Dietary flavanols and procyanidin oligomers from cocoa (Theobroma cacao) inhibit platelet function

Karen J Murphy, Andriana K Chronopoulos, Indu Singh, Maureen A Francis, Helen Moriarty, Marilyn J Pike, Alan H Turner, Neil J Mann, and Andrew J Sinclair

ABSTRACT

Background: Flavonoids may be partly responsible for some health benefits, including antiinflammatory action and a decreased tendency for the blood to clot. An acute dose of flavanols and oligomeric procyanidins from cocoa powder inhibits platelet activation and function over 6 h in humans.

Objective: This study sought to evaluate whether 28 d of supplementation with cocoa flavanols and related procyanidin oligomers would modulate human platelet reactivity and primary hemostasis and reduce oxidative markers in vivo.

Design: Thirty-two healthy subjects were assigned to consume active (234 mg cocoa flavanols and procyanidins/d) or placebo (≤6 mg cocoa flavanols and procyanidins/d) tablets in a blinded parallel-designed study. Platelet function was determined by measuring platelet aggregation, ATP release, and expression of activation-dependent platelet antigens by using flow cytometry. Plasma was analyzed for oxidation markers and antioxidant status.

Results: Plasma concentrations of epicatechin and catechin in the active group increased by 81% and 28%, respectively, during the intervention period. The active group had significantly lower P-selectin expression and significantly lower ADP-induced aggregation compared with the placebo group. Plasma ascorbic acid concentrations were significantly higher in the active than in the placebo group (P < 0.05), whereas plasma oxidation markers and antioxidant status did not change in either group.

Conclusions: Cocoa flavanol and procyanidin supplementation for 28 d significantly increased plasma epicatechin and catechin concentrations and significantly decreased platelet function. These data support the results of acute studies that used higher doses of cocoa flavanols and procyanidins.

KEY WORDS Cocoa, antioxidant, catechin, epicatechin, procyanidin, flavanols, platelet activation, P-selectin, CD62P, platelet function, cardiovascular disease, polyphenols, F2-isoprostanes, thiobarbituric acid–reactive substances, TBARS

INTRODUCTION

Nutrition has long been thought to have a profound effect on the development of coronary artery disease. The consumption of phytochemicals, particularly flavonoids, is thought to contribute to this low risk (1). Flavonoids are one subclass of polyphenols and include 7 major groups (Figure 1). The identification of plant-derived antioxidants has led to many in vitro studies and in vivo clinical trials that investigated flavonoids and other polyphenols in foods and beverages (2–9), including cocoa and chocolate (10–13). Cocoa contains monomeric flavanols (epicatechin and catechin) and oligomeric procyanidins (14). Flavonoids (including cocoa flavanols) are thought to decrease certain risk factors for cardiovascular disease (CVD) through a variety of mechanisms, including reduced LDL oxidation (15, 16), improved endothelium-dependent relaxation (17, 18), and modulation of cytokines and eicosanoids involved in the inflammatory response (19–22). Many of the physiologic effects of flavanols and procyanidins, especially from cocoa, may prevent cellular oxidation and scavenge reactive oxygen species. Platelet aggregation is the critical event occurring during the initiation of coronary thrombosis, and cocoa epicatechin and catechin have been reported to modulate platelet function, thus reducing the risk of clot formation (11–13, 23, 24). The rate and extent of absorption and metabolism of polyphenols are determined largely by the chemical structure and the glycosylation, acylation, conjugation, polymerization, and solubility of the compound (25, 26). Monomeric flavonoids are absorbed in the small intestine; however, polymeric procyanidins in particular may be degraded by intestinal and colonic microflora, followed either by absorption of the metabolites or excretion in the feces. After absorption, the monomers (and dimers) may be methylated, sulfated, or glucuronidated in the liver (25, 26).

Most flavonoids, with the exception of epicatechin and catechin, are present in the diet as glycosides (sugar moieties attached). The position of the sugar linkage determines the extent of absorption (26). Aglycones (no sugar) passively diffuse through the small intestine and peak in plasma at 1–2 h after ingestion, whereas glycosidic flavonoids require hydrolysis by colonic microflora to cleave the sugar and release the aglycone for absorption (26). Acylated flavonoids, including epicatechin and catechin, can pass through the mucosal cells of the small intestine and be absorbed with little deconjugation or hydrolysis. Some cells and tissues may in fact accumulate flavonoid conjugates to biologically active concentrations because of high affinity binding to receptors or cell targets (26).

