Dark Chocolate Effect on Platelet Activity, C-Reactive Protein and Lipid Profile: A Pilot Study

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Background: Dark chocolate (DC) is one of the richest sources of flavonoids. Since DC has been demonstrated to have beneficial effects on the cardiovascular system, our study examined its effect on platelet reactivity, inflammation, and lipid levels in healthy subjects.

Methods: In 28 healthy volunteers, we analyzed the effect of one week of DC (providing 700 mg of flavonoids/day). The primary outcome was to determine the effects of DC consumption on platelet activity measured by flow cytometry (adenosine diphosphate [ADP]- and arachidonic acid [AA]-induced total and activated glycoprotein (GP) IIb/IIIa as well as P-selectin expression). In addition to this, we measured the effect of DC on high-sensitivity C-reactive protein (hsCRP), high-density lipid cholesterol (HDL) and low-density lipid cholesterol (LDL) levels.

Results: Following seven days of regular DC ingestion, LDL fell by 6% (120 ± 38 vs 112 ± 37 mg/dL, $P < 0.018$) and HDL rose by 9% (66 ± 23 vs 72 ± 26 mg/dL, $P < 0.0019$). ADP- and AA-induced activated GPIIb/IIIa expression was reduced by DC [27.3 ± 27.8 vs 17.4 ± 20.5 mean fluorescence intensity (MFI), $P < 0.006$; and 9.2 ± 6.5 vs. 6.1 ± 2.2 MFI, $P < 0.005$, respectively]. DC reduced hsCRP levels in women (1.8 ± 2.1 vs. 1.4 ± 1.7 mg/dL, $P < 0.04$).

Conclusions: One week of DC ingestion improved lipid profiles and decreased platelet reactivity within the total group while reducing inflammation only in women. Regular dark chocolate ingestion may have cardioprotective properties. Further long-term research is warranted to evaluate the effect of flavonoids on cardiovascular health and to determine whether DC's beneficial effects are related to flavonoids or some yet unknown component. This research is based on a larger study which was presented at the American Heart Association Scientific Sessions 2007.

Key Words: dark chocolate, flavonoid, hsCRP, lipid profile, platelet reactivity

Flavonoids are polyphenolic compounds ubiquitous in fruits and vegetables. They appear in especially high concentrations in the form of flavonols in cocoa and are known to have beneficial antioxidant effects in vitro.[1,2] In addition, they are known to suppress inflammation by inhibiting cyclooxygenase-2, an enzyme that upregulates during inflammation and certain types of tumor formations.[3] Recent re-

Key Points

- Dark chocolate (DC) is one of the richest sources of flavonoids.
- Regular DC ingestion may have cardioprotective properties.
- Further long-term research is warranted to evaluate the effect of flavonoids on cardiovascular health and to determine whether DC's beneficial effects are related to flavonoids or some yet unknown component.

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views also suggest that some flavanols inhibit atherogenesis by interacting with beta-platelet derived growth factor, a potent component that contributes to atherogenesis.[4] Dark chocolate is a potent source of flavonoids. Flavonoids have been proposed as a key protective dietary component capable of reducing the risk of coronary heart disease. Beneficial effects include the reduction of elevated blood pressure in persons with hypertension,[5,6] a reduction in low-density lipoprotein (LDL) cholesterol,[7] an increase in vasodilation or improved endothelial function by increasing nitric oxide bioactivity,[8,9] as well as reversed endothelial dysfunction and inhibition of platelet activation and function.[10]

