Natural cocoa consumption: Potential to reduce atherogenic factors?

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Received 25 August 2014; received in revised form 19 December 2014; accepted 19 December 2014

Abstract

Short-term consumption of flavanol-rich cocoa has been demonstrated to improve various facets of vascular health. The purpose of the present study was to determine the effect of 4 weeks of natural cocoa consumption on selected cardiovascular disease (CVD) biomarkers in young (19–35 years) women of differing body mass indices (BMI; normal, overweight or obese). Subjects (n=24) consumed a natural cocoa-containing product (12.7 g natural cocoa, 148 kcal/serving) or an isocaloric cocoa-free placebo daily for 4 weeks in a random, double-blind manner with a 2-week washout period between treatment arms. Fasted (>8-h) blood samples were collected before and after each 4-week period. Serum was analyzed to determine lipid profile (chemistry analyzer) and CVD biomarkers (26 biarkers). EDTA-treated blood was used to assess monocytes (CD14, CD16, v11b and CD62L), while citrate-treated blood was used to measure changes in endothelial microparticles (EMPs; CD42a−/−/45−/144+) by flow cytometry. Natural cocoa consumption resulted in a significant decrease in haptoglobin (P=.034), EMP concentration (P=.017) and monocyte CD62L (P=.047) in obese compared to overweight and normal-weight subjects. Natural cocoa consumption regardless of BMI group was associated with an 18% increase in high-density lipoprotein (HDL-c) (P=.034), and a 24% decrease in monocyte CD62L expression in (P=.047) following 4 weeks of natural cocoa consumption. Collectively, these findings indicate that acute natural cocoa consumption was associated with decreased obesity-related disease risk. More research is needed to assess the stability of the observed short-term changes.

Keywords: Flavanol-rich cocoa; Monocyte adhesion molecule; Endothelial microparticle; Inflammation; Chronic disease

1. Introduction

Over the past decade, nutrition researchers have linked short-term consumption of flavanol-rich cocoa to alterations in vascular health and antioxidant defense [1,2]. Animal models have reported that long-term consumption to reverse inflammation and dietary endotoxinemia caused by high-fat feeding [3]. The most common effects attributed to short-term natural cocoa intake are improved flow-mediated dilation [4,5], increased vascular compliance [6–8], increased high-density lipoprotein cholesterol (HDL-c) [1,4,7] and decreased monocyte adhesion molecule expression (CD11b and CD62L) [6]. Increased HDL-c coupled with reduced monocyte adhesion may reflect an overall reduction in the capacity to form foam cells, which are a first stage in the development of atherosclerosis [9]. An increase in antioxidant capacity also has the potential to reduce the risk of foam cell formation by delaying the oxidation of low-density lipoprotein (LDL) in the subendothelial space. A decrease is oxidized LDL (oxLDL) decreases the stimuli responsible for increased cell-surface monocyte adhesion molecule expression [10]. Elevated endothelial microparticles (EMPs) are associated with obesity, metabolic syndrome and consumption of a high-fat meal and are an emerging risk factor for cardiovascular disease (CVD) [11,12]. A recent review article indicated the potential for daily flavanol-rich cocoa consumption to be used as an antiobesity treatment [7]. However, there is a critical gap in the existing literature with regard to the capacity to utilize short-term flavanol-rich cocoa consumption as a countermeasure to reduce cardiovascular and metabolic disease risk in individuals of differing body mass indices (BMI).

Of all the naturally occurring compounds found on flavanol-rich cocoa, catechins and polyphenols have been implicated as the primary compounds responsible for improved vascular health [1]. Obese individuals are known to have elevated systemic inflammation, and when combined with poor dietary choices, chronic damage to the endothelial wall is common [9,11,12]. Our lab and others have used EMP as an index of endothelial wall damage [13,14]. Specifically, our laboratory has focused on the evaluation of novel disease risk biomarkers with recent efforts focused on circulating biomarkers of systemic inflammation [14–19], altered EMP and alterations in monocyte adhesion molecule expression [14,19]. All of these
measures could be considered preclinical indicators of the risk of the accumulation of arterial plaques. The purpose of the present study was to examine the effects of 4 weeks of daily flavanol-rich natural cocoa consumption on CVD biomarkers, EMPs and monocytes in women of differing obesity statuses who were weight stable.

