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Regular consumption of cocoa powder with milk increases HDL cholesterol and reduces oxidized LDL levels in subjects at high-risk of cardiovascular disease

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KEYWORDS
Cocoa powder;
Oxidized LDL;
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Abstract *Background and Aims:* Epidemiological studies suggest that regular consumption of cocoa-containing products may confer cardiovascular protection, reducing the risk of coronary heart disease (CHD). However, studies on the effects of cocoa on different cardiovascular risk factors are still scarce.

The aim of this study was too evaluate the effects of chronic cocoa consumption on lipid profile, oxidized low-density lipoprotein (oxLDL) particles and plasma antioxidant vitamin concentrations in high-risk patients.

Methods and Results: Forty-two high-risk volunteers (19 men and 23 women, mean age 69.7 ± 11.5 years) were included in a randomized, crossover feeding trial. All received 40g of cocoa powder with 500 mL of skimmed milk/day(C + M) or only 500 mL/day of skimmed milk (M) for 4 weeks in a random order. Before and after each intervention period, plasma lipids, oxLDL and antioxidant vitamin concentrations were measured, as well as urinary cocoa polyphenols metabolites derived from phase II and microbial metabolisms. Compared to M, C + M intervention increases HDLc [2.67 mg/dL (95% confidence intervals, CI, 0.58–4.73;

Abbreviations: oxLDL, oxidized Low-Density Lipoprotein; HDLc, High Density Lipoprotein cholesterol; LDLc, Low Density Lipoprotein cholesterol; CHD, Coronary Heart Disease; SFA, Saturated Fatty Acids; MUFA, MonoUnsaturated Fatty Acids; PUFA, PolyUnsaturated Fatty Acids; mDP, mean Degree of Polymerization.

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$P = 0.008$)] and decreases oxLDL levels [−12.3 U/L (CI, −19.3 to −5.2; $P = 0.001$)]. No changes between intervention groups were observed in vitamins B1, B6, B12, C and E, or folic acid concentrations. In addition, subjects who showed higher increments in urinary cocoa polyphenol metabolites exhibited significant increases in HDLc and significant decreases in oxLDL levels ($P < 0.05$; all).

Conclusions: Consumption of cocoa power with milk modulates the lipid profile in high-risk subjects for CHD. In addition, the relationship observed between the urinary excretion of cocoa polyphenol metabolites and plasma HDLc and oxLDL levels suggests a beneficial role for cocoa polyphenols in lipid metabolism.

Introduction

Atherosclerosis is considered as a low-grade chronic inflammatory process resulting from interactions between plasma lipoproteins, cellular components and the extracellular matrix of the arterial wall [1]. High-density lipoproteins(HDL) exert anti-inflammatory functions, whereas oxidized low-density lipoproteins(oxLDL), very low-density lipoproteins and lipoprotein(a) are atherogenic lipoproteins that play a critical role in pro-inflammatory reactions. Therefore, an increase in serum HDLc concentrations, a reduction in LDLc levels, and an inhibition of oxidation of LDL particles may prevent the onset and progression of atherosclerosis.

A healthy diet and lifestyle modifications are the first step in the management of cardiovascular disease [2]. Indeed, consumption of polyphenol-rich foods has been associated with a reduced risk of coronary heart disease (CHD) [3]. Among these, cocoa (*Theobroma cacao*) and its derivatives represent a rich source of dietary flavonoids [4]. Evidence based on epidemiological studies suggests that consumption of cocoa-containing products may confer cardiovascular protection, reducing the risk of CHD mortality [5]. In addition, cocoa products may reduce blood pressure (BP) [6], increase plasma antioxidant capacity [7], inhibit oxidation of LDL particles in humans *ex vivo* [8] and reduce biomarkers of oxidation such as F2-isoprostanes and malondialdehyde [9].

However, most of these studies have been performed in healthy volunteers, and outcomes in lipid metabolism from cocoa-feeding trials remain scarce. Indeed, some studies have reported that consumption of chocolate reduces serum LDLc levels [10], whereas others have observed a neutral effect on serum total and LDLc concentrations [11,12]. Other studies carried out in healthy subjects [12] and patients with hypercholesterolemia [13] agree that cocoa consumption may actually increase HDLc levels.

To further evaluate the benefits of cocoa, we performed a randomized, crossover, controlled clinical trial to evaluate the effects of regular consumption of cocoa on classical risk factors for CHD in subjects at high risk of this disease.