It has been suggested that platelet function can be inhibited by the ingestion of dark chocolate.[11] One of the ingredients found to be effective in inhibiting platelet aggregation is epigallocatechin gallate (EGCG), a catechin flavonoid found mostly in green tea and dark chocolate.[12] A possible explanation is that flavonoids have the ability to competitively inhibit binding to the platelet-derived growth factor (PDGF)[4] as well as the thromboxane A2 (TXA_2) receptor,[13] therefore effectively negating arachidonic acid (AA) and collagen-induced platelet responses. Platelet function is influenced by the inhibition of TXA_2 formation through the suppression of AA liberation and TXA_2 synthase activity[14]; the antioxidant effectiveness of catechins on lipid peroxidation was found to be mediated by trapping lipid peroxyl radicals and therefore interfering with the free-radical chain-lipid peroxidation reaction.[15] A recent study showed that a snack bar enriched with phytosterol was effective in reducing plasma total and LDL cholesterol levels in a population with hypercholesterolemia[7]; this finding is consistent with data from another study.[16] Other data have clarified that cocoa polyphenols increase high-density lipoprotein (HDL) cholesterol, whereas chocolate fatty acids modify the fatty acid composition of LDL, making it more resistant to oxidative damage in a healthy population.[17] Catechins, flavanoid compounds derived from dark chocolate, have been shown to reduce plasma cholesterol levels and the rate of cholesterol absorption in rats.[18] EGCG seems to play a major dose-dependent role in decreasing cholesterol absorption.

Elevated levels of C-reactive protein have been shown to be associated with an increased risk for cardiovascular events.[19–21] There is little information describing the effect of flavonoids on high-sensitivity C-reactive protein (hsCRP) levels. One study reported hsCRP levels to be unchanged in persons with diabetes after green tea consumption.[22] Another study reported reduced hsCRP levels in patients with coronary artery disease treated with statins and a flavonoid-rich extract of chokeberry fruits.[23] No data have been reported regarding hsCRP in healthy subjects consuming dark chocolate.

The aim of this prospective trial was to assess the effect of dark chocolate consumption on inhibiting platelet activation, lipid levels, and hsCRP in healthy volunteers.

Methods

Design

We conducted a single center controlled trial at Sinai Hospital of Baltimore. All subjects were told to consume 100 grams of Lindt™ (Lindt & Sprüngli, Kilchberg, Switzerland) 70% dark chocolate per day during one week. Blood samples were drawn before and after treatment; at the Sinai Center for Thrombosis Research, blood tests were performed and the subjects were screened for adverse reactions. The Institutional Review Board at Sinai Hospital of Baltimore approved this study conducted between September 2006 and April 2007. All participants gave informed consent.

Participants

Of the 80 individuals who applied for this study, 35 met the inclusion criteria and were enrolled. Seven subjects were later excluded from analysis either because they developed viral infections during the time of the study or were not able to comply with the study requirements. Inclusion criteria included being a nonsmoker between the ages of 18 and 60 with a body mass index (BMI) of 19 to 30. Exclusion criteria included any subjects having used vitamin C $>$1000 mg, vitamin E $>$400 IU, beta carotene $>$1000 IU, vitamin A $>$5000 IU, selenium $>$200 mcg, folic acid $>$1 mg, aspirin or nonsteroidal anti-inflammatory drugs (NSAIDs) within two weeks prior to screening. In addition, the ingestion of green tea, dark chocolate, red wine, red grape juice or other flavonoid-rich foods was not allowed for two weeks prior to the study's initiation as well as during the study, unless provided by study protocol. Consumption of alcohol or caffeine 24 hours prior to the start of each study visit was prohibited. Women participants who were pregnant or nursing a child, or individuals who suffered from an infection, had donated blood within eight weeks prior to screening, or had any coagulation, bleeding or blood disorder were not allowed to participate in the study. Subjects with a history of cancer, cardiovascular disease, or drug or alcohol abuse were excluded. No other dietary modifications were made. Due to the short period of dark chocolate consumption, patients were not advised to participate in additional physical exercise to compensate for the caloric intake.

Intervention

Subjects were asked to consume 100 grams of Lindt™ dark chocolate daily for one week; the prescribed amount contained 70% cocoa and provided 700 mg of flavonoids.[12] Patients were asked not to consume any dark chocolate or flavonoid-rich products two weeks prior to the study. Each subject's blood was drawn pre- and post-treatment for analysis.

