between the two groups. Plasma epicatechin concentrations were markedly increased at 2 weeks in the high-flavonoid group (204.4 ± 18.5 nmol/L, $p \leq 0.001$) but not in the low-flavonoid group (17.5 ± 9 nmol/L, $p = 0.99$).

Conclusion: Flavonoid-rich dark chocolate improves endothelial function and is associated with an increase in plasma epicatechin concentrations in healthy adults. No changes in oxidative stress measures, lipid profiles, blood pressure, body weight or BMI were seen.

INTRODUCTION

Several studies provide evidence suggesting that dietary flavonoids may be protective against cardiovascular disease [1–5]. Other epidemiological studies demonstrate that flavonoid-rich diets high in fruits and/or vegetables also lower the risk of coronary heart disease [7–9]. This beneficial cardiovascular effect of flavonoids has been attributed to their natural antioxidant properties and their role in conserving tocopherols in biological membranes [10].

Recent advances in analytical methodology have shown that dark chocolate, a food of plant origin (*Theobroma cacao*), is a

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rich source of flavonoids [11–13]. Cocoa flavonoids are classified as flavanols and include the monomers, (−)-epicatechin, (+)-catechin, and procyanidins, the oligomers of these monomeric units. Specific cardioprotective effects recently ascribed to the cocoa flavonoids include: decreased susceptibility of low density lipoprotein (LDL) oxidation and sparing of alpha-tocopherol *in vitro* and *ex vivo* [14–17], and inhibition of platelet activation and aggregation [18, 19]. Furthermore, an increase in plasma antioxidant capacity and a decrease in plasma oxidation products are associated with elevated epicatechin concentrations [20, 21]. Schramm *et al.* [22] recently reported that the cocoa flavonoids decrease plasma leukotriene-prostacyclin ratios in human plasma and aortic endothelial cells. Other experimental investigations have shown that cocoa flavonoids activate endothelial nitric oxide synthase (eNOS) and enhance endothelium-dependent relaxation *in vitro* [23], inhibit human cytokine transcription and secretion [24], and inhibit mammalian 15-lipoxygenase activity [25].

These properties may impede atherogenesis since oxidative modification of LDL is believed to contribute to endothelial dysfunction and vascular disease [26, 27]. Macrophage scavenger receptors internalize oxidized LDL particles resulting in foam cell formation in the arterial wall. Oxidized LDL induces an inflammatory response with the production of endothelial leukocyte adhesion molecules and cytokines [28]. Nitric oxide (NO), an endogenous vasodilator, has many antiatherosclerotic properties including the inhibition of LDL oxidation, adhesion molecule expression, smooth muscle proliferation and contraction, platelet activation and aggregation, monocyte adhesion, and endothelin production [29]. Increased oxidative stress reduces the bioavailability of NO either by decreased NO production and/or increased NO degradation.

Endothelial dysfunction is characterized by dysregulation of NO metabolism and other important vasoactive molecules with resultant impairment of endothelium-dependent vascular relaxation. It can be demonstrated by diminished brachial artery flow-mediated dilation (FMD) which correlates with abnormal coronary artery endothelial responsiveness [30]. Endothelial dysfunction is associated with atherosclerosis and many cardiovascular risk factors including hypertension, hypercholesterolemia, smoking, increased age, and diabetes. It is not known whether cocoa flavonoids can improve endothelial function and measures of oxidative stress in human subjects. The present study was initiated to investigate these questions. A secondary aim of the study was to evaluate the effects of flavonoid-rich dark chocolate on plasma epicatechin concentrations, lipoprotein profiles and blood pressure.

SUBJECTS AND METHODS

Subjects

Twenty-two healthy volunteer subjects (n = 11F and 11M) ages 21–55 years were enrolled in the study. Subjects were in good general health and English-speaking. Exclusion criteria included known cardiovascular disease, diabetes, hyperlipidemia, thyroid disorders, smoking, ± 20% ideal body weight, pregnancy, vegetarianism or extreme physical activity. Twenty-one subjects were included based on the above criteria. One subject was excluded due to hypercholesterolemia. The protocol was approved by the Institutional Review Board of the University of California, San Francisco and written informed consent was obtained.

Study Design

The study was a randomized, double-blind, placebo-controlled design conducted over a 2 week period. Subjects were assigned to daily intake of high-flavonoid (DOVE® Dark Chocolate, Mars, Incorporated, Hackettstown, NJ) or to low-flavonoid dark chocolate bars (46 g, 1.6 oz.) provided by the American Cocoa Research Institute (Vienna, VA). The nutrient composition of the chocolate bars is shown in Table 1.