Methods

Subjects

A total of 47 high-risk subjects (age $\geq$ 55 years) were recruited for the study in the outpatient clinic of our institution. The subjects included had diabetes mellitus or three or more of the following risk factors: tobacco smoking, hypertension, plasma LDLc $\geq$ 160 mg/dL, plasma HDLc $\leq$ 40 mg/dL for men or $\leq$50 mg/dL for women, overweight or obesity [body mass index (BMI) $\geq$ 25 Kg/m^2] and/or family history of premature CHD. Exclusion criteria included documented cardiovascular events and history of allergic reactions to any cocoa components. The Institutional Review Board of the hospital approved the study protocol, and all participants gave written consent. This trial was registered in the Current Controlled Trials at the International Standard Randomized Controlled Trial Number Register in London, as ISRCTN75176807.

Study design

The study was designed as a 4-week randomized, crossover and controlled clinical trial. After a 2-week lead-in diet, subjects received two sachets of 20 g of soluble cocoa powder(C) per day, one for breakfast and another for an afternoon snack or after dinner (total/day: 40 g) with 250 mL of skimmed milk each (total/day:500 mL) (C + M intervention) or only 500 mL/day of skimmed milk(M intervention) for 4 weeks in a random order. Half received C + M as the first intervention, and the other half, only M. None received multivitamin or vitamin E supplements. The nutritional composition of the soluble cocoa powder (defatted and sugar-free) used in the study is detailed in Table 1. The total phenolic and total proanthocyanidin content of the soluble cocoa powder was determined by using the Folin–Ciocalteu [14] and Bathe–Smith methods [15], respectively. Individualized phenolic compounds were determined by HPLC. The mean degree of flavanol polymerization (mDP) in the soluble cocoa powder was 8, as estimated by thiolysis.

Diet monitoring

All participants in the study followed an isocaloric Mediterranean-type diet and were asked to exclude all other cocoa-containing foods. At the study onset and after each intervention period, a 3-day validated food recall questionnaire was used to assess nutrient intake. Energy and nutrient intake was calculated from Spanish food composition tables [16], using the Professional Diet Balancer software (Cardinal Health Systems, Inc., Edina, MN). Throughout the study, dieticians assessed any adverse effects from the interventions and gave advice on possible remedies.

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Table 1 Nutritional composition of the soluble cocoa powder used in the study.[a]

Parameter	Mean value
Macronutrients	
Total carbohydrates (g)	21.5
Starch (g)	6.4
Dietary fibre (g)	3.6
Total fat (g)	2.10
SFA (g)	1.3
MUFA (g)	0.69
PUFA (g)	0.07
Cholesterol (mg)	26.3
Proteins (g)	6.8
Micronutrients	
Potassium (mg)	492
Phosphorous (mg)	349
Calcium (mg)	285
Magnesium (mg)	119
Sodium (mg)	72.8
Iron (mg)	17.6
Zinc (mg)	1.6
Copper (μg)	1.1
Vitamin B1 (mg)	0.11
Vitamin B2 (mg)	0.18
Vitamin B3 (mg)	0.72
Vitamin B5 (mg)	0.60
Vitamin B9 (μg)	8.0
Theobromine (g)	0.44
(+)-Catechin (mg)	10.41
(−)-Epicatechin (mg)	46.08
Procyanidin B2 (mg)	36.54
Vanillin (mg)	37.79
Isoquercetrin (mg)	2.23
Quercetin (mg)	0.22
Quercetin-3-arabinoside (mg)	0.70
Quercetin-3-glucuronide (mg)	0.10
Total polyphenols (mg)	495.2
Total proanthocyanidins (mg)	425.7

[a] Mean value for 40 g of soluble cocoa powder. SF: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. Total polyphenols were determined using the Folin—Ciocalteau reagent and total proanthocyanidins by using the Bathe—Smith method.

Measurements

Before and after each intervention period, all clinical procedures were undertaken in accordance with a previous operation manual. Weight and height were measured with calibrated scales and a wall-mounted stadiometer, respectively. Trained personnel measured BP in triplicate with a semi-automatic oscillometer (Omron HEM-705CP, The Netherlands).