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2 Supported in part by Mars Inc (Hackettstown, NJ), who also kindly supplied the cocoa and placebo tablets for the study.

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Received July 30, 2002.

Accepted for publication November 6, 2002.
Monomeric flavanols appear to be absorbed in a dose-dependent manner, whereby maximum plasma concentrations peak ~2 h after ingestion and return to baseline within 24 h (27, 28). The stability of oligomers in the stomach (29–33) and the absorption of oligomeric flavanols, including cocoa procyanidins, is still in its infancy (29); however, Deprez et al (30) reported that dimeric and trimeric compounds are absorbed through an intestinal epithelium cell monolayer. Similarly, Holt et al (31) also reported the presence of dimeric procyanidins in human plasma after consumption of a cocoa beverage.

Previous studies that investigated platelet function and dietary supplementation of cocoa flavanols and procyanidins (11, 12) have shown that they decrease platelet activation and aggregation ex vivo at high doses (≈900 mg/d). Similarly, Holt et al (13) found that after the consumption of 25 g chocolate, supplying 220 mg flavanols and procyanidins, there was a decrease in primary hemostasis (as measured by the time to occlude an aperture in a collagen membrane) 2 h after consumption. The present study was designed to determine the effect of 4 wk of supplementation with cocoa flavanols and procyanidins (and related oligomers) at a lower dose (234 mg/d) on thrombotic risk factors, which included platelet aggregation and activation. The effect of supplementation on plasma oxidation markers and antioxidant status was also investigated.

SUBJECTS AND METHODS

Subjects and dietary intake

Thirty-two healthy, nonsmoking adults (17 men, 15 women) with no history of heart disease or hemostatic disorders participated in this 28-d double-blind, randomized, placebo-controlled study. The subjects were separated into 2 groups that were sex matched and randomly assigned to consume the active tablet (CocoaPro; Mars Inc, Hackettown, NJ), containing 39 mg cocoa flavanols and procyanidins per tablet, or the placebo tablet (< 1 mg cocoa flavanols and procyanidins per tablet).

Subjects completed a simple questionnaire that screened for the personal and family history of heart disease or thrombosis, diabetes, high cholesterol, or blood pressure and for the use of any medication (particularly statins and blood pressure–lowering and antiinflammatory medications). Subjects were screened about their physical activity levels, dietary supplement use, and dietary intakes of polyunsaturated fatty acid–rich foods, alcohol, and cocoa and chocolate products. Those subjects with high physical activity levels, taking medication, taking dietary supplements, and with high dietary intakes of alcohol, seafood, and cocoa and chocolate products were also excluded from the study. Similarly, subjects with a history of CVD, bleeding disorders, and blood total cholesterol and LDL-cholesterol concentrations > 6.5 and 3.5 mmol/L, respectively, were excluded from the study. Subjects completed two 7-d weighed food records before the study and once during the study, which were assessed by using the DIET VERSION 4 software (Xyris Software, Pty Ltd, Highgate Hill, Australia) with the NUTTAB 95 database. On days 0 and 28, a fasting venous blood sample and anthropometric measurements were taken. All subjects gave written informed consent before commencing the study, which was approved by the Human Research Ethics Committee at RMIT University (Melbourne, Australia) and the Ethics in Human Research Committee at Charles Sturt University (Wagga Wagga, Australia).

Anthropometry and cocoa tablets

Each subject’s height and weight were recorded to calculate body mass index (BMI; in kg/m²). Subjects had their percentage of body fat calculated with a bioimpedance fat analyzer/scale (TBF-501 Tanita Corp, Arlington Heights, IL). Waist and hip measurements were taken with a metric tape measure to calculate the waist-to-hip ratio. Systolic and diastolic blood pressure and pulse were measured with a digital auto-inflating blood pressure monitor (Lumiscope Co, Inc, East Brunswick, NJ).

