Dark Chocolate Improves Coronary Vasomotion and Reduces Platelet Reactivity

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Background—Dark chocolate has potent antioxidant properties. Coronary atherosclerosis is promoted by impaired endothelial function and increased platelet activation. Traditional risk factors, high oxidative stress, and reduced antioxidant defenses play a crucial role in the pathogenesis of atherosclerosis, particularly in transplanted hearts. Thus, flavonoid-rich dark chocolate holds the potential to have a beneficial impact on graft atherosclerosis.

Methods and Results—We assessed the effect of flavonoid-rich dark chocolate compared with cocoa-free control chocolate on coronary vascular and platelet function in 22 heart transplant recipients in a double-blind, randomized study. Coronary vasomotion was assessed with quantitative coronary angiography and cold pressor testing before and 2 hours after ingestion of 40 g of dark (70% cocoa) chocolate or control chocolate, respectively. Two hours after ingestion of flavonoid-rich dark chocolate, coronary artery diameter was increased significantly (from 2.36 ± 0.51 to 2.51 ± 0.59 mm, P < 0.01), whereas it remained unchanged after control chocolate. Endothelium-dependent coronary vasomotion improved significantly after dark chocolate (4.5 ± 11.4% versus −4.3 ± 11.7% in the placebo group, P = 0.01). Platelet adhesion decreased from 4.9 ± 1.1% to 3.8 ± 0.8% (P = 0.04) in the dark chocolate group but remained unchanged in the control group.

Conclusions—Dark chocolate induces coronary vasodilation, improves coronary vascular function, and decreases platelet adhesion 2 hours after consumption. These immediate beneficial effects were paralleled by a significant reduction of serum oxidative stress and were positively correlated with changes in serum epicatechin concentration. (Circulation. 2007;116:2376-2382.)

Key Words: atherosclerosis ■ endothelium ■ nutrition ■ oxidative stress ■ platelets

Dark chocolate contains high levels of flavonoids that exert antioxidant properties. An emerging body of evidence suggests a potential beneficial impact of a great variety of different flavonoid-rich food and beverages on cardiovascular events, for cocoa in particular. Interestingly, flavonoid-rich chocolate or cocoa drinks have been shown to improve peripheral vascular function in healthy volunteers and patients with increased cardiovascular risk. The cardiovascular effect of flavonoid-rich dark chocolate, however, remains elusive.

Atherosclerotic cardiovascular events are characterized by arterial occlusion, vasospasm, and thrombosis. A rapidly progressing form of coronary atherothrombosis is transplantation-associated arteriosclerosis, which limits long-term survival after transplantation. In addition to traditional risk factors, high oxidative stress and reduced antioxidant defense play a crucial role in the pathogenesis of transplantation-associated arteriosclerosis. Antioxidant strategies thus hold the potential to retard early progression of the disease.

Increased oxidative stress and impaired nitric oxide (NO) bioavailability are the key features of vascular dysfunction and can be detected as abnormal coronary vasomotion in response to endothelium-dependent stimuli such as acetylcholine or the cold pressor test (CPT). In patients with intact vascular function, acetylcholine and CPT induce vasodilation, whereas with dysfunctional endothelium, a paradoxical vasoconstriction occurs. Patient showing paradoxical vasoconstriction, particularly heart transplant recipients, are at increased risk for cardiovascular events. We report here the
results of a double-blind, controlled study examining the effect of flavonoid-rich dark chocolate or flavonoid-free control chocolate on coronary endothelial vasomotion and platelet function in heart transplant recipients.

Methods

Subject Selection
Twenty-two heart transplant recipients (18 men, 4 women) who were scheduled for a regular coronary angiogram and who did not require immediate coronary intervention were included in this randomized, double-blind, controlled, investigator-initiated trial. Exclusion criteria were acute or chronic heart failure (at least New York Heart Association class II), renal failure (creatinine >200 μmol/L), liver disease (alanine aminotransferase or aspartate aminotransferase >150 IU), symptomatic hypotension or hypertension >160/100 mm Hg, known allergy to compounds of the dark or control chocolate, acute infectious diseases, diseases with systemic inflammation, participation in another study within the last month, and concomitant vitamin use. Dark and control chocolates were prepared by Nestlé (Lausanne, Switzerland). Both were wrapped identically, and randomization was done by an organization independent of the study group (InterCorNet, Zürich, Switzerland). Chocolate was distributed to the patient by a nurse not involved in the study.