Subjects were instructed to maintain their usual diet except to refrain from flavonoid-rich food and beverages (list provided to subjects), alcoholic beverages, vitamin supplements, and non-steroidal anti-inflammatory drugs 2 days before each visit (baseline and 2 weeks later). Participants were instructed to fast overnight for 12 hours before each morning visit between 6:00 and 8:00 am (except the last visit when the last chocolate bar was consumed 2 hours before their appointment). They were also instructed to maintain their usual physical activity throughout the study period. Each chocolate sample was provided in coded foil wrapped containers. Both high- and low-flavonoid chocolate bars were similar in physical appearance and taste. Subjects were instructed to consume their chocolate daily after lunch (and before dinner) except for the day of their last visit when the chocolate was to be consumed 2 hours before their morning appointment. This time point was chosen because plasma epicatechin concentrations are at their highest 2 hours after chocolate consumption [21, 22, 31]. In a separate experiment, we asked two non-study volunteers to consume 120 g of

Table 1. Nutrient Composition of High-Flavonoid and Low-Flavonoid Dark Chocolate[1]

Contents	High-Flavonoid (46 g)	Low-Flavonoid (46 g)
Total Procyanidins (mg)	213	Trace
Epicatechin (mg)	46	Trace
Total Fat (g)	15	15
Saturated Fat (g)	9	9
Total Cholesterol (mg)	5	5
Total Carbohydrates (g)	27	27
Sugar (g)	21	21
Protein (g)	2	2
Calories	240	240

[1] Analyses information was provided by the American Cocoa Research Institute (Vienna, VA).

flavonoid-rich dark chocolate to assess plasma epicatechin concentrations after high dose consumption that might have physiological relevance.

A validated food frequency questionnaire (Block 98.2 BDDS, Block Dietary Data System, Berkeley, CA) [32] was completed at baseline to assess habitual dietary patterns. Three-day food records were also completed each week for the 2 week study and were analyzed using nutrition software, ESHA Food Processor for Windows, Version 7.11 (ESHA Research, Salem, OR). At each early morning visit, brachial artery endothelial function and blood pressure were measured followed by height and weight measurements. Venous blood was collected into vacutainer tubes containing EDTA or sodium heparin. Samples were analyzed for total cholesterol, triacylglycerol, LDL, high density lipoprotein (HDL), LDL oxidation, epicatechin, total antioxidant capacity, and 8-isoprostanes. Plasma was immediately processed and stored at −80°C until analysis. Plasma samples for 8-isoprostanes were immediately transferred to eppendorf tubes containing butylated hydroxytoluene (BHT) at a final concentration of 20 μmol/L, quickly frozen and stored at −80°C.

Endothelial Function

Endothelium-dependent FMD of the brachial artery was assessed at each early morning visit after 5–10 minutes of rest supine in a darkened room. All studies were performed by a single investigator blinded to treatment assignment using a 15 MHz linear array vascular transducer and a Sequoia C256 ultrasound system (Acuson, Mountain View, CA) as previously described [33]. Scans were recorded both digitally and on a Super VHS videotape for off-line imaging analyses using the Brachial Ultrasound Workstation (Medical Imaging Applications, Iowa City, IA). FMD was expressed as the peak change in arterial diameter from baseline within 2 minutes of hyperemia. Blood flow was determined from Doppler flow velocity and the cross sectional area of the artery.

Blood Pressure

Blood pressure was determined using the Dinamap (Johnson & Johnson Medical Inc., Tampa, FL.) automated blood pressure device with the subject supine after a 5-min. period of rest. Cuff pressure on the left arm was inflated 2 minutes after the initial reading. Both systolic and diastolic pressures were calculated as the mean value of 2 determinations, 2 minutes apart.

Lipid Profile

Blood was drawn into EDTA tubes and immediately stored on ice until centrifugation. Cholesterol was determined in plasma and lipoprotein fractions by an enzymatic technique and triacylglycerol was determined by a glycerokinase reaction [34]. Plasma levels of HDL cholesterol were determined after precipitation of LDL and VLDL with magnesium chloride and dextran sulfate. LDL cholesterol levels were calculated using the Friedwald equation [35] where LDL cholesterol = total cholesterol-(HDL cholesterol + triacylglycerol/5) with lipid concentrations measured as mmol/L.

Plasma Epicatechin

The bioavailability of dark chocolate polyphenols was assessed by identifying (−)-epicatechin in the plasma by HPLC with electrochemical detection (ECD) according to slight modification of the method described by Milbury [36]. Briefly, 20 μL vitamin C-EDTA (200 mg vitamin C and 1 mg EDTA in 1 mL 0.4 mol/L NaH_2PO_4) and 20 μL glucuronidase/sulfatase type II (Sigma, St. Louis, MO) was added to 200 μL plasma and incubated at 37°C for 45 minutes. Flavonoids were extracted by addition of 500 μL acetonitrile with immediate vortexing. The mixture was centrifuged at 10,000 × *g* for 5 minutes at room temperature and 500 μL supernatant removed, dried under purified nitrogen air, and reconstituted in 100 μL of the aqueous HPLC mobile phase. After centrifugation at 14,000 × *g* for 5 minutes at room temperature, 50 μL supernatant was injected into the HPLC column for separation, detection, and analysis. Plasma epicatechin concentration was calculated based on a standard curve created by authentic epicatechin (Sigma, St. Louis, MO) through the same procedures as the plasma samples.