Outcomes and Measurements

The primary outcome was the absolute change in platelet activity measured by flow cytometry, HDL, LDL and hsCRP after dark chocolate. Blood samples were obtained in a fasting state between the hours of 7 and 10 AM and transferred to a 3.8% trisodium citrate Vacutainer® blood-collecting tube (Becton-Dickinson, Franklin Lakes, NJ) for platelet function analysis, and to a serum separator tube for all other measurements. Basic metabolic profile, HDL, LDL, triglyceride, and total cholesterol measurements were performed using a Roche Hitachi Modular P800 chemistry analyzer (Diamond Diagnostics, Holliston, MA) and hsCRP measurements by using an Immulite® 2500 Analyzer (Diagnostics Products Corporation [DPC], Los Angeles, CA). Flow cytometry inter- and intra-assay variation was between 5 and 7% each. The coefficient of variation for hsCRP was reported as 5% at the upper reference level (3 mg/dL) by Siemens. (Taken from Siemens product insert sheet for Immulite® 2500 hsCRP; PIL5KCRP-3, 2007-05-21, page 6) The coefficient of variation (COV) for LDL was noted to be between 0.8% to 1.2%, and the COV for HDL between 0.6% to 1.3%, as reported by Roche. (Taken from Roche product insert sheets for the LDL and HDL assays; LDL04843541002, page 7 and HDL04866584003, page 7).

Platelet surface receptor expression was determined by flow cytometry using monoclonal antibodies as previously described.[24,25] The antibody CD41a, which binds to total glycoprotein (GP) IIb/IIIa, was used to identify platelets. P-selectin was expressed as percent positive cells (ie, the ratio of CD62P [CY-Chrome™; Becton Dickinson (BD) Biosciences, San Jose, CA] versus CD41a [R-PE] positive cells) as previously described, activated GP IIb/IIIa was expressed as log mean fluorescence intensity (MFI).[25]

Statistical Analysis

We calculated that a sample size of 28 persons was needed to have 95% power to detect a moderate decrease (mean of 5 mg/dL) in LDL cholesterol, with an overall α value of 0.05. The SigmaStat® (SYSTAT Software, Inc., San Jose, California) and MedCalc software package (MedCalc Software, Mariakerke, Belgium) were used for data analysis. All data are expressed as mean $\pm$ SD. To compare baseline platelet activation, lipid profile, and hsCRP before and after dark chocolate, the nonparametric paired test (Wilkinson test) was used. Differences were considered significant at $P < 0.05$. Analysis of variance (ANOVA) was used to determine whether the effect of the treatment was dependent on variables such as gender or age, measured by the comparison of the absolute changes before and after treatment with dark chocolate.

Results

Subjects

Between August 2006 and April 2007, 80 subjects were screened. A total of 35 subjects (12 men, 23 women) were enrolled in the study. Seven subjects were excluded from the study because of acquired infection or noncompliance to the dietary requirements, leaving 28 with data that were analyzed. The mean age of the subjects was 45 $\pm$ 11 years (range 24 to 60), and the BMI was 23.4 $\pm$ 3.1 (range 19 to 30). A total of 28 subjects (9 males, 19 females) completed the end of the study (Table 1). Besides constipation and nausea in two study subjects, no adverse events were reported. No significant weight change was observed during the short duration of the study.