2. Materials and methods

2.1 Subjects

The University of North Texas committee for the protection of human subjects approved all procedures used in this report, and the study was carried out in accordance with the Declaration of Helsinki. After being explained the study requirements, interested subjects gave oral consent to participate and signed an informed consent form. We screened 75 women who matched one of the three desired BMI groups. Of the 75 that we screened, 35 met all study requirements and 30 of those had schedules compatible with the study timeline. These 30 individuals were assigned to one of three groups based on BMI: normal weight (BMI = 20.0–24.9 kg/m²), overweight (BMI = 25.0–29.9 kg/m²) and obese (BMI = 30.0–39.9 kg/m²). We excluded subjects with a BMI > 40 kg/m². Every subject completed both conditions in a double-blind, crossover design. Condition orders were randomized to minimize carry over and account for potential order effects. Twenty-four of the 30 subjects completed all study requirements and samples. There was 0% attrition in the normal-weight group and 30% attrition in both the overweight and obese groups. These attrition percentages are consistent with what we have observed in other human studies in our laboratory and what others commonly report in the literature [14,17,18,20,21].

2.2 Subject screening

Subjects were assessed inclusion/exclusion criteria using a medical history form, a graded exercise test (Medgraphics Ultima metabolic cart, St. Paul, MN, USA), a whole body dual-energy X-ray absorptiometry (DXA) scan (GE Lunar Prodigy, Piscataway, NJ, USA) and a basal metabolic test (MedGem, Golden, CO, USA). Subject characteristics are presented in Table 1. In general, the obese subjects had greater body weight, BMI, percent fat, fat mass, lean mass and basal metabolic rate than overweight and normal-weight subjects. Also, obese subjects had lower levels of fitness than normal-weight and overweight subjects. Overall, the fitness level of all the subjects was low and no subjects reported any regular physical activity habits prior to or during completion of the study.

2.3 Natural cocoa treatment

The active and placebo bars used for the present study were manufactured and provided by The Hershey Company (Hershey, PA, USA). According to the manufacturer, the bars did not differ in their respective macronutrient composition, but did differ in magnesium, potassium and catechins in accordance with natural cocoa content. Bars were provided to the study staff packaged in either silver or white wrappers, and the study conditions were not revealed until all tests were completed. Table 2 summarizes the various nutritional components found in the bars. Subjects were asked to avoid consuming chocolate in any form during participation in the study and were asked to consume the bars at the same time each day. Table 3 summarizes the polyphenol content of the natural cocoa and placebo bars. Total flavanols were assessed using three different methods (each analytical method is optimized to assess a specific subgroup of flavanols). The flavanol monomers, epicatechin and catechin, were measured by atmospheric-pressure chemical ionization mass spectrometry [22], and the flavanol dimer through decaemer polymers (DP10-20) was measured using mass spectrometry [23,24]. Total flavanols were measured using the colorimetric 4-dimethylaminomphenylhydrazide (DMAC) method. The DMAC assay is unique in that it measures all flavanols including monomers (epicatechin and catechin), gallated flavanols and flavanoid polymers (DP10 to DP10) and polymers greater than DP10 [25].

2.4 Blood sample collection

Venous blood samples were collected before and after each 4-week supplement condition. Subjects reported to the laboratory between 0600 and 0900 h following an overnight fast (>8 h) and abstention from physical activity (>15 h). Subjects were encouraged to drink plenty of water during the fast in order to ensure hydration and ease of blood sample collection. After 30 min of seated rest, a trained technician collected a venous blood sample into three evacuated tubes (Greiner Vacutainer, Monroe, NC, USA) treated with serum-clotting chemical, EDTA or sodium citrate. Sample tubes were mixed 10 times by inversion. EDTA and citrate tubes were held at room temperature until analysis, while serum tubes were centrifuged to separate serum, which was frozen at −80°C until the end of the human subjects trials.

2.5 Traditional disease risk factors

In the present study, total cholesterol, HDL-c, triglycerides and glucose were measured using a series of enzymatic assays (Pointe Scientific) on an automated chemistry analyzer (ChemWell T; Awareness Technologies, Palm City, FL, USA). All samples were analyzed in triplicate.