The study was designed to restrict exogenous dietary polyphenols and substitute with a concentrated source of flavanols and other dietary polyphenols.
procyanidins from cocoa. It was estimated before the intervention that the subjects were consuming \( \leq 1 \text{ g total polyphenols/d, specifically from red wine, oranges, apples and their juices, onions, licorice, and chocolate. Diets were then modified to reduce dietary polyphenols (other than the cocoa source) to } \approx 200–400 \text{ mg/d. During the intervention it was estimated that the active group consumed 200–400 \text{ mg polyphenols/d and an additional 234 mg cocoa flavanols and procyanidins/d, whereas the placebo group consumed 200–400 \text{ mg polyphenols/d and an additional } \leq 6 \text{ mg cocoa flavonols and procyanidins/d.}

Subjects were given the tablets on day 0 and were asked to consume 6 tablets/d for 28 d. Subjects were asked not to consume the tables with hot beverages, but to either chew or swallow them whole. The level chosen (234 mg/d) was approximately equivalent to a serving of dark chocolate (\( \approx 250 \text{ mg cocoa flavanols and procyanidins/50 g} \text{) and was similar to usual dietary intakes of chocolate and cocoa (33). A known surplus of tablets was intentionally supplied to the volunteers. Subjects were asked to return the containers and unused tablets at the final visit. The tablets were counted, and the actual consumption of cocoa flavanols and procyanidins was calculated. All subjects consumed the recommended amount of tablets per day. The total cocoa flavanol and procyanadin intakes over the intervention period for the active group were 6.55 and 0.18 g, respectively, for the placebo group.

**Laboratory methods**

**Sample collection**

Twenty-seven milliliters of venous blood was drawn into evacuated tubes containing EDTA, serum, or sodium citrate (Interpath, Heidelberg West, Australia). Plasma was separated by centrifuging the blood at 1800 \( \times \) g for 10 min at 4 \( ^\circ \)C and stored at \(-70\)\(^\circ\)C in the dark until analyzed (for 2–4 wk). Blood collected for the measurement of isoprostanes and platelet function is described below.

**Plasma (+)-catechin and (-)-epicatechin**

Plasma concentrations of epicatechin were measured as described by Richelle et al (27). Samples were analyzed by using reversed-phase HPLC with electrochemical (coulometric) detection (RP-HPLC-ECD). A reversed-phase Alltima C18 \( 5\mu \text{m, 150 mm } \times \) 4.6 mm column with a C18, 5 \( \mu \text{m guard column (Alltech Associates Inc, Deerfield, IL) was used for separation. The mobile phase consisted of 2 solvent solutions that were mixed according to the detection method used: solvent A, 40% methanol:60% 100 mmol sodium acetate/L in water, pH 5.8; and solvent B, 7% methanol:93% 100 mmol sodium acetate/L in water, pH 5.2.

**Ascorbic acid, retinol, uric acid, trapping radical antioxidant potential, and \( \alpha-, \beta-, \text{ and } \gamma- \text{tocopherol**}

Ascorbic acid was measured colorimetrically (540 nm) with a Varian 635 ultraviolet-visible spectrophotometer (Varian, Mulgrave, Australia) as described by Varley et al (34). Retinol and \( \alpha-, \beta-, \text{ and } \gamma- \text{tocopherol were analyzed according to the method of Catignani and Bieri (35) with a Hewlett-Packard HPLC with a diode array (ultraviolet-visible spectrum) fitted with a reversed-phase Phenomenex Explore Luna C18 (2) (5 \( \mu \text{m, 250 mm } \times \) 4.6mm; Phenomenex, Torrance, CA). The separation system consisted of acetonitrile, dichloromethane, and methanol (Merck, Darmstadt, Germany) in a ratio of 7:2:1 (by vol) in isocratic flow. \( \alpha-, \beta-, \text{ and } \gamma- \text{Tocopherol were detected at 298 nm, and retinol was detected at 325 nm. Serum uric acid was measured with a Hitachi Autoanalyzer system 747 (Hitachi, Tokyo) with the use of Roche Diagnostic uric acid kits (Roche Diagnostics Corp, Indianapolis). The autoanalyzer was calibrated by using calibrator for automated systems. Precinorm L was used as the normal quality control, and precipath L was used as the pathologic quality control (Roche Diagnostics Corp). EDTA plasma (500 \( \mu \text{L}) was analyzed for plasma antioxidant status by using the trapping radical antioxidant potential (TRAP) assay, as previously described by Wang et al (28).