Fasting blood samples were also collected at baseline and after each intervention. Analytes determined in serum or plasma as appropriate were: blood glucose using the glucose-oxidase method; cholesterol and triglyceride levels using enzymatic procedures; HDLc level after precipitation with phosphotungstic acid and magnesium chloride; apolipoproteins A1 and B using turbidimetry; lipoprotein(a) by using ELISA techniques and homocystein by fluorescence polarization immunoassays. Circulating oxLDL in plasma was measured by ELISA, using the mAb-4E6 antibody (Mercodia AB, Uppsala, Sweden). Other blood parameters were evaluated, including red cell, leukocyte and platelet counts, haemoglobin, fibrinogen, transferrin, creatinine, electrolytes, uric acid, and serum aspartate and alanine aminotransferases. Plasma and intra-erythrocyte levels of folic acid levels, as well as serum vitamins B1, B6, B12, C and E concentrations were determined.

Cocoa flavonoid derived from intestinal and hepatic metabolism (phase II metabolism) of cocoa (−)—epicatechin(i.e., epicatechin—*O*—glucuronides, epicatechin sulfates, *O*—methyl epicatechin—*O*—glucuronides, and *O*—methyl epicatechin sulfates) and intestinal microbial metabolism of cocoa flavonoids(hydroxyphenylvalerolactones, hydroxyphenylpropionic acids, hydroxyphenylacetic acids, hydroxycinnamic acids and hydroxybenzoic acids) were measured in 24-h urine samples using liquid chromatography tandem mass spectrometry [17], as nutritional biomarkers of compliance. In order to do a targeted study of microbial metabolites, hydrolyzed urine samples were screened as previously described [17].

Statistical analysis

Statistical analysis was performed using the SPSS Statistical Analysis System (version 15.0; SPSS Inc., Chicago, IL). Descriptive statistics were used for the baseline characteristics of the participants. Before analysis, we transformed variables with skewed distribution to their natural logarithm for analysis. One-factor analysis of variance (ANOVA) for repeated measures with the Bonferroni post hoc test was used to compare changes in outcome variables in response to the intervention treatments. To exclude the presence of a carryover effect for the two periods, we compared the differences in the parameters obtained from the group who started with C + M intervention with the parameters obtained from those who started with M intervention. In subgroup analysis, the relationship between qualitative and quantitative variables was determined by Pearson's chi-square and unpaired Student *t* tests, when indicated. Within- and between-group differences were expressed as means and 95% confidence intervals (CI). P was considered significant when < 0.05.

Results

Subject characteristics and diet monitoring

From the 47 eligible subjects 5 declined to participate. Thus, 42 volunteers were finally included in the study (19 men and 23 women, mean age 69.7 ± 11.5 years). Almost 70% were overweight or obese (body mass index-BMI $\geq 25\,Kg/m^2$), half of the volunteers were hypertensive and more than one third had dyslipidemia. Table 2 summarizes the baseline characteristics of the subjects included divided by gender. No changes in the medications received were reported by the subjects throughout the study. Except for one subject reporting constipation during the C + M period, no other participants reported any side effects during the two phases of the study. The constipation reported was resolved by increasing fibre

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Table 2 Baseline characteristics of the study participants divided by gender.

Characteristics	Men ($n = 19$)	Women ($n = 23$)
Age, mean (SD), years	69 (11)	70 (12)
Family history of CHD, No. (%)	3 (16)	4 (17)
Current smokers, No. (%)	4 (21)	3 (13)
Type 2 diabetes mellitus, No. (%)	10 (53)	12 (52)
Hypertension, No (%)	15 (79)	18 (78)
Dyslipidemia, No. (%)	12 (63)	14 (61)
BMI†, mean (SD), Kg/m^2	26.6 (2.6)	28.3 (5.9)
Overweight or obesity (BMI $>$ 25 g/m^2), No. (%)	16 (84)	19 (83)
Medications, No. (%)		
ACE inhibitors	10 (53)	14 (61)
Diuretics	6 (32)	7 (30)
Other antihypertensive agents	2 (11)	2 (9)
Statins	10 (53)	13 (57)
Other lipid-lowering agents	2 (11)	2 (9)
Insulin	1 (5)	0 (0)
Oral hypoglycemic drugs	10 (53)	12 (52)
Aspirin or other antiplatelet drugs	6 (32)	5 (22)
Occupations, No. (%)		
Unskilled	4 (21)	6 (26)
Skilled manual	6 (32)	8 (35)
Skilled non-manual	5 (26)	6 (26)
Directive and professional	4 (21)	3 (13)
Education level, No. (%)		
Primary school	11 (58)	15 (65)
First degree high school	6 (32)	7 (30)
High school or university	2 (11)	1 (4)