All patients gave signed informed consent. The study was performed at the University Hospital of Zurich, was approved by the local ethics committee, and was conducted according to the Declaration of Helsinki.

Experimental Protocol
Each patient was instructed to refrain from eating flavonoid-rich food for 24 hours and to fast for 8 hours before baseline examinations, which were always performed in the morning. These included clinical examination, blood sampling, and a routine coronary angiography. Quantitative coronary angiography was assessed at rest, after the CPT (immersion of the right hand in ice-cold water for 2 minutes), and after intra coronary administration of 100 μg of nitroglycerin. Blood pressure and heart rate were recorded continuously during the coronary angiogram.

After removal of the angiography catheters, the sheath remained in place. After the first assessment, the participants were randomized to receive either 40 g of dark chocolate or the control chocolate, which had to be ingested within 10 minutes.

Two hours after chocolate ingestion, a second assessment of the coronary vasomotion was performed, again with quantitative coronary angiography and the CPT. Clinical parameters were measured and blood samples taken.

Study Chocolate and Control
Commercially available “Nestlé Noir Intense” was used for dark chocolate. This chocolate contains 10.5 g of sugar and 17.9 g of fat per 40 g serving with a 70% cocoa content. The catechin and epicatechin concentrations were 0.27 and 0.9 mg/g, respectively (as measured by high-performance liquid chromatography), with total polyphenol content (as measured by the Folin-Ciocalteau method) of 15.6 mg of epicatechin equivalents per gram (data from Nestlé Research Center, Lausanne, Switzerland). Nestlé also supplied the isoflavone, isocarbohydrate, flavonoid-free chocolate for the control group.

Quantitative Coronary Analysis
Coronary angiography was performed with a biplane Philips Igris Allura System (Philips Medical Systems, The Netherlands). Images were digitally recorded and were analyzed offline with CAAS QCA software (Pie Medical Imaging, Maastricht, the Netherlands) for automatic contour detection of the coronary arteries and analysis of their diameter; this software has been described and validated previously.15,16 End-diastolic images of coronary arteries were evaluated at baseline, after CPT with immersion of the right hand into ice water for 2 minutes, and after nitroglycerin application (100 μg), as described previously.17,18 In all patients, measurements were performed in 2 proximal, smooth, nonbranching coronary vessel segments without evidence of luminal wall irregularities or diffuse caliber reduction and stenosis. Each segment was defined by 2 anatomic references to ensure measurements were always at the same segment after each procedure. The artery diameter was calibrated against the contrast-filled tip of the catheter. An investigator blinded to treatment group performed all measurements.

Shear Stress–Dependent Platelet Function
Shear stress–dependent platelet function was assessed with a cone and platelet analyzer. In brief, citrated whole blood (200 μL) was circulated in polystyrene wells at a shear rate of 1875 s⁻¹ for 2 minutes with a rotating polytetrafluoroethylene cone, as described previously.21–23 Wells were washed, stained with May–Grünwald, and analyzed with a microscope connected to an image-analysis system. Results are expressed as the percentage of surface covered by platelets and as the size of the platelet aggregates (ImageJ 1.31, National Institutes of Health, Bethesda, Md).

Biomarkers of Oxidative Stress
Serum samples were analyzed at the Antioxidant Research Laboratory of the Unit of Human Nutrition INRAN in Rome, Italy, a laboratory of the human nutrition unit that specializes in research on antioxidant properties of diet and development of markers of redox status and oxidation. The analyses were performed before unblinding of the study.