2.6 Novel CVD biomarker

CVD risk biomarkers were measured using multiplex technique (EMD Millipore Milliplex, Billerica, MD, USA). The panel of 26 serum CVD risk biomarkers included the following: α2-macroglobulin, adipin, AGP, C-reactive protein, fetuin A, fibrinogen, haptoglobin, st-selectin, platelet factor 4, serum amyloid P, von Willebrand factor, BNP, CK-MB, CXCL6/GPC-2, CXCL16, endocan-1, FABP3, FABP4, LIGHT, oncostatin, plasminogen activation factor, troponin I, II–|, II-L, IL-8 and TGF-α. These were biomarkers were measured in duplicate on the same day using a Human CVD Panel 1 (HC1DMAG-67K), Human CVD Panel 3 (HCNDDMAG-67K) and a Human High-Sensitivity Cytokine Panel (HSTCMAG2BSPMX4). After following the manufacturer’s protocol for sample processing, samples were acquired using a Luminex MagPix (Austin, TX, USA). All samples were assayed in duplicate on the same day in order to minimize intra-assay and interassay coefficient of variations. After acquisition, unknown values were calculated using a 7-point standard curve method on Milliplex Analyst software (v. 5.1; EMD Millipore).

2.7 Flow cytometry for monocytes

Monocytes were measured to determine relative concentration (CD14 and CD16) and expression of cell-surface adhesion molecules (CD11b and CD62L) using four-color immunofluorescent assays. All antibodies were titrated prior to analysis to ensure adequate signal-to-noise ratios (data not shown). Consistent with previous methods utilized in our laboratory [14,21,26], we separated peripheral blood mononuclear cells (PBMCs) from 10 ml of EDTA-treated whole blood. After separation using Leukosep tubes (Gierer) and Ficoll-paque, cells were measured for concentration and viability

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Subject characteristics.</th>
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<tbody>
<tr>
<td>Characteristic</td>
<td>Normal (n=10)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>21±2</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>161.1±5.4</td>
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<tr>
<td>Weight (kg)</td>
<td>56.2±7.5</td>
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<tr>
<td>BMI</td>
<td>21.9±3.9</td>
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<tr>
<td>DXA body fat (%)</td>
<td>28.6±5.7</td>
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<tr>
<td>Fat mass (kg)</td>
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<tr>
<td>Lean mass (kg)</td>
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<tr>
<td>Bone mass (kg)</td>
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<tr>
<td>SBP (mm Hg)</td>
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<tr>
<td>DBP (mm Hg)</td>
<td>71±10</td>
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<tr>
<td>RMR (kcal/d)</td>
<td>1174±171</td>
</tr>
<tr>
<td>VO2max (ml/kg/min)</td>
<td>30.5±5.5</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>173.7±48</td>
</tr>
<tr>
<td>HDL-c (mg/dl)</td>
<td>43.6±4.2</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>69.6±5.9</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>88.3±23</td>
</tr>
</tbody>
</table>

Values reported as mean±S.D. Body composition measurements were made from a standard whole-body DXA scan. All serum blood measurements were made following a fast of at least 8 h.

* Greater than normal (P<.05).

b Greater than normal and overweight (P<.05).
CD62L expression differed at baseline as a function of BMI group. The initial differences were also altered as a function of BMI group following consumption of natural cocoa. Baseline haptoglobin was significantly greater in obese (29.3±4.6 ng/ml) compared to overweight (10.3±2.8) and normal-weight (8.4±1.8) subjects (P<.0001). Following natural cocoa consumption, haptoglobin significantly declined by 13% in the obese group, compared to no change in either the overweight or normal-weight groups (P=.034; Fig. 1A). Baseline EMP concentration was significantly greater in obese (103.6±4.0×10^5 EMPs/ml) compared to overweight (85.5±4.0×10^5 EMPs/ml) and normal-weight subjects (67.8±6.6×10^5 EMPs/ml) (P=.034). Following natural cocoa consumption, we found significant decreases in EMPs for obese (−31%) and overweight (−13%), but not normal-weight (+5%) subjects (P=.017; Fig. 1B). Baseline monocyte CD62L expression on the total monocyte populations was significantly greater in obese (105.8±6.5 gMFI) compared to overweight (91.1±7.4 gMFI) and normal-weight (87.8±6.6 gMFI) subjects (P=.032). After consumption of natural cocoa, total monocyte CD62L expression decreased in the obese (−18%) compared to no change (+1%) in overweight and an increase in normal-weight (+12%) subjects (P=.047; Fig. 2A). The changes in obese and normal-weight subjects reached statistical significance. Baseline CD62L expression on proinflammatory monocytes was significantly greater in obese (116.4±7.2 gMFI) compared to overweight (101.4±7.3 gMFI) and normal-weight (87.8±4.6 gMFI) subjects (P=.030). Following natural cocoa consumption, proinflammatory monocyte CD62L expression decreased in obese (−20%) compared to an increase in overweight (+18%) and normal-weight (+28%) subjects (P=.049; Fig. 2B). The changes in obese, overweight and normal-weight subjects reached statistical significance.