Abbreviations: CHD, coronary heart disease; BMI, body mass index; ACE, angiotensin-converting enzyme.
†Calculated as weight in kilograms divided by the square of height in meters.

intake during both intervention periods. The daily energy and nutrient intake estimated from the 3-day food recall questionnaire did not differ after each intervention period (Table 3). Compliance was assessed according to the participants' reports and none were classified as non-compliant. In addition, as another measure of compliance, cocoa polyphenol metabolites were determined in 24-h urine before and after each intervention. The analysis of 24-h urine revealed significant increases of phase II metabolites, including glucuronide and sulfate conjugates of (−)−epicatechin, *O*−methyl−epicatechin, 5-(3,4-dihydroxyphenyl)-γ-valerolactone and 5-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone, after C + M. Consumption of cocoa powder with milk per day resulted in a significant increase in the total amount of phase II (−)−epicatechin metabolites(TAEM)(i.e., Σ epicatechin−*O*−glucuronides, epicatechin sulfates, *O*−methyl epicatechin−*O*−glucuronides, and *O*−methyl epicatechin sulfates) in 24 h [mean increase of 15.21 μmol/d (CI, 9.44−20.97 μmol/d); $P < 0.001$], in comparison to that observed after the M intervention. Phase II metabolites of phenylvalerolactones(i.e., Σ 2 glucuronide conjugates and a sulfate conjugate of 5-(3,4-dihydroxyphenyl)-γ-valerolactone, a glucuronide conjugate and 2 sulfate conjugates of 5-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone) significantly increased in 24 h urine after C + M compared to M intervention [mean increase of 588.12 μmol/d (95% CI, 291.5−884.7 μmol/d); $P < 0.001$]. Considering microbial-derived phenolic metabolites, vanillic, 3,4-dihydroxyphenylacetic and 3-hydroxyphenylacetic acids, and 5-(3,4-dihydroxyphenyl)-γ-valerolactone, significantly increased after C + M compared to the M intervention. The mean increase in the total excretion of microbial-derived phenolic metabolites in 24 h was 75.10 μmol/d (CI, 34.02−116.19 μmol/d);$P<0.01$].

Lipids, lipoproteins, oxidized LDL and other risk factors

The concentration of HDLc was significantly higher after C + M intervention compared to M intervention (5% increase; $P = 0.008$) (Table 4), whereas oxLDL levels exhibited a significant decrease after C + M intake (14% decrease; $P = 0.001$). No significant changes were registered for total plasma cholesterol, triglycerides, LDLc, apoliprotein A1 and B and lipoprotein concentrations, or LDLc/HDLc ratio. Body weight slightly increased after both interventions, with a mean increase of 0.50 Kg (CI, 0.20−0.80 Kg; $P < 0.002$) after the C + M compared to M intervention. No significant changes were observed in BP, or in heart rate between the two interventions. No differences were observed in the effects of both interventions between subjects starting with the C + M intervention compared to those starting with the M intervention.

Other biochemical parameters

Table 4 shows the other biochemical parameters and vitamins determined. No significant changes were found in

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Table 3 Daily energy and dietary intakes in the 42 subjects studied at baseline and after both interventions (The data represent the daily energy and dietary intakes, exclusive of the intake of the tested products).

	Baseline	C + M intervention	M intervention	*p*
	Mean (CI)	Mean (CI)	Mean (CI)	
Energy (kcal/d)	1721 (1585–1857)[a]	1662 (1503–1820)[a]	1706 (1566–1846)[a]	0.411
Proteins (g/d)	86.9 (79.9–93.9)[a]	94.2 (87.8, 100.6)[a]	96.5 (89.4, 103.6)[b]	0.199
Carbohydrates (g/d)	202.9 (182.7–223.3)[a]	187.2 (165.6–208.7)[a]	198.6 (177.5–219.7)[a]	0.182
Dietary fibre(g/d)	24.9 (21.0–28.8)[a]	19.6 (16.9–22.4)[a]	20.8 (17.7–23.9)[a]	0.144
Total fat (g/d)	63.2 (56.7–69.6)[a]	58.5 (51.5–66.1)[a]	58.2 (52.5–63.9)[a]	0.185
SFA (g/d)	17.3 (14.9–19.6)[a]	14.3 (12.4, 16.2)[a]	14.4. (12.5–16.3)[a]	0.198
MUFA (g/d)	30.9 (27.5, 34.4)[a]	30.0 (27.5, 34.4)[a]	29.7 (26.8–32.6)[a]	0.523
PUFA (g/d)	9.6 (8.4, 10.8)[a]	9.5 (7.8, 11.2)[a]	9.3 (8.0–10.5)[a]	0.717
Cholesterol (mg/d)	289.1 (241.3, 336.9)[a]	290.5 (251.2, 329.8)[a]	264.5 (234.2–294.8)[a]	0.401
Vitamin C (mg/d)	171.1 (135.3, 206.9[a]	171.8 (142.7, 200.8)[a]	169.7 (136.5–203.0)[a]	0.917
Vitamin A (mg/d)	7440.9 (5541.7–9340.3)[a]	8377.0 (6229.1–10525.9)[a]	7019.2 (5234.6–8803.8)[a]	0.482
Vitamin E (μg/d)	8.6 (7.4–9.9)[a]	9.0 (7.6–10.4)[a]	8.4 (7.7–9.2)[a]	0.551
Total polyphenols (mg/d)	307.1 (228.4–386.4)[a]	334.3 (267.6–400.9)[a]	349.1 (273.6–424.6)[a]	0.368