Ex Vivo LDL Oxidation

The effect of chocolate polyphenols on the resistance of LDL against Cu^{2+}-induced oxidation was measured according to a slight modification of the method described by Esterbauer *et al.* [37]. To preserve the oxidation properties of LDL, sucrose was added to EDTA-containing plasma samples at a final concentration of 0.6% and stored at −80°C for a maximum of 8 weeks [38]. Briefly, LDL (density = 1.019–1.063 g/mL) was separated from plasma according to Havel *et al.* [39]. One mL plasma was mixed with 0.52 g KBr and centrifuged at 330,000 × *g* for 90 minutes at 4°C using a Beckman NVT-90 rotor in a Beckman L8-M centrifuge (Palo Alto, CA). The LDL band was aspirated from the centrifuge tube and EDTA was removed using a PD-10 column according to the manufacturer's instructions (Amershan Pharmacia Biotech, Sweden). LDL protein was determined with a BCA protein assay kit (Pierce; Rockford, IL). The oxidation reaction was initiated by 10 μmol/L $CuSO_4$ at a final concentration of 100 μg/mL LDL protein in a volume of 1 mL. Formation of conjugated dienes was monitored by absorbance at 234 nm at 37°C over 3 hours using a Shimadzu UV1601 spectrophotometer (Japan) equipped with a six-position automated sample changer. Results from the LDL oxidation and the conjugated diene formation are expressed in terms of lag time (defined as the intercept at the abscissa in the diene-time plot) [40].

Oxygen Radical Absorbance Capacity (ORAC)

The total antioxidant capacity of plasma was assessed by ORAC assay using a Fluo Star Optima Microplate reader (BMG Labtechnologies, Inc. Durham, NC) and a modification of the method described by Huang *et al.* [41]. Heparinized plasma samples were used for ORACtotal and ORACpca. Plasma ORACpca samples were prepared with 0.5 M perchloric acid (1:1, v/v) and centrifuged at 10,000 × *g* for 10 minutes to obtain the protein free supernatant. The ORAC value (μmol Trolox Equivalent) represents the area under the quenching curve of fluorescein initiated by 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) in the presence of plasma antioxidants relative to that of Trolox, a water-soluble vitamin E analog (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid). One ORAC unit equals the net protection produced by 1 μmol/L Trolox.

Plasma 8-Isoprostanes

8-isoprostanes, a biomarker of *in vivo* peroxidation of arachidonic acid independent of cyclooxygenase activity, was measured by an enzyme immunoassay (EIA) microtitre plate assay kit (Cayman, Ann Arbor, MI) based on the method developed by Maclouf *et al.* [42] and using a Fluo Star Optima Microplate reader (BMG Labtechnologies, Inc. Durham, NC). Following Folch extraction and KOH digestion, total 8-isoprostanes from 0.5 mL plasma were extracted by use of a solid phase extraction (SPE) 8-isoprostanes affinity column (Cayman Chemical Company). Recoveries averaged >90% with a variance of <20%. Subsequently, total 8-isoprostanes were measured by EIA. This assay is based on the competition between 8-isoprostane and an 8-isoprostane-acetylcholinesterase conjugate (8-isoprostane tracer) for a limited number of 8-isoprostane-specific rabbit antiserum binding sites. The assay employs an Ellman's reaction producing an absorbance at 412 nm proportional to the amount of 8-isoprostane tracer bound to the well and inversely proportional to free 8-isoprostane.

Statistical Analysis

Differences between the two groups in the baseline dietary characteristics shown in Table 3 were examined by independent sample t-tests, but if the assumption of equal variance was not met, the separate variances t-test (Welch test) was used. Statistical significance of the outcomes was determined by applying a repeated measures analysis of variance (RMANOVA) at alpha = 0.05 with one between subject's factor (group) with two levels (experimental and control) and one within subject's factor (time) with two levels (baseline and post intervention). This design allowed for three tests: the main effect of group, the main effect of time, and the interaction of group by time. If the test of the interaction was significant, tests of simple main effects were done to examine the difference between baseline and post intervention within each group. All results are shown as mean ± SEM. Eta-squared (eta^2) represents the percent of explained variance.