Table 1. Demographics of subjects who successfully finished the study requirements

	Total group (n = 28) Mean ± SD
Demographics	
Age (yr)	42 ± 12
Male, n (%)	9 (32%)
White (%)	27 (97%)
Body mass index (kg/m^2)	23.5 ± 3.16
Systolic blood pressure (mm Hg)	118 ± 12
Diastolic blood pressure (mm Hg)	74 ± 10
Laboratory data	
White blood cell count (10^6/mL)	5.47 ± 1.96
Platelets (10^6/mL)	213 ± 68
Red blood cell count (10^6/mL)	4.64 ± 0.81
Hemoglobin (g/dL)	14 ± 2
Hematocrit (%)	39.37 ± 7.02
Glucose (mg/dL)	89 ± 10
Blood urea nitrogen (mg/dL)	13.5 ± 4.3
Creatinine (mg/dL)	0.77 ± 0.13

Flow Cytometry

The effect of dark chocolate on platelet GP IIb/IIIa and P-selectin expression in healthy volunteers is shown in Table 2. AA- and adenosine diphosphate (ADP)-stimulated GP IIb/IIIa expression was not significantly affected. Both AA- and ADP-stimulated expression of active IIb/IIIa receptors were significantly lowered with dark chocolate treatment by 33% ($P < 0.005$) and 37% ($P < 0.006$), respectively. A nonsignificant reduction in AA- and ADP-induced P-selectin expression was observed after dark chocolate ingestion.

Inflammatory Markers

The effect of dark chocolate on hsCRP is shown in Table 3. After the ingestion of dark chocolate for one week, levels of hsCRP were reduced by 16%, but these changes were not statistically significant in the total group ($P < 0.2$). When examined by gender however, we found that hsCRP was significantly decreased by 23% in women, ($P < 0.04$). On Figure 1 we display the effects of dark chocolate on hsCRP.

Table 2. Platelet surface markers and platelet activity tests measured with flow cytometry[a]

	Pre-DC treatment		Post-DC treatment		Absolute change	Pre-post DC	ANOVA age[b]	ANOVA gender[c]
	Mean ± SD	95% CI	Mean ± SD	95% CI	Mean ± SD	*P*	*P*	*P*
Total IIb/IIIa expression (MFI)								
1 mmol/L AA	618 ± 144	557–679	586 ± 162	518–655	−31 ± 0.183	0.253	NS	NS
1 μmol/L ADP	818 ± 225	723–913	676 ± 360	524–828	−141 ± 435	0.092	NS	NS
Activated IIb/IIIa expression (MFI)								
1 mmol/L AA	9.2 ± 6.5	6.4–11.9	6.1 ± 2.2	5.1–7.0	−3.1 ± 5.4	0.005	NS	NS
1 μmol/L ADP	27.3 ± 27.7	15.6–39.0	17.4 ± 20.6	8.7–26.1	−9.9 ± 16.4	0.006	NS	NS
P–selectin (% pos. cells)								
1 mmol/L AA	11.8 ± 12.2	6.7–17.0	11.2 ± 7.4	8.1–14.3	−0.6 ± 13.5	0.807	NS	NS
1 μmol/L ADP	26.4 ± 11.6	21.5–31.3	22.9 ± 12.4	17.7–28.2	−3.5 ± 11.9	0.253	NS	NS

Pre–DC treatment stands for baseline values, post–DC treatment equals values after seven days of dark chocolate treatment. It is evident that after treatment with dark chocolate activated IIb/IIIa marker expression on platelets were significantly reduced as measured by MFI.

[a]DC, dark chocolate; ANOVA, analysis of variance; MFI, mean fluorescence intensity; AA, arachidonic acid; ADP, adenosine diphosphate.

[b]Analysis of variance of subjects split in three groups by age (group 1, aged 18–30; group 2, aged 31–45; and group 3, aged 46–60 years) showed no significant difference between the three age-matched groups).

[c]Analysis of variance of subjects compared by gender showed no significant difference between women or men.

Lipid Profile

An 8% increase in HDL levels was observed after dark chocolate consumption ($P < 0.0019$); LDL levels decreased by 6% ($P < 0.018$). Triglycerides (TGL) and total cholesterol (TC) levels were not significantly altered by dark chocolate (Table 3). Using ANOVA, there was no influence of gender on lipid measurements in either treatment group (Fig. 2).