3.2. Natural cocoa effects not associated with BMI group

Of the tested outcome variables, we found two variables (HDL-c and endocan-1) that presented an effect of natural cocoa that was not associated with a specific BMI group. For HDL-c, we found that natural cocoa consumption resulted in a significantly greater increase (+18%) compared to placebo (+4%) (P=.020; Fig. 3A). We found a trend for endocan-1 where following natural cocoa consumption there tended to a decrease (−13%) compared to only a minor decrease with placebo (−1%) (P=.067; Fig. 4A).

3.3. BMI group differences, no natural cocoa effect

Total cholesterol (Fig. 3B) and glucose (Fig. 3D) were similar across all three BMI groups at baseline and over time regardless of condition. For triglycerides, obese (105±18 mg/dl) were greater than overweight (58±10 mg/dl) and normal-weight (73±7 mg/dl) subjects (P=.034; Fig. 3C). Total cholesterol (Fig. 3B), triglycerides (Fig. 3C) and glucose (Fig. 3D) were not significantly altered following either condition. sE-selectin trended toward a significant BMI group effect, but no effect of condition. Obese (35.4±9.4 mg/ml) trended toward having greater sE-selectin than overweight (9.9±1.5 mg/ml) and normal-weight (7.6±1.9 mg/ml) subjects (P=.061; Fig. 4B).

4. Discussion

Previously published research has focused almost exclusively on the effect of flavanol-rich cocoa consumption on various facets of endothelial health and antioxidant defense [4,7,8,27], which has implications for the development of atherosclerosis. We categorized the key findings of the present study into responses that are either dependent or independent of BMI group. Haptoglobin, EMP concentration, total monocyte CD62L expression and proinflammatory monocyte CD62L expression all differed at baseline as a function of BMI group and demonstrated a variable natural cocoa response as a
function of BMI group. Only HDL-c or endocan-1 had no baseline difference among the three BMI groups, and the natural cocoa effect was not specific to a certain BMI group. To our knowledge, the present work is the first published study to report that short-term natural cocoa consumption was associated with alterations in disease risk biomarkers, EMPs and monocyte adhesion molecules.

It was an interesting and unexpected finding that not all of our outcome variables changed following natural cocoa consumption as a function of baseline BMI group. Our observed reductions in EMP and monocyte CD62L expression have implications for preclinical risk of developing atherosclerosis. Specifically, our laboratory has previously demonstrated that within 3 h after consuming a high-fat meal, there is an increase in circulating EMP concentration [14], indicating that damage to the endothelium has occurred. Others have speculated that habitual consumption of high-fat foods not only causes obesity, but also damages the endothelial wall to the point that there is an increased risk of developing atherosclerosis [7,12]. It was recently reported that patients with metabolic syndrome had 51% greater EMP concentration than matched controls [11]. In the present study, we found that at baseline obese subjects had 48% greater EMP concentration than normal-weight subjects and that cocoa consumption reduced the difference between obese and normal-weight subjects to 4%. An increase in monocyte expression of CD62L (also known as L-selectin) has been linked to an increase in atherogenesis; however, CD62L differences have not been consistently reported between obese and nonobese subjects [9,28]. In the present study, we found that obese subjects had 25% greater monocyte CD62L expression than normal-weight subjects and that the difference was reduced to 2% following the consumption of natural cocoa for 4 weeks.

The exact mechanism underlying the observed effects of natural cocoa consumption on cellular outcomes is not fully known or understood. Based on the existing literature, natural cocoa consumption improves vascular compliance/health and increases antioxidant capacity [1,3,4,6–8]. The later change may explain some of the key findings of this study. For example, obesity is associated with an increased oxidative stress, which in turn increases the accumulation of oxLDL in the subendothelial space [10,29,30]. In excessive amounts, oxLDL increases the recruitment of monocytes, which require increased CD62L expression to modulate trafficking into the subendothelial space [10,29]. Monocyte trafficking into and out of the various peripheral tissue compartments is a normal function; however, in the case of atherogenesis, this function is disrupted causing monocytes to improperly accumulate in the subendothelial space [10]. It is worth noting that changes in monocyte CD62L expression and the formation of EMPs are linked events since they are both associated with trauma to the endothelial wall. Thus, if monocyte CD62L expression increased without a corresponding increase in EMPs, then the increase in CD62L simply may be a result of normal diurnal changes in cell trafficking. In the present study, the obese subjects experienced a decrease in both monocyte CD62L expression