RESULTS

Demographics and dietary intake of the subjects at baseline were similar in the low-flavonoid and high-flavonoid groups with the exception of polyunsaturated fat (Tables 2 and 3). Dietary intake of other nutrients, including antioxidant vitamins (C, E), folate, carotenoids, calcium, magnesium and zinc, were comparable in the two groups at baseline. This was determined by analysis of the food frequency questionnaires. Subjects with regular intake of any vitamin supplements discontinued use prior to the study. Subjects also avoided flavonoid-rich foods and beverages 2 days before the study visits as indicated by food records and in corroboration with low plasma epicatechin concentrations at baseline. Excellent compliance in all participants was documented by the return of all empty sample wrappers and by plasma epicatechin concentrations at 2 weeks.

Brachial artery measurements at baseline were also similar between the low-flavonoid and high-flavonoid dark chocolate groups (10.7 ± 0.7% *versus* 10.2 ± 1.3%, $p = 0.75$). The mean change in FMD was significantly different in the high-flavonoid group as compared to the low-flavonoid group after the 2 week consumption period ($p = 0.024$) (Fig. 1). The mean decrease in FMD seen in the low-flavonoid group following the 2 week intervention was not significant ($p = 0.17$, $eta^2 = .08$); whereas, the mean increase in FMD seen in the high-flavonoid group after 2 weeks almost achieved significance at $p = 0.05$, $eta^2 = .17$.

A significant increase in plasma epicatechin concentrations is seen in the high-flavonoid group at 2 weeks after intervention ($p < 0.001$) but not in the low-flavonoid group ($p = 0.99$) (Fig. 2). In the separate experiment with two non-study volunteers who consumed a single dose of 120 g of flavonoid-rich dark chocolate, plasma epicatechin was not detected at baseline, but the concentrations peaked at 200 and 227 nmol/L two hours after consumption. This 2.6-fold increase in the dose of chocolate, compared to the 46 g used in the current study, had no further effect on increasing plasma epicatechin concentrations. No significant differences in the change over time between the two groups were noted in plasma oxidation measurements:

Table 2. Baseline Demographics of Study Subjects

Characteristic	Low-Flavonoid (n = 10)	High-Flavonoid (n = 11)
Age (years)*	32.5 ± 2.9	31.8 ± 3.2
Male, n (%)	5 (50)	6 (54.5)
Female, n (%)	5 (50)	5 (45.5)
Caucasian n (%)	6 (60)	5 (45.4)
Asian/Pacific Islander n (%)	4 (40)	6 (54.6)

* Values represent mean ± SEM.

Table 3. Dietary Intake of Study Subjects at Baseline

	Low-Flavonoid (n = 10)	High-Flavonoid (n = 11)
Total calories/day	1443 ± 105	1923 ± 280
Saturated fat (g)	14 ± 1	22 ± 3
Monounsaturated fat (g)	21 ± 2	29 ± 4
Polyunsaturated fat (g)	11 ± 1	20 ± 3*
Cholesterol (mg)	155 ± 16	219 ± 45
Fat (% of total calories)	33 ± 2	37 ± 2
Protein (% of total calories)	16 ± 1	15 ± 0.8
Carbohydrates (% of total calories)	51 ± 2	47 ± 3
Servings (daily)		
Fruits and Fruit Juices	1.8 ± 0.3	1.3 ± 0.2
Fats, oils, sweets, snacks	2.3 ± 0.2	3.5 ± 0.6
Vegetables	1.9 ± 0.3	1.2 ± 0.1
Bread, cereals, rice, pasta	2.0 ± 0.2	2.3 ± 0.2
Sweets	1.3 ± 0.2	1.8 ± 0.4
Alcoholic beverages	0.1 ± 0.2	0.2 ± .09

Values represent mean ± SEM. * Significance at $p < 0.05$.

LDL oxidation, 8-isoprostanes, or oxygen radical absorbance capacity (ORAC) (Table 4) or other study endpoints (Table 5).

DISCUSSION

This is the first clinical trial to demonstrate improved endothelial function in healthy adults following short-term consumption of flavonoid-rich dark chocolate. This improvement is associated with increased plasma epicatechin concentrations. Flow-mediated dilation of the brachial artery is an endothelium-dependent function associated with release of nitric oxide and possibly endothelium-derived prostanoids [43]. Thus, it is possible that the elevated plasma epicatechin concentrations in the high-flavonoid group increased endothelium-derived vasodilators and improved endothelial function. This mechanism is consistent with a recent study in ten healthy subjects, who exhibited increased (32%) plasma prostacyclin concentrations with a concomitant increase in plasma epicatechin at 2 hours following consumption of high-procyanidin chocolate (37 g)

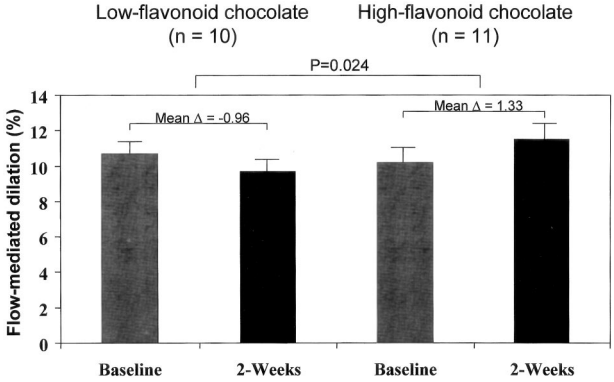

Fig. 1. Flow-mediated dilation (FMD %) of the brachial artery in the low-flavonoid and high-flavonoid dark chocolate groups.