Discussion

It is well known that dark chocolate contains abundant amounts of polyphenols, especially catechins. Our data suggest that the consumption of dark chocolate in moderate amounts is capable of decreasing LDL and increasing HDL significantly, even in a healthy population. This is likely achieved by decreasing cholesterol absorption. Dark chocolate may also protect LDL particles from undergoing oxidation by providing antioxidant protection in the form of polyphenols.[17]

Dark chocolate improved HDL and LDL values and decreased AA- and ADP-induced activated GPIIb/IIIa expression in all subjects; but hsCRP levels were only significantly reduced in women.

The effects of dietary flavanols on platelet activity have been previously described.[26] As shown in our research, the reduction of activated GPIIb/IIIa expression on platelets after

Table 3. Total cholesterol, LDL, HDL, triglycerides, and hsCRP levels before and after chocolate ingestion[a]

	Pre-DC treatment		Post-DC treatment		Absolute change	Pre-post DC	Anova age[b]	Anova gender[c]
	Mean ± SD	95% CI	Mean ± SD	95% CI	Mean ± SD	*P*	*P*	*P*
Lipid profile (mg/dL)								
Total cholesterol	194 ± 37	179–209	194 ± 35	180–209	0 ± 19	0.73	NS	NS
Low density lipoprotein (LDL)	119 ± 38	104–135	112 ± 38	97–128	−7 ± 15	0.018	NS	NS
High density lipoprotein (HDL)	66 ± 23	56–75	71 ± 26	61–82	5 ± 7	0.0019	NS	NS
Triglycerides	90 ± 46	71–109	85 ± 42	68–102	−5 ± 36	0.81	NS	NS
High sensitivity C–reactive protein (mg/L)	1.5 ± 1.8	0.7 ± 2.3	1.2 ± 1.4	0.6 ± 1.8	−0.2 ± 0.7	0.20	NS	0.04

LDL cholesterol was significantly reduced by 7 mg/dL (−6%) and HDL increased by 5 mg/dL (+9%) after 1 week of dark chocolate ingestion. High–sensitivity C-reactive protein (hsCRP) levels were not significantly reduced. Compare with Figure 2 where hsCRP level changes are shown by gender subanalysis.

[a]DC, dark chocolate; ANOVA, analysis of variance.

[b]ANOVA analysis of subjects split in three groups by age (group 1, aged 18–30; group 2, aged 31–45; and group 3, aged 46–60 years) showed no significant difference between the three age matched groups comparing LDL, HDL, and hsCRP.

[c]ANOVA analysis of subjects compared by gender showed no significant difference between women or men regarding lipid profile but significant difference in hsCRP change when comparing women with men.

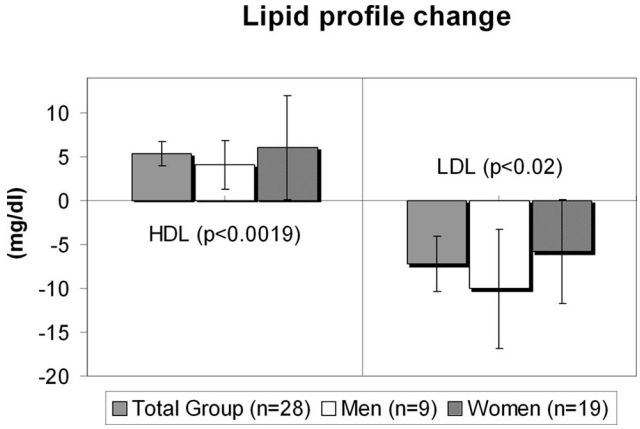

Fig. 1 **This figure shows the effect of dark chocolate on HDL and LDL levels split by total group and gender. *P*-values shown represent the total group. Bars shown over each graph represent SEM (standard error of mean).**

the ingestion of dark chocolate may be an effect of the flavanols contained in the dark chocolate.