**Fig. 1.** Serum haptoglobin (A) and circulating endothelial microparticle concentration (A). Serum (haptoglobin) and sodium citrate (EMPs) blood samples were collected following an overnight fast (8 h) in normal-weight, overweight and obese subjects. Obesity groups were determined according to BMI. Additional samples were collected following 4 weeks of daily placebo of flavanol-rich cocoa consumption (12.7 g of natural cocoa per day; 355.6 g for 28-day treatment period). Samples were analyzed using image-based flow cytometry to determine EMP concentration. * indicates obese greater than normal weight and overweight (Pb.05). ● indicates post supplementation less than baseline (P=.05).

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and EMP concentration following natural cocoa consumption, which represents a potential improvement in vascular health. In normal-weight and overweight subjects, we observed an increase in monocyte CD62L expression without a corresponding increase in EMPs following natural cocoa consumption. This later finding suggests that the observed increase in CD62L expression for nonobese subjects may simply be due to diurnal shifts and has no direct relationship to increased disease risk. The conclusion is further supported by the fact that haptoglobin and endocan-1 were decreased and HDL-c was increased following a period of natural cocoa consumption. Haptoglobin, sE-Selectin, endocan-, and HDL-c have been previously associated with vascular inflammation in sedentary individuals [31–33]. In the present study, we observed significant differences at baseline for sE-selectin and a trend for endocan-1 between the three BMI groups. The targeted biomarkers in the present study have been linked to CVD, but, to our knowledge, not compared in individuals of differing obesity status. Thus, demonstrating that obese subjects have elevated haptoglobin, elevated sE-selectin and suppressed endocan-1 is novel. Specifically, at baseline obese subjects had 300% more haptoglobin, 275% more sE-selectin and 17% less endocan-1 compared to normal-weight subjects. Given the previously reported links between haptoglobin, sE-selectin, endocan-, and atherogenesis, our findings with respect to obesity-associated differences are reasonable [31–33]. Further examination of these biomarkers revealed that 4 weeks of cocoa consumption resulted in only a minor, 17% reduction in haptoglobin among obese subjects who still presented serum haptoglobin that was 250% greater than normal-weight subjects. Natural cocoa consumption was associated with an 18% increase in HDL across combined BMI groups, which translated to about a 10-mg/dl increase in HDL across the three obesity groups. It is worth noting that we did not observe any statistically significant changes in total cholesterol, although with the increased in HDL, it can be inferred that LDL cholesterol likely decreased. This is consistent with the 5% to 15% increase in HDL that has been previously reported in the existing literature following short-term cocoa consumption [1,5,7]. Overall, we observed an increase of HDL that is consistent with a reduced risk of CVD across subjects with a range of BMIs. The observed changes in CVD biomarkers following natural cocoa consumption were less pronounced than that observed for cellular risk factors (CD62L expression and EMP concentration). Despite the small magnitude of change the biomarker responses in the obese group, along with the observed cellular responses, they provide support for the notion that short-term natural cocoa consumption may transiently reduce risk of atherosclerosis. Another interpretation of the observed biomarker response is that alone natural cocoa consumption may not exert a powerful enough effect and thus may need to be combined with one or more lifestyle modifications to facilitate a stronger effect.

In summary, the key findings of the present study reinforced the notion that small lifestyle changes, such as the daily incorporation of natural cocoa, have the potential to transiently alter cellular disease...
the study design, data collection, data analysis/interpretation, writing of the report or the decision to submit the article for publication.

Disclosures

The authors report no conflict of interest associated with the completion of the present study.

Acknowledgments

Each listed author fulfilled the following criteria for authorship: (1) the conception and design of the study, or acquisition of data or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be submitted.

The present study was funded, in part, by a grant to the University of North Texas from The Hershey Company. The authors were not directly compensated for completing this research. Beyond providing support for the project, The Hershey Company did not contribute to factors in a clinical relevant manner. To a lesser extent, we found that daily natural cocoa consumption lowered selected CVD biomarkers; however, these changes were much smaller and may have less clinical relevance. Future studies should seek to further explore and identify disease risk outcomes that are affected by daily consumption of natural cocoa per day; 35.6 g for 28-day treatment period). * indicates obese different than normal weight and overweight (P<.05).

References