TRAP Assay
The TRAP (total radical-reducing antioxidant potential) method is based on the protection provided by antioxidants against the fluorescence decay of R-phycocerythrin (lag phase) during a controlled peroxidation reaction.24 Briefly, 25 μL of diluted sample was added to 130 μL of phosphate buffer (pH 7.4), 15 μL of diluted R-phycocerythrin, and 30 μL of Azo-bis-(2-aminodipropene); the reaction kinetics at 38°C were recorded for 60 minutes by a Teco GENios standard fluorescent plate reader spectrometer (Teco Italia SRL, Milan, Italy). TRAP values were calculated from the length of the lag phase due to the sample compared with that of trolox and expressed as μmol/L (micromoles of peroxyl radicals trapped by 1 L of serum). Intra-assay and interassay coefficients of variation were <3% and <9%, respectively.

FRAP Assay
The FRAP (ferric-reducing antioxidant potential) assay is based on the protection provided by antioxidants against the fluorescence decay of Fe³⁺-TPTZ complex to the ferrous form at low pH monitored at 595 nm. The absorbance change is then recorded and expressed as μmol/L (micromoles of peroxyl radicals trapped by 1 L of serum). Intra-assay and interassay coefficients of variation were <1% and <3%, respectively.

F₂-Isoprostane Analysis
Total 8-iso-prostaglandin F₂α (8-iso-PGF₂α) was quantified in serum samples with a direct 8-iso-PGF₂α enzyme immunoassay kit (Assay Designs Inc, Ann Arbor, Mich). The kit uses a polyclonal antibody to competitively bind the 8-iso-PGF₂α in the sample or an alkaline phosphatase molecule that has 8-iso-PGF₂α covalently attached to it. After a short incubation period, the enzyme reaction is stopped, and absorbance is read at 405 nm. The intensity of the bound yellow color is inversely proportional to the concentration of 8-iso-PGF₂α in either standards or samples. Total 8-iso-PGF₂α is expressed as picograms per milliliter. Intra-assay and interassay coefficients of variation were <11% and <10%, respectively.

Assessment of Serum Flavonoid Concentrations
The serum samples were sent to the Nestlé Research Centre, Lausanne, Switzerland, where the analyses were performed before...
the study was unblinded. The bioavailability of dark chocolate flavonoids was assessed by identifying catechin and epicatechin in serum by high-performance liquid chromatography with colorimetric array electrochemical detection. Briefly, serum was centrifuged at 14 000 rpm for 5 minutes at 4°C. A total of 200 μL of serum was mixed with 12 μL of 10% ascorbic acid:40 mmol/L KH2PO4–0.1% EDTA, 20 μL of 50 mmol/L potassium phosphate (pH 7.4), 20 μL of 1.0 μg/mL catechin gallate as internal standard, 500 U of β-d-glucuronidase type X-A from Escherichia coli (Sigma Chemical Co, St. Louis, Mo), and 4 U of sulfatase type VIII from abalone entails (Sigma Chemical Co). The mixture was incubated at 37°C for 45 minutes. The reaction was stopped by the addition of 2 mL of ethyl acetate followed by shaking for 20 minutes and centrifugation at 4°C at 2000g for 5 minutes. The supernatant was transferred to a clean tube, and the ethyl acetate extraction was repeated. A total of 10 μL of 0.02% ascorbic acid:0.005% EDTA was added to the pooled supernatant fraction and subjected to a thorough vortex to mix. The supernatant was then evaporated to dryness with nitrogen for 2 hours at room temperature. The samples were reconstituted in 200 μL of methanol:water (1:2 vol), subjected to a vortex, sonicated for 10 minutes, and centrifuged (14 000 rpm, 5 minutes, 4°C). A total of 20 μL of the supernatant was injected into the high-performance liquid chromatography system. Intra-assay and interassay coefficients of variation were 7.27% and 6.22%, respectively.

**Statistical Analysis**

All data are expressed as mean±SD, except data in figures, which are mean±SE. Differences between the 2 patient groups for baseline characteristics and coronary vasomotion were compared with ANOVA. Within each group, comparisons were made by paired Student t test. A probability value of less than 0.05 was considered statistically significant. For the correlation between changes in serum epicatechin levels and coronary vasomotion, Spearman’s correlation coefficient analysis was used.