**Plasma lipid and lipoprotein lipids**

Plasma total cholesterol, triacylglycerol, and HDL were measured with the use of an enzymatic colorimetric test on a Hitachi Autoanalyzer system 705 with Boehringer Mannheim test kits (Boehringer Mannheim, Nunawading, Australia). HDL cholesterol was measured after precipitating all plasma lipoproteins except HDL with polyethylene glycol 6000. LDL cholesterol was calculated by using the Friedewald formula (36).

**TBARS and F\(_2\)-isoprostanes**

Thiobarbituric acid–reactive substances (TBARS) were measured according to the method of Halliwell and Chirico (37) by using a Shimadzu liquid chromatograph (LC 10AD) with a Shimadzu RF-535 fluorescence detector (Shimadzu, Tokyo) fitted with a LiChrosorb RP-18 column (150 mm \( \times \) 4.5 internal diameter) (Merck, Germany) and a mobile phase of 50 mmol potassium dihydrogen phosphate/L in methanol (65:35; by vol). Data were quantified with the use of Millennium Integration software (Waters, Milford, MA). F\(_2\)-isoprostanes were measured from 5 mL whole EDTA blood containing glutathione in 0.9% saline (0.1 g/mL blood) to prevent the artificial formation of F\(_2\)-isoprostanes. Butylated hydroxytoluene (4 mg/mL plasma) in ethanol was added to plasma to prevent artificial F\(_2\)-isoprostane production. F\(_2\)-isoprostanes were measured by using a combination of silica and reversed-phase extraction cartridges, HPLC, and gas chromatography–electron capture negative ionization mass spectrometry as described by Mori et al (38).

**Platelet function**

Mean platelet volume (MPV) and platelet count were measured by using whole blood collected in EDTA-containing tubes with the use of a Coulter STKR hematology analyzer (Coulter Electronics Inc, Hialeah, FL). Blood collected in citrate-containing tubes was used to determine the extent of platelet aggregation and to measure the release of ATP from platelets. Whole blood platelet aggregation was measured with an impedance aggregometer (Chrono-Log Corp, Philadelphia) equipped with MacLab software (ADInstruments Pty, Ltd, Castle Hill, Australia) for data quantitation and analysis. Citrated whole blood was diluted with saline, incubated, and mixed with agonists [1 mmol arachidonic acid (AA)/L, 8 \( \mu \text{mol ADP/L, or 2 } \mu \text{g collagen/mL; Chrono-Log Corp}], and aggregation was recorded for 6 min. Additional aliquots of citrated whole blood were diluted with modified tyrode’s buffer and incubated for 5 min in the presence of 3, 10, or 25 \( \mu \text{mol ADP/L. Subsequently, aliquots of activated blood were then incubated in the dark with monoclonal antibodies, phycoerythrin conjugated CD41 (Immunotech, Marseille) which were used to identify platelets because it has specificity for the glycoprotein IIb portion of the glycoprotein IIb-IIIa antigen present on resting and activated platelets), fluorescein isothiocyanate conjugated CD62P (Immunotech;
an activation-dependent antibody directed against P selectin, a component of the α-granule membrane of resting platelets that becomes expressed on the platelet surface membrane upon activation), or one of the isotype controls, immunoglobulin G1 (IgG1). Samples were fixed with paraformaldehyde and incubated to prevent further artifactual in vitro platelet activation. Modified tyrode’s buffer was added to terminate the fixation, and samples were analyzed on an EPICS Elite flow cytometer (Coulter Electronics) equipped with a 15-mW argon laser, with excitation at 488 nm. The fluorescence of fluorescein isothiocyanate and phycoerythrin was detected by using 525- and 575-nm band pass filters, respectively. Single platelets were identified by gating on both phycoerythrin positivity (CD41 binding) and characteristic light scatter. Because single platelets are smaller and less complex than other blood cells, including aggregated platelets, their forward scatter and side scatter are lower in comparison with other cells. Once identified, the expression of P selectin was determined by analyzing 20,000 free platelets, which were collected at a rate between 1300 and 1600 events/s. Activated platelets were defined as CD41-positive events that expressed P selectin.

The data are reported as a proportion of maximum CD62P expression. The results of a preliminary study (results not published) suggest that 25 μmol/L maximally activates CD62P expression. Therefore, the mean fluorescence intensity of platelets activated with 25 μmol ADP/L was arbitrarily assigned 100 units of fluorescence, and the submaximal ADP concentrations were expressed as a proportion of the maximum activation.