C + M, cocoa + milk intervention; M, milk intervention; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Changes in outcome variables in response to the intervention treatment were determined by repeated-measures ANOVA. Values in a row with different superscript letters are significantly different, $P < 0.05$ (Bonferroni post hoc test).

the plasma concentrations of vitamins B1, B6, B12, C and E, or in plasma and intra-erythrocyte folic acid concentrations after C + M consumption. Similarly, levels of fibrinogen and homocystein did not differ between interventions.

Correlation between changes in polyphenols, lipids and oxLDL measurements

Twenty-eight out of 42 participants (67%) showed an increase in plasma HDLc concentration, as well as a decrease

Table 4 Changes in body weight, blood pressure, and biochemical parameters at baseline and after the two interventions [means (95% confidence intervals -CI-]. Changes in outcome variables in response to the intervention treatment were determined by repeated-measures analysis of variance.

	Baseline	C + M intervention	M intervention	*p*
	Mean (CI)	Mean (CI)	Mean (CI)	
Weight (Kg)	74.0 (69.5–78.4)[a]	74.1 (69.4–78.7)[a]	73.6 (69.2–78.1)[b]	0.030
BMI (Kg/m^2)	27.6 (26.0–29.1)[a]	27.7 (26.2–29.3)[a]	27.4 (25.8–28.9)[b]	0.042
Systolic blood pressure (mm Hg)	138 (130–146)[a]	138 (131–142)[a]	135 (126–1437[a]	0.366
Diastolic blood pressure (mm Hg)	84 (80–88)[a]	82 (77–87)[a]	81 (76–86)[a]	0.320
Heart rate (bpm)	73 (67–78)[a]	74 (69–79)[a]	75 (69–80)[a]	0.415
Glucose (mg/dL)	121 (109–133)[a]	130 (111–148)[a]	124 (108–139)[a]	0.114
Total Cholesterol (mg/dL)	225 (212–238)[a]	222 (208–236)[a]	221 (207–233)[a]	0.305
Triglycerides (mg/dL)	127 (107–145)[a]	118 (98–138)[a]	119 (102–136)[a]	0.174
LDLc (mg/dL)	176 (165–188)[a]	174 (165–181)[a]	170 (160–182)[a]	0.370
HDLc (mg/dL)	51.8 (47.6–56.0)[a]	54.6 (50.2–59.0)[b]	52.0 (47.4–56.5)[a]	0.027
LDLc/HDLc ratio	3.29 (2.98–3.61)[a]	3.15 (2.79–3.51)[a]	3.22 (2.89–3.54)[a]	0.353
Lipoprotein a (mg/dL)	38.6 (20.9–56.1)[a]	39.3 (24.4–54.1)[a]	40.7 (24.2–57.2)[a]	0.618
OxidizedLDL (U/L)	96.1 (90.5–101.7)[a]	82.0 (73.9–90.2)[b]	94.3 (88.1–100.5)[a]	0.001
Fibrinogen(g/L)	4.17 (3.82–4.53)[a]	4.21 (3.90–4.52)[a]	4.09 (3.84–4.34)[a]	0.444
Vitamin B1 (μg/dL)	54.5 (45.8–63.3)[a]	55.9 (49.7–62.1)[a]	60.2 (51.1–69.2)[a]	0.242
Vitamin B6 (nmol/L)	46.6 (17.8–75.5)[a]	65.3 (22.8–107.7)[a]	51.8 (12.1–91.5)[a]	0.075
Vitamin B12 (pg/mL)	454 (404–505)[a]	487 (436–540)[a]	488 (443–532)[a]	0.131
Vitamin C (μg/ml)	36.1 (27.6–44.7)[a]	32.7 (26.6–38.7)[a]	38.7 (30.9–46.5)[a]	0.229
Vitamin E (μg/ml)	41.7 (33.0–50.5)[a]	42.6 (33.2–52.1)[a]	33.4 (27.6–39.3)[a]	0.175
Folic acid (serum) (ng/mL)	10.4 (8.6–12.3)[a]	9.4 (7.9–10.9)[a]	10.4 (8.7–12.1)[a]	0.076
Folic acid (erytrocyte) (ng/mL)	466 (430–502)[a]	483 (439–527)[a]	487 (443–533)[a]	0.355
Homocystein (mmol/L)	14.4 (12.3–16.4)[a]	12.8 (11.4–14.2)[a]	12.3 (11.0–13.7)[a]	0.075