The sample-size calculation was based on the results of our previous study examining endothelial function in the forearm circulation of young healthy smokers1 and on previous experience using quantitative coronary angiography at our institution23; we considered an improvement in vasomotion to be a reduction in coronary vasoconstriction during the CPT 2 hours after dark chocolate consumption from initially 20±8% to 10%. Assuming a normal distribution, a 95% chance for significance was calculated with 20 patients (2-tailed significance level of 0.05). Statistics were performed with SPSS 11.0.4 for Mac OS X (SPSS Inc, Chicago, Ill).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

Twenty-two patients were included in the present study and randomized to receive either flavonoid-rich chocolate or control chocolate. Clinical and laboratory characteristics of the study population are given in Tables 1 and 2, respectively. Although the majority of clinical characteristics and coronary risk factors, as well as medications used, did not differ between the groups, baseline glucose and hemoglobin A1c concentrations were increased significantly and body mass index tended to be higher in the control group, which reflects the higher incidence of patients with diabetes mellitus in the control group (4 of 11 in the control group and 0 of 11 in the dark chocolate group, respectively).

Coronary artery diameter was analyzed with quantitative coronary angiography. Endothelium-dependent vasomotion in response to CPT and endothelium-independent vasodilation in response to nitroglycerin, respectively, were assessed in patients undergoing routine posttransplantation coronary evaluation. At baseline, endothelium-dependent and nitroglycerine-induced vasomotion were similar in the 2 groups. Most interestingly, 2 hours after ingestion of dark chocolate, coronary artery diameter increased significantly from 0.57 mm to 0.59 mm (P=0.018; Figure 1B). Endothelium-independent coronary vasomotion (percent change of artery diameter induced by CPT after chocolate ingestion compared with baseline coronary artery diameter) improved significantly 2 hours after dark chocolate ingestion (4.5±11.4% versus −4.3±11.7% in the control group, P=0.018; Figure 1B). Endothelium-independent vasomotion induced by intracoronary injection of nitroglycerin did not differ between the groups (20.8±12.6% after dark chocolate versus 15.8±19.9% after control; P=0.36).