Statistical analysis

Four subjects were excluded from the study (one was found not to meet inclusion criteria, 2 withdrew because of family illnesses, and 1 failed to consume the specified number of tablets during the final week of the intervention. For each variable, the postintervention (day 28) value minus the preintervention (day 0) value was compared between groups. Data were not normally distributed; therefore, data were log transformed. Because the data still remained not normally distributed, a nonparametric Friedman’s rank test on analysis of variance ranks was used to test the difference in variable change between the active and placebo groups. If significance was determined with Friedman’s rank test, Wilcoxon’s signed-rank test was used to compare the change between day 28 and day 0 within each treatment group (STATVIEW software for Macintosh, 1992; Abacus Concepts, Inc, Berkeley, CA). Significance was set at P < 0.05 unless otherwise stated.

RESULTS

No side effects from the active or placebo tablets were reported; however, one subject failed to consume the recommended number of tablets and was subsequently excluded from the study, as described above. There was no significant difference between groups in age, weight, percentage body fat, BMI, WHR, systolic and diastolic blood pressures, and pulse (Table 1). These variables were not expected to change, but were recorded on days 0 and 28 to control for any confounding factors. Similarly, there were no significant differences between the dietary intakes (Table 2) or the plasma lipoprotein lipids (data not shown) of either group. Plasma epicatechin and catechin on day 28 were greater in the active group than in the placebo group (P = 0.006 and P = 0.005, respectively) (Table 3).

The mean platelet count was significantly higher (P = 0.0001) and the MPV was significantly lower (P = 0.004) in the active group than in the placebo group on day 28 (Table 4). There were no significant differences in AA-induced platelet aggregation (data not shown) between the active and placebo groups on day 28 or changes within each group from days 0 to 28. Although the change was modest, the active group had significantly lower collagen-induced aggregation (P = 0.031) and ADP-induced aggregation (P = 0.042) than did the placebo group on day 28. There was also a small but significant decrease in collagen-induced ATP release in the active group (P = 0.006) from days 0 to 28.

The proportion of maximum CD62P expression (P selectin) with the use of 3 μmol ADP/L (P = 0.06) and 10 μmol ADP/L (P = 0.036) was significantly lower in the active group than in the placebo group on day 28 (Table 4). Within the active group, CD62P expression with the use of 3 μmol ADP/L significantly decreased from days 0 to 28 (P = 0.008).

The active group had a significantly higher concentration of plasma ascorbic acid than did the placebo group on day 28 (P = 0.04) (Table 5) and there was a significantly lower concentration of plasma uric acid in the placebo group than in the active group on day 28 (P = 0.006). There were no significant differences in plasma TRAP, F₂-isoprostanes, TBARS, or α-, β-, and γ-tocopherol between days 0 and 28 in either group.

DISCUSSION

Previous studies have shown that short-term ingestion of cocoa powder modulates platelet function (11–13, 28), particularly a
reduction in platelet aggregation and activation. The aim of this study was to determine whether a lower dose of cocoa powder consumed over a 4-wk period would also influence platelet function. The present study found that platelet function was modified, as shown by reduced platelet aggregation, a decreased mean platelet volume (an indicator of lowered activation status), a reduction in the amount of platelet degranulation as measured by ATP-release, and increased plasma ascorbic acid.

Because the changes in platelet function were quite modest (although significant), the biological significance of these results is uncertain. In the future, dose-response studies should be conducted with larger numbers of subjects. Furthermore, it may be important to study “at-risk” subjects with thrombotic disorders (to investigate platelet function) or subjects with increased oxidative stress (to investigate oxidative indexes). There are many potential platelet pathways, which could be influenced by flavonols and procyanidins. In general, platelets may become activated in response to many factors, such as vascular injury and agonistic triggers, some of which are released from stimulated platelets. On activation they undergo a shape change and secrete α granules and other components (39). α Granules additionally initiate the secretion of dense granules, which stimulate the release of ADP, ATP, calcium, and serotonin from the platelet. This synchrony of release causes a conformational change in the platelet and expression of the glycoprotein Ib-IIIa complex on the cell surface, leading to platelet aggregation (39). Aggregation then stimulates the release of hydrogen peroxide from the platelet, which leads to calcium mobilization and eventually further α-granule secretion (39).