The means with the same letter (super index) are not significant by the Bonferroni post hoc test. C + M: Cocoa + milk intervention; M: Milk intervention. BMI: Body mass index; LDLc: low-density lipoprotein cholesterol; HDLc: high-density lipoprotein cholesterol.

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in oxLDL concentration after the C + M intervention, whereas only seven (17%) exhibited the opposite (Chi square 13.03; $P < 0.001$). In addition, subjects exhibiting these conditions also showed higher increments in the urinary excretion of cocoa (−)−epicatechin metabolites. Thus, subjects who presented increased HDLc levels and decreased oxLDL after C + M, showed a significant increase in urinary levels of total amount of phase II (−)−epicatechin metabolites(TAEM) [+13.37 μmol/d (CI, 3.38−23.36 μmol/d; $P = 0.010$), compared to their counterparts.

On the other hand, on comparing the subjects showing an increase in TAEM in urine (high excretors) to those with no changes (low excretors), plasma HDLc concentrations [mean increase of 8.88 mg/dL (CI, 3.50−14.26 mg/dL; $P = 0.002$)] (Fig. 1) significantly increased and plasma oxLDL [mean decrease of −17.14 U/L (−30.52 to −3.75 U/L); $P = 0.017$] significantly decreased.

Regarding cocoa metabolites derived from intestinal microbial metabolism, the participants showing an increase in the urinary excretion of 3-hydroxyphenylacetic and vanillic acids after C + M, also presented a significant increase in plasma HDLc concentration [mean increase of 3.94 mg/dL (CI, 0.02−7.86 mg/dL; $P = 0.049$) and 7.73 mg/dL(CI, 0.46−8.99 mg/dL; $P = 0.031$), respectively] compared to their non-excretor counterparts after C + M treatment(Fig. 2). Moreover, subjects with a decrease in plasma oxLDL levels exhibited a significant increase in the urinary levels of 3-hydroxyphenylacetic and vanillic acids [mean increase of 19.63 μmol/day (CI, 1.73−37.52 μmol/day; $P = 0.041$), and 48.81 μmol/day (CI, 5.80−91.81 μmol/day; $P = 0.027$), respectively].

Discussion

The results of the current study show that consumption of soluble cocoa powder over 4 weeks significantly increased plasma HDLc levels and decreased oxLDL concentrations in high-risk subjects. In addition, these changes appeared to be associated with the increases in the urinary levels of phase II and microbial-derived metabolites of cocoa flavonoids, suggesting a relationship between the polyphenol cocoa intake, changes in lipid profile, and serum oxLDL measurements.

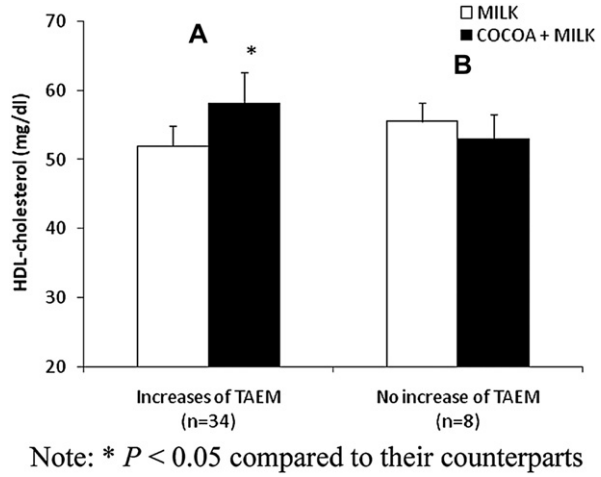

Figure 1 Plasma HDLc concentrations in subjects who showed an increase in total amount of (−)-epicatechin metabolites (TAEM) in urine (A) ($n = 34$) after C + M (dark column) and M (white column) interventions, compared to those who did not showed changes in TAEM (B) ($n = 8$).