Epicatechins, one of the polyphenolic components found in dark chocolate, have been shown to be absorbed intestinally and reach plasma peak concentrations between 17.3 to 228.8 nmol/L two hours after chocolate consumption.[7] Other sources describe the average plasma peak concentration of polyphenols ranging between 250 to 700 nmol/L and occurring two to three hours after ingestion.[12]

Our study is the first to describe a significant decrease in hsCRP following the consumption of dark chocolate in premenopausal women. Prior data have shown a reduction of CRP in menopausal women following the ingestion of soy isoflavones, similar in structure to dark chocolate flavonoids.

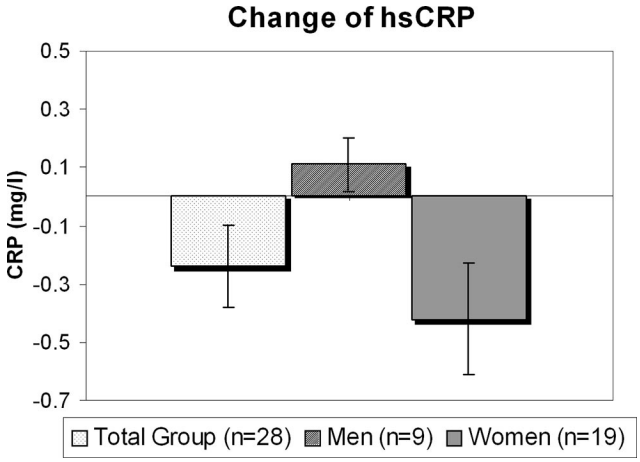

Fig. 2 **Treatment with dark chocolate analyzed by gender in this graph shows significant reduction of hsCRP-levels by 0.2 mg/L (−23%) in females (n = 19) only ($P < 0.047$). For males (n = 9) and the total group *P* was not significant ($P < 0.38$, respectively $P < 0.20$). Bars shown over each graph represent SEM (standard error of mean).**

Dietary isoflavones are also thought to be cardioprotective because of their structural similarity to estrogen. The reduction of concentrations of circulating inflammatory markers by estrogen may be one of the mechanisms by which premenopausal women are protected against cardiovascular disease.[27,28]

The additional caloric intake of 528 calories contained in 100 grams of dark chocolate (containing 33 g carbohydrates, 41 g fat and 6.4 g proteins) has to be considered, and adaptations to the regular diet in regards to further caloric input are necessary should individuals decide to consume dark chocolate on a daily basis. There is a need to find a way of delivering flavonoids, and perhaps other beneficial compounds found in chocolate, without the high caloric intake. Other potential harmful ingredients in dark chocolate, such as caffeine, should also be considered for their effects.

There may be additional, as yet unknown ingredients in dark chocolate that might contribute to the beneficial effects in addition to flavonoids. The long-term effect of dark chocolate on cardiovascular disease is a subject of current ongoing investigation.

Limitations

Despite the small number of subjects enrolled in our study, we were able to report statistically significant reductions in LDL and an increase in HDL, as well as hsCRP reduction in women. Larger powered studies are necessary to confirm our findings.

To limit other flavonoid intake besides the dark chocolate, our subjects had to be instructed not to consume any other flavonoid-rich foods. Compliance regarding the prescribed consumption of the flavonoid-rich chocolate was tested by asking the subjects whether they consumed it, as 100 grams of dark chocolate were too much to be consumed at once in the study center; this could have introduced a margin of error.

We did not measure flavonoid concentration in plasma, as this has been done previously,[29–31] but recognize that this would be helpful in currently ongoing long-term studies.

Additionally, there was a high baseline HDL in women in our study. Likely this was due to the fact that we only enrolled healthy men and women. No subjects were chosen or excluded from the study due to their HDL level.

Finally, a considerable limitation of our study was its short duration. We chose to look at a brief time period (seven days) to avoid confounding problems that might have resulted from the increased caloric intake from the chocolate. This clearly needs to be looked at in more detail in subsequent long-term studies.

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Please see Drs. Manfredi Rizzo and Kasper Berneis' editorial on page 1194 of this issue.