Change in platelet shape is also stimulated by protein kinase C, which induces the conversion of ATP to ADP and simultaneous conversion of inactive to active myosin fibers (39). Protein kinase C is directly stimulated by the release of diacylglycerol from the phosphoinositol pathway conversion of phosphoinositol phosphate to inositol phosphate (39). Protein kinase C and inositol phosphate then stimulate calcium release from the dense tubular system. The activation of the phosphoinositol phosphate pathway is stimulated by phospholipase C and by the synthesis of thromboxane 2 (TXA2) from membrane-bound AA (39). Cyclic adenosine monophosphate (cAMP) concentrations are increased by the adenylyl cyclase conversion of ATP, leading to calcium uptake by the dense tubular system, reducing the amount of cytosolic calcium available for the calcium-dependent pathway activation of the platelet (39).

Numerous in vitro and in vivo studies have shown that flavonoids inhibit primary hemostasis and many pathways associated with platelet activation and aggregation (including eicosanoid synthesis, hydrogen peroxide, calcium mobilization, inositol phosphate inhibition, and modulation of cAMP concentrations) (11–13, 22, 40, 41). Pignatelli et al (40) showed that quercetin and catechin (in vitro) synergistically inhibited platelet function by blunting the release of hydrogen peroxide from platelets, subsequently reducing phospholipase C activation, calcium mobilization, and inositol phosphate

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### TABLE 2

Daily nutrient intakes of active and placebo groups on days 0 and 28 after 4 wk of cocoa flavanol and procyanidin supplementation (234 mg/d)

<table>
<thead>
<tr>
<th></th>
<th>Active group (n = 13)</th>
<th>Placebo group (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 28</td>
</tr>
<tr>
<td>Energy (MJ)</td>
<td>9.8 ± 3.6</td>
<td>10.7 ± 5.5</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>95 ± 31</td>
<td>106 ± 65</td>
</tr>
<tr>
<td>Total fat (g)</td>
<td>85 ± 36</td>
<td>97 ± 51</td>
</tr>
<tr>
<td>Saturated fat (g)</td>
<td>35 ± 16</td>
<td>40 ± 24</td>
</tr>
<tr>
<td>Monounsaturated fat (g)</td>
<td>31 ± 13</td>
<td>34 ± 17</td>
</tr>
<tr>
<td>Polyunsaturated fat (g)</td>
<td>12 ± 5</td>
<td>13 ± 6</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>252 ± 109</td>
<td>263 ± 105</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>297 ± 108</td>
<td>316 ± 170</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>990 ± 343</td>
<td>1041 ± 527</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>130 ± 70</td>
<td>115 ± 77</td>
</tr>
<tr>
<td>β-Carotene (µg)</td>
<td>2507 ± 1709</td>
<td>3352 ± 2372</td>
</tr>
<tr>
<td>Retinol (µg)</td>
<td>367 ± 146</td>
<td>397 ± 213</td>
</tr>
<tr>
<td>Total vitamin A equivalents (µg)</td>
<td>781 ± 308</td>
<td>957 ± 444</td>
</tr>
</tbody>
</table>

*± SD. There were no significant differences for any variable (Friedman’s rank test).*

### TABLE 3

Plasma concentrations of (−)-epicatechin and (+)-catechin in the active and placebo groups on days 0 and 28 after 4 wk of cocoa flavanol and procyanidin supplementation (234 mg/d)

<table>
<thead>
<tr>
<th></th>
<th>Active group (n = 13)</th>
<th>Placebo group (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 28</td>
</tr>
<tr>
<td>(−)-Epicatechin</td>
<td>64 ± 69</td>
<td>116 ± 164</td>
</tr>
<tr>
<td>(+)-Catechin</td>
<td>71 ± 60</td>
<td>91 ± 84</td>
</tr>
</tbody>
</table>

*± SD.*

*1 Change from days 0 to 28 significantly different from that in the placebo group, *P* < 0.01 (nonparametric Friedman’s rank test).