Cardiovascular disease is the leading cause of death and disability in developed countries. Diet is the major factor contributing to the onset and development of atherosclerosis and consequently CHD. High intake of calories and certain fats increases the risk for CHD [1], but diet also provides macro- and micronutrients that seem fundamental in controlling cardiovascular disease. Additionally, it is important to understand how macro- and micronutrients can interact with biological systems to enhance health. OxLDL plays a key role in the development of atherosclerosis. Oxidation of LDL lipids and apolipoproteins leads to a change in the conformation of the lipoprotein particles that facilitates their uptake by macrophages resident in the arterial wall, thereby promoting the atherosclerotic process [18]. However, despite the results of epidemiological studies showing an inverse association between customary intakes of dietary antioxidants and CHD risk, clinical trials with antioxidant vitamin supplements have shown no benefit or indeed may increase mortality [19]. Since recent results from the INTERHEART study support the notion that antioxidants in a normal diet protect against CHD [20], probably lifelong consumption of the complex mixtures of antioxidants present in vegetables might be more beneficial than large doses of a single antioxidant given for a finite period. The latter might deplete endogenous antioxidant pools, thus turning an antioxidant effect into a pro-oxidant one *in vivo*, as shown for vitamin E.

We used antibodies directed against MDA-lysine epitopes on LDL to measure oxLDL. The same assay was used in studies showing lower oxLDL in subjects adhering to Mediterranean-type diets [21]. OxLDL measured by this method correlates directly with LDLc and inversely with HDLc [22]. The mean decrease in oxLDL was −12.3 U/L after the C + M compared to the M intervention. In a recent study using the same oxLDL assay [23], the mean difference in oxLDL between CHD patients and healthy controls was quite similar, 17 U/L. However, current state of the art does not allow estimation of the weight of the CHD risk associated with a given change in oxLDL.

To date, few human feeding trials have been conducted to study the effect of cocoa consumption on lipid metabolism. Some studies have shown that consumption of cocoa powder significantly inhibited susceptibility of LDL to oxidation in healthy humans [8] and others that cocoa products increased serum total antioxidant capacity and HDLc concentrations, but only modestly reduced LDL oxidation susceptibility [11]. Similarly, in one study performed in patients with hypercholesterolemia, consumption of cocoa powder increased plasma HDLc concentrations and decreased oxLDL and apo B concentrations [13]. In the current study, 4-week consumption of soluble cocoa powder also significantly increased the concentration of HDLc and reduced oxLDL levels in high-risk subjects. Differences in outcomes among the different trials could be attributed to the cocoa polyphenol dose intake, duration of intake, gender, age and initial serum lipid concentrations or health status of volunteers.

The absorption of flavanols, the main polyphenols from cocoa, in the human body is well documented. Monomeric flavanols and dimeric procyanidins are directly absorbed in

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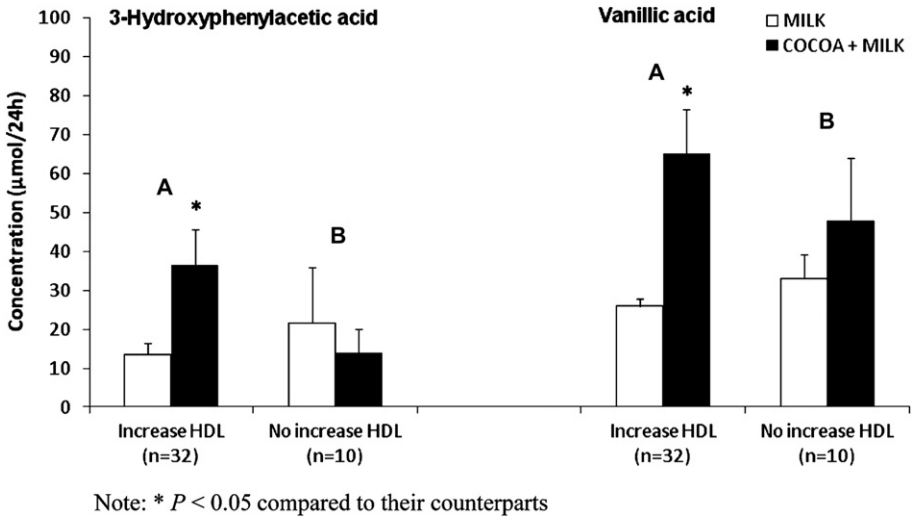