### Table 1. Baseline Clinical Characteristics (n=22)

<table>
<thead>
<tr>
<th></th>
<th>Control (n=11)</th>
<th>Dark Chocolate (n=11)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>55.6±11.2</td>
<td>51.5±14.7</td>
<td>0.46</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>133.5±11.9</td>
<td>135.1±13.5</td>
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</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>84.8±8.0</td>
<td>85.1±15.9</td>
<td>0.96</td>
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<tr>
<td>Heart rate, bpm</td>
<td>88.6±13.9</td>
<td>83.6±9.5</td>
<td>0.33</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>27.4±4.7</td>
<td>24.0±3.6</td>
<td>0.06</td>
</tr>
<tr>
<td>Years since transplantation</td>
<td>6.3±6.0</td>
<td>8.9±5.1</td>
<td>0.28</td>
</tr>
<tr>
<td>Former smoker</td>
<td>6/11</td>
<td>3/11</td>
<td>...</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>4/11</td>
<td>2/11</td>
<td>...</td>
</tr>
<tr>
<td>Hypertension</td>
<td>4/11</td>
<td>3/11</td>
<td>...</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>4/11</td>
<td>0/11</td>
<td>...</td>
</tr>
<tr>
<td>Former MI/PAD</td>
<td>6/11</td>
<td>3/11</td>
<td>...</td>
</tr>
<tr>
<td>Family history</td>
<td>4/11</td>
<td>1/11</td>
<td>...</td>
</tr>
<tr>
<td>Total risk factors</td>
<td>2.0±1.3</td>
<td>1.1±1.3</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Values represent mean±SD or absolute numbers. P for comparison of groups by ANOVA. MI indicates myocardial infarction; PAD, peripheral artery disease.
To investigate the concept of increased antioxidative properties of dark chocolate as the potential mechanism leading to vasodilation and reversal of vascular dysfunction, we measured the flavonoid concentration in serum at baseline and 2 hours after chocolate intake. In addition, changes in oxidative stress parameters and antioxidant status were measured. Serum epicatechin concentration increased significantly after dark chocolate ingestion (from 0.0127±0.0046 to 0.0604±0.0191 μg/mL, P<0.001), whereas it did not change after ingestion of control chocolate (from 0.0338±0.0052 to 0.023.1±0.0181 μg/mL, P=0.48; Figure 2). Catechin concentrations did not change significantly after chocolate or control ingestion (from 0.007±0.0055 to 0.0214±0.0315 μg/mL, P=0.22, and from 0.0092±0.0107 to 0.0141±0.0071 μg/mL, P=0.19, respec-
In the present study, we demonstrate that flavonoid-rich chocolate markedly improves coronary vasodilation, indicating a significant effect was evident on the inflammatory parameter (C-reactive protein) before and after dark chocolate consumption (6.3 ± 5.9 versus 7.6 ± 6.2 mg/L, P = 0.029), whereas they did not change significantly after control chocolate consumption. Serum epicatechin concentration increased significantly 2 hours after dark chocolate ingestion (P < 0.001), with no change after control. Two hours after chocolate consumption, 8-isoprostanes were reduced significantly (P = 0.029), whereas they did not change significantly 2 hours after control chocolate consumption. TRAP and FRAP both tended to improve after chocolate consumption (P = 0.069 and 0.096, respectively). *P < 0.05 or less for comparison between baseline and 2 hours after chocolate; error bars represent SE; n = 22.

Figure 2. Changes in epicatechins and oxidant status parameters. Mean percent changes in serum epicatechin concentrations, lipid oxidation, and oxidant status parameters 2 hours after chocolate consumption. Serum epicatechin concentration increased significantly 2 hours after dark chocolate ingestion (P < 0.001), with no change after control. Two hours after chocolate consumption, 8-isoprostanes were reduced significantly (P = 0.029), whereas they did not change significantly after control chocolate consumption. TRAP and FRAP both tended to improve after chocolate consumption (P = 0.069 and 0.096, respectively). *P < 0.05 or less for comparison between baseline and 2 hours after chocolate; error bars represent SE; n = 22.

Figure 3. Platelet adherence before and after dark chocolate/conrol ingestion. Effect of dark chocolate on platelet function. Bars represent percentage of adhering platelets at baseline and 2 hours after control (white bars) or dark chocolate (gray bars) consumption. Shear stress–dependent platelet adhesion at high shear rate conditions decreased from 4.9 ± 1.1% to 3.8 ± 0.8% (P = 0.04) in the dark chocolate group, whereas it remained unchanged in the control group (4.4 ± 1.3% to 4.1 ± 1.4%; P = 0.12). *P < 0.05 or less.
lowering the plasma level of F₂-isoprostanes. Indeed, in the present study, 8-isoprostane levels were significantly reduced just 2 hours after chocolate intake, and serum antioxidant chain–breaking (TRAP) and –reducing (FRAP) potentials were increased, all of which are essential biomarkers of redox balance in vivo. As such, both activation of NO synthase and reduction of oxidative stress might have improved endothelial dysfunction and reduced platelet activation.

The possibility that ingredients of control chocolate might have influenced the results is very unlikely. Indeed, the control preparation was manufactured specifically for the present trial to provide a chocolate bar with exactly the same amount of fat and carbohydrate as dark chocolate but no cocoa liquor.

It is noteworthy that the effect of dark chocolate on coronary vasomotion was observed in addition to current optimal therapy for heart transplantation patients, such as a statin, β-blocker, angiotensin-converting enzyme–inhibitor, or angiotensin receptor blocker if indicated. Given that several of these medications, specifically statins and blockers of the renin angiotensin system, are known to have beneficial effects on the vascular endothelium, coronary vasomotion, and platelet adhesion, the present results imply that the true effect of dark chocolate might provide a clinically relevant benefit not only in healthy subjects but also in patients treated with cardiovascular drugs.