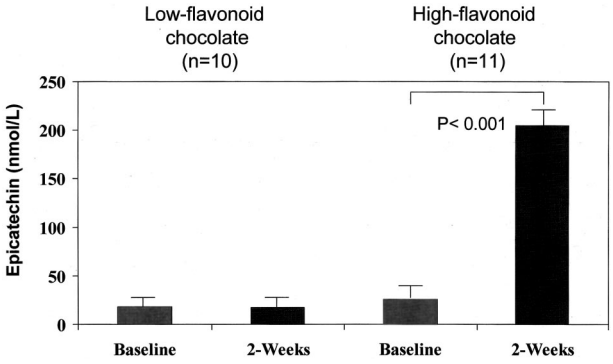

Fig. 2. Effect of low-flavonoid and high-flavonoid dark chocolate on plasma epicatechin. Mean ± SEM.

[22]. This is also supported by results in cultured human aortic endothelial cells following procyanidin treatment that showed increased production of 6-keto-prostaglandin $F_{1\alpha}$ [22]. Cocoa extracts rich in procyanidins, activate endothelial nitric oxide synthase (NOS) and induce endothelium-dependent relaxation in isolated rabbit aortic rings [23]. These studies support our findings of improved endothelial function following short-term intervention with flavonoid-rich chocolate.

In the current study, no changes in biomarkers of antioxidant and oxidative stress were demonstrated following the intervention. Our results are consistent with an acute chocolate feeding study by Wang *et al.*, which showed that plasma antioxidant capacity, concentrations of 8-isoprostanes and 2-thiobarbituric acid reactive substances (TBARS) were unchanged at 2 and 6 hour time points after chocolate consumption [21]. Another study by Rein and colleagues [20] did report a significant increase in plasma total antioxidant capacity and a decrease in TBARS at 2 hours after ingestion of 80 g flavonoid-rich chocolate concomitant with a significant increase in plasma epicatechin (257 nmol/L) in thirteen healthy subjects. This concentration of epicatechin was approximately 50 nmol/L higher than our levels after 2 weeks intervention with 46 g. Osakabe *et al.* [15] also found a reduction in LDL susceptibility to oxidation in healthy subjects after consumption of cocoa powder daily for 2 weeks. In two recent long-term studies, dietary supplementation for 4–6 weeks with flavonoid-rich chocolate and cocoa powder [16, 17] increased LDL oxidation lag time, but did not affect plasma ORAC or urinary F_2 isoprostanes.

Although, previous studies have demonstrated an increase in plasma epicatechin concentrations following consumption of flavonoid-rich products, the findings relative to measurements of oxidative stress are conflicting with our results. We saw higher baseline epicatechin concentrations (17–25 nmol/L) in our study subjects as compared to Wang's study (1–4 nmol/L). Some variances in the baseline measurements among the studies may be the result of differences in the baseline diets or in the sensitivity for detecting low concentrations of epicatechin

Table 4. Biomarkers of Antioxidant and Oxidative Stress Status

	Low-Flavonoid (n = 10)		High-Flavonoid (n = 11)	
	Baseline	2 Weeks	Baseline	2 Weeks
LDL oxidation (lag, min)	50 ± 2.3	52 ± 2.8	53 ± 3.6	55 ± 3.1
8-isoprostanes (pg/mL)	240.3 ± 32.7	308.2 ± 64.8	221.1 ± 39.1	221.8 ± 40.8
ORAC (μmol Trolox Eq)				
Total	12,017 ± 531	11,939 ± 638	12,154 ± 534	12,193 ± 398
Pca	1,102 ± 58.7	1,020 ± 60.5	1,136 ± 51	1,109 ± 39.1

Values represent mean ± SEM.
ORAC = Oxygen radical absorbance capacity.