*2 Significantly different from day 0 within group, *P* < 0.01 (Wilcoxon’s signed-rank test).*
TXB2 and 12-hydroxyheptadrienoic acid (a product of the metabolism. Mower et al (41) showed, in vitro, a reduction in mechanism was due to reduced TXA2 as a result of reduced AA sized that cocoa flavanols and procyanidins inhibit platelet Similarly, Rein et al (12) and Mower et al (41) also hypothe-
sing cAMP concentrations through increased prostacyclin. Schramm et al (22) speculated that cocoa fla-
reducing plasma leukotriene and increasing prostacyclin con-
siderations of flavonoids were due to cyclooxygenase pathway inhi-
hibition. In addition, Rein et al (11) showed that short-term ingestion of cocoa flavanols and procyanidins suppressed platelet activation as measured by P selectin expression and speculated that the mechanism of action was by the desensitization of platelets to agonists by competing with the platelet receptor or by interfering with signal transduction. In the present study there were several indicators that suggested that the ingestion of cocoa powder for 28 d was associated with modest reductions in platelet function, including 1) reduced mean platelet volume, 2) decreased expression of P selectin (a platelet surface protein), 3) decreased platelet aggregation to ADP and collagen, and 4) decreased collagen-induced ATP release. These global measures of platelet function do not allow us to pinpoint where the active components of cocoa powder are acting on the platelet.

Mean platelet volume was recently identified as an important determinant of platelet reactivity, and increases in MPV have been linked to several cardiovascular conditions (44–47). Kelly et al

TABLE 4
Platelet count, platelet volume, platelet aggregation, and ATP release from platelets and activation (P selectin expression) in the active and placebo groups on days 0 and 28 after 4 wk of cocoa flavanol and procyanidin supplementation (234 mg/d)\textsuperscript{1}

<table>
<thead>
<tr>
<th></th>
<th>Active group (n = 13)</th>
<th>Placebo group (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 28</td>
</tr>
<tr>
<td>Mean platelet count (\times 10^{11}/L)</td>
<td>253.1 ± 61.2</td>
<td>244.4 ± 51.5\textsuperscript{2}</td>
</tr>
<tr>
<td>Mean platelet volume (fL)\textsuperscript{3}</td>
<td>7.63 ± 0.87</td>
<td>7.56 ± 0.87\textsuperscript{2}</td>
</tr>
<tr>
<td>Aggregation (t/s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP-induced</td>
<td>0.07 ± 0.04</td>
<td>0.05 ± 0.03\textsuperscript{2,3}</td>
</tr>
<tr>
<td>Collagen-induced</td>
<td>0.12 ± 0.04</td>
<td>0.10 ± 0.03\textsuperscript{2,3}</td>
</tr>
<tr>
<td>ATP release (t/s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>0.57 ± 0.48</td>
<td>0.55 ± 0.49</td>
</tr>
<tr>
<td>Collagen</td>
<td>1.09 ± 0.58</td>
<td>0.97 ± 0.38\textsuperscript{2}</td>
</tr>
<tr>
<td>P selectin expression (proportion of maximum)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 μmol ADP/L</td>
<td>82.7 ± 6.1</td>
<td>75.9 ± 6.1\textsuperscript{3}</td>
</tr>
<tr>
<td>10 μmol ADP/L</td>
<td>92.3 ± 4.0</td>
<td>89.8 ± 4.0\textsuperscript{1}</td>
</tr>
</tbody>
</table>

\textsuperscript{1} \textsuperscript{1} ± SD.
\textsuperscript{2} Change from days 0 to 28 significantly different from that in the placebo group, \(P < 0.05\) (nonparametric Friedman’s rank test).
\textsuperscript{3} Significantly different from day 0 within group, \(P < 0.05\) (Wilcoxon’s signed-rank test).

TABLE 5
Biomarkers of antioxidant status and oxidative stress in the active and placebo groups on days 0 and 28 after 4 wk of cocoa flavanol and procyanidin supplementation (234 mg/d)\textsuperscript{1}

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 28</td>
</tr>
<tr>
<td>Ascorbic acid (μmol/L)</td>
<td>30 ± 19</td>
<td>51 ± 20\textsuperscript{2,3}</td>
</tr>
<tr>
<td>TRAP (TE)</td>
<td>334 ± 113</td>
<td>388 ± 136</td>
</tr>
<tr>
<td>Uric acid (μmol/L)</td>
<td>335 ± 91</td>
<td>340 ± 89\textsuperscript{2}</td>
</tr>
<tr>
<td>Retinol (μg/mL)</td>
<td>1.4 ± 0.4</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>α-Tocopherol (μg/mL)</td>
<td>9.8 ± 2.0</td>
<td>10.8 ± 2.1</td>
</tr>
<tr>
<td>β- and γ-tocopherol (μg/mL)</td>
<td>0.93 ± 0.32</td>
<td>0.87 ± 0.21</td>
</tr>
<tr>
<td>TBARS (TBA-MDA adduct)</td>
<td>0.99 ± 0.66</td>
<td>1.21 ± 1.00</td>
</tr>
<tr>
<td>F₂-isoprostanes (μg/mL)</td>
<td>0.38 ± 0.15</td>
<td>0.47 ± 0.24</td>
</tr>
</tbody>
</table>