Activation of platelets is an essential step in acute coronary syndromes. The fact that shear stress–dependent platelet adhesion was reduced 2 hours after flavonoid-rich chocolate consumption in heart transplantation patients extends our previous findings in young healthy smokers, in whom a similar effect on platelet function was found. A high shear rate mimics severely stenotic or disrupted plaques, and thus, reduction of shear stress–dependent platelet adhesion is indicative of clinically relevant protective effects.

It is a limitation of the results of the present study that they are based on a short-term intervention only. The precise effects of long-term chocolate ingestion are not yet known, even though in a cross-sectional analysis, cocoa intake was inversely related to blood pressure and to cardiovascular and all-cause mortality. Because chocolate also contains fat and sugar, careful conclusions should be drawn, because these compounds might influence health-relevant factors such as insulin resistance, weight, or serum lipids. Furthermore, most chocolate products are manufactured with milk, a compound known to influence antioxidant capacity in serum. Even if the flavonoid-content in milk chocolate were the same as in its dark counterpart, the antioxidant effect of cocoa is potentially attenuated in the serum if chocolate is ingested as milk chocolate or in combination with milk.

The sample size of this kind of study design presents other potential limitations. The fact that baseline characteristics did not match perfectly with respect to blood glucose and diabetes mellitus is important, because blood glucose might influence endothelial function. However, the diabetic patients were all in the control group, and therefore, the improvement in vascular and platelet function seen 2 hours after chocolate consumption is unlikely to be influenced by this potential confounder. Glucose level did not change during the course of the study in either group (online Data Supplement Figure). Moreover, both chocolates contained exactly the same amount of sugar and fat.

Because the present study was done in collaboration with industry with respect to chocolate manufacturing and the analysis of polyphenol levels in patients and chocolate, concerns about a commercial confounding might arise. However, the present study was fully investigator-initiated and was without industrial funding. The collaboration with the manufacturer of the chocolate used was necessary to obtain control chocolate, to allow for a double-blind study design. We are convinced that this collaboration did not influence the study results.

In conclusion, our results suggest a short-term effect of flavonoid-rich dark chocolate in terms of inducing coronary vasodilatation and improving coronary vasomotion and shear stress–dependent platelet adhesion, which results in the potential to beneficially affect atherothrombosis. This beneficial potential provides a strong rationale to further investigate the clinical effects of cocoa in cardiovascular disease.

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We thank Astrid Hirt, Priska Kaiser, Rosy Hug, and Ines Bühlér (University Hospital Zurich) for technical assistance.

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Disclosures
The Swiss National Science Foundation was not involved in any part of the study. Nestlé provided chocolate but was not involved in funding or in any part of the study, except in the analysis and interpretation of serum and chocolate polyphenol concentrations. Dr Cooper is an employee of the Nestlé Research Center. Dr Lüscher has been a consultant for MARS Inc and has received research grants not related to the present study. The remaining authors report no conflicts.

References
During the last few decades, consumption of cocoa has increased, probably because of its potential beneficial effects on human health. Recent research revealed that cocoa exerts beneficial cardiovascular effects, most likely mediated by its polyphenols, a heterogeneous group of antioxidant molecules. Cardiovascular events are characterized by vasoconstriction, impaired vasomotion, and platelet activation, which lead to atherothrombosis. Our results suggest a short-term effect of flavonoid-rich dark chocolate in terms of inducing coronary vasodilation and improving coronary vasomotion and shear stress–dependent platelet adhesion and thus suggest the potential of dark chocolate to beneficially affect atherothrombosis. We believe that the observed improvement of these important cardiovascular surrogates is remarkable and that cacao thus holds the potential to play an important role in a well-balanced nutrition plan. However, to make more definitive statements, further studies are needed, especially because placebo-controlled prospective studies of morbidity and mortality are lacking.

**CLINICAL PERSPECTIVE**


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