Table 5. Baseline and 2 Week Study Endpoints

Endpoint	Low-Flavonoid (n = 10)		High-Flavonoid (n = 11)	
	Baseline	2 Weeks	Baseline	2 Weeks
BMI (kg/m^2)	21.9 ± 0.5	21.8 ± 0.5	23.2 ± 0.5	23.1 ± 0.5
Weight (lbs)	140 ± 9	140.7 ± 9	150.2 ± 6.9	150.8 ± 6.7
Total cholesterol (mmol/L)	4.9 ± 0.2	5.1 ± 0.2	4.2 ± 0.2	4.3 ± 0.2
LDL cholesterol (mmol/L)	2.5 ± 0.1	2.7 ± 0.2	2.1 ± 0.1	2.0 ± 0.1
HDL cholesterol (mmol/L)	1.7 ± 0.2	1.7 ± 0.2	1.5 ± .09	1.6 ± 0.1
Triacylglycerol (mmol/L)	1.2 ± 0.1	1.3 ± .05	1.2 ± 0.1	1.5 ± 0.1
Blood Pressure (mmHg)				
Mean Systolic	112.8 ± 2.8	110 ± 2	121.0 ± 5.4	120 ± 4
Mean Diastolic	66.1 ± 1.7	66 ± 2	68.1 ± 2.5	69 ± 2
Brachial artery resting diameter (mm)	3.79 ± 0.1	3.77 ± 0.2	3.76 ± 0.2	3.81 ± 0.2

Values represent mean ± SEM.
To convert cholesterol values from mmol/L to mg/dL, divide by 0.02586, and to convert triacylglycerol values, divide by 0.0113.

using different HPLC coupled with electrochemical (coulometric detection) methodologies. In addition, the increment of epicatechin concentrations 2 hours after chocolate consumption compared to baseline was approximately eightfold greater in our study using 46 g chocolate, 65-fold in the study by Wang *et al.* (53 g chocolate), and twelvefold in the Rein *et al.* study (80 g chocolate). The variability in oxidative stress measures may be related to the duration of the intervention as well as the type and amount of the chocolate or cocoa powder and flavonoids consumed. A recent 6-week study on the effects of increased intake of fruits and vegetables, other plant sources of flavonoids, with varied intakes of unsaturated fatty acids (linoleic or oleic acid) also showed no measurable effects on lipid peroxidation (*ex vivo* LDL oxidation, TBARS, 8-isoprostanes) or lipoprotein metabolism [44]. It is acknowledged that adequate assessment of oxidative stress requires several independent measurements [45].

It is notable that the subjects' lipid profiles did not change adversely following the flavonoid-rich or low-flavonoid chocolate intervention. In previous long-term studies of chocolate supplementation, neutral and/or beneficial cholesterol effects have been observed [46, 47]. The high content of stearic acid in cocoa butter which is readily converted to oleic acid [48] could help explain these effects. Dietary supplementation with isoflavonoids, a class of flavonoids derived from soybean-based foods, similarly has no effect on lipoprotein parameters in normocholesterolemic individuals [49, 50].

CONCLUSION

In summary, flavonoid-rich dark chocolate improves endothelium-dependent vasodilation. This effect is associated with increased plasma epicatechin concentrations in healthy adults, though it is possible that flavonoids are a marker for some other bioactive constituent of chocolate. This could be evaluated by the administration of purified flavonoids. It is also acknowledged that the long-term clinical significance of the changes in endothelial function detected in the study are not known. Our findings suggest a possible cardioprotective effect by flavonoid-rich chocolate, independent of changes in measures of oxidative stress and lipid profiles. Further larger, long-term clinical trials with food sources rich in flavonoids, including chocolate, are certainly warranted.

REFERENCES

1. Knekt P, Kumpulainen J, Järvinen R, Rissanen H, Heliövaara M, Reunanen A, Hakulinen T, Aromaa A: Flavonoid intake and risk of chronic diseases. Am J Clin Nutr 76:560–568, 2002.
2. Arts ICW, Jacobs DR, Harnack LJ, Gross M, Folsom AR: Dietary catechins in relation to coronary heart disease death among postmenopausal women. Epidemiology 12:668–675, 2001.
3. Yochum L, Kushi LH, Meyer K, Folsom AR: Dietary flavonoid intake and risk of cardiovascular disease in postmenopausal women. Am J Epidermiol 149:943–949, 1999.