Figure 2 Changes in urinary excretion of microbial-derived phenolic metabolites in subjects who showed an increase in plasma HDLc concentration (A) after cocoa + milk (dark column) and milk (white column) interventions compared to those who did not showed changes in lipid profile (B).

the small intestine. Oligomers with a mean degree of polymerization (mDP) $>$ 3 and polymeric flavanols (proanthocyanidins or condensed tannins) are not absorbed in their native forms. These compounds reach the colon where they are metabolized by the intestinal microbiota into various phenolic acids and other compounds that can be further absorbed and reach the liver. Microbial metabolites circulate and are excreted in their conjugated forms (sulfate and glucuronide conjugates). Some of the absorbed polyphenols could be bound to LDL particles and inhibit their oxidation [24]. In humans, a positive correlation has been found between the reduction in the susceptibility of LDL to oxidation and the concentration of wine polyphenols in LDL particles [25].

The HDL-increasing effect of polyphenols has also been reported. Long-term administration of hesperidin and glucosyl hesperidin significantly increased HDLc levels in spontaneously hypertensive rats [26]. In a meta-analysis, soy protein containing isoflavones significantly increased HDLc, but the changes were related to the level and duration of intake and the sex and initial serum lipid concentrations of the subjects [27]. Although the exact mechanism implicated in the rise in HDLc levels by cocoa polyphenols remains to be elucidated, polyphenols such as genistein can increase the expression and secretion of apolipoprotein (Apo) A1, the major protein component of HDL particles [28]. Our study also showed that cocoa powder-enhanced plasma HDLc concentrations positively correlated with the urinary excretion of cocoa polyphenol metabolites. These findings suggest that absorbed flavanoids in cocoa powder may favour an increase in plasma HDLc concentrations. The potential formation of micelles in the intestine, thus modifying fat absorption, is one hypothetical mechanism. Similar to other polyphenol-rich foods such as wine or virgin olive oil, other possibilities include increases of the synthesis of Apo A1 and Apo A2, increases in the efflux cholesterol promoter, ATP-binding cassette transporter (ABC) A1, increases in phospholipid transfer protein activity and decreases in cholesteryl ester transfer protein (CETP) activity [29].

Conversely, no significant differences were observed between the two interventions in plasma vitamins or intra-erythrocyte folic acid concentrations. However, it should be indicated that plasma concentrations of some vitamins (C and folic acid) were slightly lower after cocoa intake. Recently, new research concluded that choosing between adding full-fat or skimmed milk to tea may influence the antioxidant capacity of the beverage [30]. Therefore, we wonder whether the addition of skimmed milk to cocoa powder limits the beneficial effects of the latter and also reduces the absorption of some vitamins.

With regard to side effects, none of the participants reported any important side effects related to the cocoa intervention. However, a slight but significant increase in body weight was observed after the intake of 40 g cocoa plus 500 mL of milk per day for 28 days. The reported energy intake of the participants was similar in both intervention periods, thus, changes in body weight should be attributed to the calories added to the diet because of the intervention. Since no changes in anthropometric variables were observed in other trials [6], further studies are needed for a more in-depth exploration of the relationship between body weight and cocoa intake.

Our study has several strengths, such as its design, which is able to provide first-level scientific evidence in real-life conditions. It also has limitations. One limitation was to ensure participant compliance with dietary instructions. Adherence to the supplemental foods, however, was good, as determined by objective measures of compliance. Additionally, a one-month period provides no information about the sustainability or long-term effects of cocoa on cardiovascular risk factors.

In summary, the findings of this work indicate that the consumption of cocoa polyphenols may contribute to an increase in HDLc and that this increase, together with the antioxidant protection of polyphenols incorporated into LDL particles, may reduce the levels of oxLDL. In addition, subjects showing higher increments in the urinary excretion cocoa polyphenol metabolites also exhibited an improved lipid profile, with higher HDLc and lower oxLDL levels. The results of this study provide further evidence for recommending regular consumption of cocoa as a useful tool against risk factors for CHD.

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