\textsuperscript{1} ± SD. TE, trolox equivalent; TBARS, thiobarbituric acid–reactive substances; TRAP, trapping radical antioxidant potential; MDA, malondialdehyde.
\textsuperscript{2} Change significantly different from that in the placebo group, \(P < 0.05\) (nonparametric Friedman’s rank test).
\textsuperscript{3} Significantly different from day 0 within group, \(P < 0.05\) (Wilcoxon’s signed-rank test).
(46) showed that stearic acid from chocolate significantly reduces MPV after 28 d of supplementation. The present study also found a modest but significant reduction in MPV, suggesting the presence of smaller, less-reactive platelets, which is consistent with the platelet aggregation data obtained.

Given the results of the present study and the findings of others, it is hypothesized that the daily consumption of small amounts of flavanols and procyanidins from cocoa or chocolate, in conjunction with usual dietary intake of flavonoids from mixed food sources, can result in an increase in plasma flavanol concentrations and an increase in the antioxidant capacity of plasma. In the present study, there was an increase in plasma ascorbic acid in the active group. The most likely explanation for this finding is that the subjects increased their intake even though this was not reported in their weighed food records. Subjects were informed on several occasions about antioxidant-rich food sources and they were asked to restrict their consumption of these foods. Epicatechin and catechin have been reported to increase plasma antioxidant status and decrease markers of lipid peroxidation in vitro and in vivo by reducing plasma TBARS and 2,2’-azobis(2-amidino-propane) dihydrochloride–induced oxidation and increasing plasma total antioxidant capacity (10, 23, 47–53). Despite an increase in plasma flavanols and ascorbic acid in the active group, there was no evidence of antioxidant protection as measured by F2-isoprostanes or TBARS. Wang et al (28) and Rein et al (10) showed a significant decrease in TBARS after 2 and 6 h of cocoa flavanol ingestion; however, in that study the plasma epicatechin concentration was twice the concentration seen in the active subjects in the present study. Lotito and Fraga (50) also measured lipid oxidation by using TBARS and showed catechins inhibited the plasma TBARS formation in vitro. The F2-isoprostanes are thought to be a reliable biomarker of whole-body oxidative stress (38). Abu-Amsha Caccetta et al (54) showed that polyphenols in dealcoholized red wine can reduce in vivo lipid peroxidation as measured by F2-isoprostanes in smoking subjects. In contrast, Wang et al (28) and Hodgson et al (55) also found no change in F2-isoprostanes after ingestion of cocoa and tea, respectively. The subjects in the present study were healthy nonsmokers and did not participate in heavy physical exercise, which may indicate minimal in vivo oxidative stress.

In summary, the present study showed that regular consumption of flavanols and procyanidins from cocoa, in a range easily accommodated in a normal diet, increased plasma concentrations of epicatechin and catechin, but there were no effects on markers of oxidation. The cocoa flavanol and procyanidin treatment modestly reduced platelet aggregation and activation. In future, dose-response studies are recommended to determine the most effective intakes of cocoa flavanols and procyanidins in terms of their effects on platelets.

We thank Roberta Holt at the University of California, Davis, for the plasma epicatechin, catechin, and TRAP analyses; Clare Hedkinson, Sue Dingey, Ariana Marsili, and Duo Li for their assistance with various aspects of the study; Sheryl Lazarus and Roger Bektash for their comments; and Duo Li for statistical advice. KJM, AJS, NJM, AHT, and MJP initiated and designed the study. KJM recruited the subjects, prepared the drafts of the paper, and conducted the interviews, dietary analyses, laboratory analyses, data collection, and statistical analyses. AJS supervised the project and secured the funding. AHT, MJP, HM, and MAF supervised the hematologic aspects of the study. AKC and IS helped with the laboratory analyses. MF, SD, CH, and AM provided assistance with blood collection. AJS, NJM, AHT, MJP, AKC, HM, and IS contributed to the drafts of the publication. There were no conflicts of interest.

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