4. Hertog MG, Kromhout D, Aravanis C, Blackburn H, Buzina R, Fidanza F, Giampaoli S, Jansen A, Menotti A, Nedeljkovic S, Pekkarinen M, Simic BS, Toshima H, Feskens EJM, Hollman PCH, Katan MB: Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. Arch Intern Med 155:381–386, 1995.
5. Geleijnse JM, Launer LJ, van der Kuip DAM, Hofman A, Witteman JCM: Inverse association of tea and flavonoid intakes with incident myocardial infraction: the Rotterdam Study. Am J Clin Nutr 75:880–886, 2002.
6. Joshipura KJ, Ascherio A, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, Hennekens CH, Spiegelman D, Willett WC: Fruit and vegetable intake in relation to risk of ischemic stroke. JAMA 282:1233–1239, 1999.
7. Liu S, Manson JE, Lee IM, Cole SR, Hennekens CH, Willett WC, Buring JE: Fruit and vegetable intake and risk of cardiovascular disease: the Women's Health Study. Am J Clin Nutr 72:922–928, 2000.
8. Joshipura KJ, Hu FB, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, Colditz G, Ascherio A, Rosner B, Spiegelman D, Willett WC: The effect of fruit and vegetable intake on risk of coronary heart disease. Ann Int Med 134:1106–1114, 2001.
9. Liu S, Lee IM, Ajani U, Cole SR, Buring JE, Manson JE: Intake of vegetables rich in carotenoids and risk of coronary heart disease in men: The Physicians' Health Study. Intl J Epidemiol 30:130–135, 2001.
10. Middleton E, Kandaswami C, Theoharides TC: The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. Pharmcol Rev 52:673–751, 2000.
11. Hammerstone JF, Lazarus SA, Schmitz HH: Procyanidin content and variation in some commonly consumed foods. J Nutr 130: 2086S–2092S, 2000.
12. Vinson JA, Proch J, Zubik L: Phenol antioxidant quantity and quality in foods: cocoa, dark chocolate, and milk chocolate. J Agric Food Chem 47:4821–4824, 1999.
13. Adamson GE, Lazarus SA, Mitchell AE, Prior RL, Cao G, Jacobs PH, Kremers BG, Hammerstone JF, Rucker RB, Ritter KA, Schmitz HH: HPLC method for quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. J Agric Food Chem 47:4184–4188, 1999.
14. Steinberg FM, Holt RR, Schmitz HH, Keen CL: Cocoa procyanidin chain length does not determine ability to protect LDL from oxidation when monomer units are controlled. J Nutr Biochem 13:645–652, 2002.
15. Osakabe N, Baba S, Yasuda A, Iwamoto T, Kamiyama M, Takizawa T, Itakura H, Kondo K: Daily cocoa intake reduces the susceptibility of low-density lipoprotein to oxidation as demonstrated in healthy human volunteers. Free Rad Res 34: 93–99, 2001.
16. Wan Y, Vinson J, Etherton TD, Proch J, Lazarus SA, Kris-Etherton PM: Effects of cocoa powder and dark chocolate on LDL oxidative susceptibility and prostaglandin concentrations in humans. Am J Clin Nutr 74:596–602, 2001.
17. Mathur S, Devaraj S, Grundy SM, Jialal I: Cocoa products decrease low density lipoprotein oxidative susceptibility but do not affect biomarkers of inflammation in humans. J Nutr 132:3663–3667, 2002.
18. Rein D, Paglieroni TG, Pearson DA, Wun T, Schmitz HH, Gosselin R, Keen CL: Cocoa and wine polyphenols modulate platelet activation and function. J Nutr 130:2120S–2126S, 2000.
19. Rein D, Paglierori TG, Wun J, Pearson DA, Schmitz HH, Gosselin R, Keen CL: Cocoa inhibits platelet activation and function. Am J Clin Nutr 72:30–35, 2000.
20. Rein D, Lotito S, Holt RR, Keen CL, Schmitz HH, Fraga CG: Epicatechin in human plasma: In vivo determination and effect of chocolate consumption on plasma oxidation status. J Nutr 130: 2109S–2114S, 2000.
21. Wang JF, Schramm DD, Holt RR, Ensunsa JL, Fraga CG, Schmitz HH, Keen CL: A dose-response effect from chocolate consumption on plasma epicatechin and oxidative damage. J Nutr 130:2115S–2119S, 2000.
22. Schramm DD, Wang JF, Holt RR, Ensunsa JL, Gonsalves JL, Lazarus SA, Schmitz HH, German JB, Keen CL: Chocolate procyanidins decrease the leukotriene-prostacyclin ratio in humans and human aortic endothelial cells. Am J Clin Nutr 73:36–40, 2001.
23. Karim M, McCormick K, Kappagoda CT: Effects of cocoa extracts on endothelium-dependent relaxation. J Nutr 130:2105S–2108S, 2000.
24. Mao T, Van de Water J, Keen CL, Schmitz HH, Gershwin ME: Cocoa procyanidins and human cytokine transcription and secretion. J Nutr 130:2093S–2099S, 2000.
25. Schewe T, Sadik C, Klotz LO, Yoshimoto T, Kuhn H, Sies H: Polyphenols of cocoa: inhibition of mammalian 15-lipoxygenase. Biol Chem 382:1687–1696, 2001.
26. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL: Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. New Engl J Med 320:915–924, 1989.
27. Steinberg D, Lewis A: Conner Memorial Lecture: Oxidative modification of LDL and atherogenesis. Circulation 95:1062–1071, 1997.
28. Libby P, Ridker PM, Maseri A: Inflammation and atherosclerosis. Circulation 105:1135–1143, 2002.
29. John S, Schmieder RE: Impaired endothelial function in arterial hypertension and hypercholesterolemia: potential mechanisms and differences. J Hypertens 18:363–374, 2000.
30. Anderson TJ, Uehata A, Gerhard MD, Meredith IT, Knab S, Delagrange D, Lieberman EH, Ganz P, Creager MA, Yeung AC, Selwyn AP: Close Relation of Endothelial Function in the Human Coronary and Peripheral Circulations. J Am Coll Cardiol 26:1235–1241, 1995.
31. Richelle M, Tavazzi I, Enslen M, Offord EA: Plasma kinetics in man of epicatechin from black chocolate. Eur J Clin Nutr 53:22–26, 1999.
32. Berkeley Nutrition Services: Questionnaire development and methodologic issues. http://www.nutritionquest.com/validation.htm (Accessed January 14, 2003).
33. Mietus-Snyder ML, Malloy MJ: Endothelial dysfunction occurs in children with three genetic hyperlipidemias: Improvement with antioxidant therapy. J Pediatr 133:35–40, 1998.
34. Kane JP, Malloy MJ, Ports TA, Phillips NR, Diehl JC, Havel RJ: Regression of coronary atherosclerosis during treatment of familial hypercholesterolemia with combined drug regimens. J Am Med Assoc 264:3007–3012, 1990.

35. Friedewald WT, Levy RI, Fredrickson DS: Estimation of low-density lipoprotein cholesterol in plasma without use of preparative ultracentrifuge. Clin Chem 18:499–502, 1972.
36. Milbury P: Analysis of complex mixtures of flavonoids and polyphenols by high-performance liquid chromatography electrochemical detection methods. Methods Enzymol 335:15–26, 2001.
37. Esterbauer H, Striegl G, Puhl H, Rotheneder M: Continuous monitoring of in vitro oxidation of human low-density lipoprotein. Free Rad Res Commun 6:67–75, 1989.
38. Ramos P, Gieseg SP, Schuster B, Esterbauer H: Effect of temperature and phase transition on oxidation resistance of low density lipoprotein. J Lipid Res 36:2113–2128, 1995.
39. Havel R, Eder H, Bragdon J: The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest 34:1354–1353, 1955.
40. Esterbauer H, Gebicki J, Puhl H, Jurgens G: The role of lipid peroxidation and antioxidants in oxidative modification of LDL. Free Rad Biol Med 13:341–390, 1992.
41. Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior RL: High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. J Agric Food Chem 50:4437–4444, 2002.
42. Maclouf J, Grassi J, Pradelles P: Development of enzyme-immunoassay techniques for the measurement of eicosanoids. In Walden TL, Hughes HN (eds): "Prostaglandin and Lipid Metabolism in Radiation Injury." Cambridge, MA: Perseus Publishing, pp 355–364, 1987.
43. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R: Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery. J Am Coll Cardiol 39:257–265, 2002.
44. Freese R, Alfthan G, Jauhiainen M, Basu S, Erlund I, Salminen I, Aro A, Mutanen M: High intakes of vegetables, berries, and apples combined with a high intake of linoleic or oleic acid only slightly affect markers of lipid peroxidation and lipoprotein metabolism in healthy subjects. Am J Clin Nutr 76:950–960, 2002.
45. Prior RL, Cao G: In vivo total antioxidant capacity: comparison of different analytical methods. Free Rad Bio Med 27:1173–1181, 1999.
46. Kris-Etherton PM, Mustad VA: Chocolate feeding studies: a novel approach for evaluating the plasma lipid effects of stearic acid. Am J Clin Nutr 60(Suppl):1029S–1036S, 1994.
47. Kris-Etherton PM, Derr JA, Mustad VA, Seligson FH, Pearson TA: Effects of a milk chocolate bar per day substituted for a high-carbohydrate snack in young men on an NCEP/AHA Step 1 diet. Am J Clin Nutr 60(Suppl):1037S–1042S, 1994.
48. Connor WE: Harbingers of coronary heart disease: dietary saturated fatty acids and cholesterol. Is chocolate benign because of its stearic acid content? Am J Clin Nutr 70:951–952, 1999.
49. Hodgson JM, Puddey IB, Beilin LJ, Mori TA, Croft KD: Supplementation with isoflavonoid phytoestrogens does not alter lipid concentrations: a randomized controlled trial in humans. J Nutr 128:728–732, 1998.
50. Simons LA, von Konigsmark M, Simons J, Celermajer DS: Phytoestrogens do not influence lipoprotein levels or endothelial function in healthy, postmenopausal women. Am J Cardiol 85:1297–1301, 2000.

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