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Randomized Controlled Trial

Cocoa ingestion protects plasma lipids in healthy males against *ex vivo* oxidative conditions: A randomized clinical trial

Manuel Barrios [a, *], Luis C. Orozco [b, 1], Elena E. Stashenko [a]

[a] Chromatography Laboratory, Research Center of Excellence, CENIVAM, Industrial University of Santander, Bucaramanga, Colombia
[b] Cardiovascular Diseases Epidemiologic Observatory, Epidemiologic Research Center Industrial University of Santander, Bucaramanga, Colombia

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SUMMARY

The effects of *in vivo* cocoa-based supplementation were studied as a preconditioning treatment for *ex vivo* acute oxidative conditions in a controlled randomized clinical trial. Subjects were 100 healthy young men at Universidad Industrial de Santander blinded to the intervention and divided into two groups: The intervention group (n = 50) receiving 30 g of cocoa powder and 50 g of dark chocolate/d for 1 week with the remaining subjects receiving placebo. Cocoa products preconditioning for 1 week resulted in modifications in the susceptibility of plasma lipids over *ex vivo* oxidative conditions with effects of i) a significant increase in the oxidative resistance of plasma lipids measured by dienes formation (4.2, CI: 0.18, 8.2; $P = 0.04$), and ii) a significant reduction in the production of toxic aldehydes as established by a decrease in the content of hexanal, quantified by gas chromatography (−0.22, CI: −0.38, −0.05; $P = 0.009$). The *in vivo* cocoa-based preconditioning demonstrated protective properties against *ex vivo* oxidative modifications, improving total plasma lipids resistance to oxidation and protecting plasma lipids against great acute oxidative insult in comparison with placebo. This trial was registered at clinical clinicaltrials.gov as NCT01347450.

1. Introduction

Cocoa contains many bioactive compounds, mainly methylxanthines (like theobromine and caffeine) and nonflavonoid and flavonoid phenolic compounds, primarily from the flavanol subfamily, including (−)-epicatechin (EC), catechins (CAs), and procyanidins (PCs). Cocoa has been described as having the highest flavanol content of all foods on a per-weight basis [1,2]. These naturally occurring compounds possess antioxidant properties *in vitro* and are considered to have potential beneficial health effects *in vivo*, in particular, the modulation or interaction with specific molecular targets linked to the pathogenesis of chronic human diseases, including cancer, obesity, diabetes, and neurodegenerative and cardiovascular diseases (CVD) [3]. Over the last decade, the majority of the research on chocolate and cocoa has mainly focused on the relationship between consumption and cardiovascular risk because CVD is the leading cause of death and disability-adjusted life years globally [3,4].

It has been proposed that oxidative stress makes a significant contribution to chronic and acute inflammatory diseases [5]. Epidemiological evidence suggests that diets rich in plant foods reduce the risk of developing chronic diseases characterized by high oxidative stress conditions [6]. Regarding CVD, flavonoid consumption has been inversely associated with coronary artery disease and mortality [3]. It is thought that flavonoids decrease cardiovascular risk by protecting lipids, proteins, and nucleic acids from oxidative damage, as well as by reducing inflammation and regulating vascular homeostasis. Lipids such as cholesterol and polyunsaturated fatty acids (PUFAs), the most readily oxidized chemicals in nature, are the primary target of oxidative attack. As a result, lipid peroxidation products (LPPs), in particular oxysterols, hydroperoxides, and endoperoxides [7], are produced and accumulated under physiological and pathological conditions [8]. The diet flavanols present in cocoa and chocolate, especially EC, have been found responsible for decreasing human plasma levels of oxidized LDL and LPPs [3].

Abbreviation: AC, Areas counts; BHT, 3,5-Di-*tert*-butyl-4-hydroxytoluene; CVD, cardiovascular diseases; GC, gas chromatography; HS-SPME, headspace solid-phase microextraction; Ox-LDL, oxidative modified LDL; PUFA, polyunsaturated fatty acids; PFPH, pentafluorophenylhydrazine; TCA, trichloroacetic acid.

* Corresponding author.
E-mail address: manuel@curavibroacustica.com (M. Barrios).

[1] Deceased January 30, 2016.

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Despite the growing body of basic research, observational studies and clinical interventions strongly suggesting that cocoa and chocolate consumption may decrease multiple risk factors for chronic diseases [9], there is very little information available regarding the potential of flavonoids, in general, to prevent or treat acute conditions. Recent preclinical studies in rodents have identified the role of short-term pretreatment with EC to prevent myocardial infarction and cerebral stroke in ischemia-reperfusion injury models [10–12]. However, evidence for the extrapolation of these effects to similar or different acute oxidative conditions such as hypoxia, endotoxemia or trauma in humans cannot be done readily.

In this study, we tested the hypothesis that the preconditioning effect of short-term cocoa and dark chocolate consumption in healthy young men would reduce plasma lipid peroxidation after severe acute oxidative conditions, replicated through an *ex vivo* oxidative model in a single-blind, controlled and randomized clinical trial.

2. Materials and methods

2.1. Materials

Cocoa powder and dark chocolate bars were obtained from Colombian cocoa beans were manufactured by Casa Luker Company, Colombia. Certified substances and analytical reagents: *trans*-2-hexenal, copper (II) chloride, trichloroacetic acid (TCA), 3,5-Di-*tert*-butyl-4-hydroxytoluene (BHT), and pentafluorophenylhydrazine (PFPH) were purchased from Aldrich Laboratories (Milwaukee, WI, USA). Sodium chloride, sodium phosphate, and potassium chloride were obtained from Merck (Darmstadt, Germany). For headspace solid-phase microextraction (HS-SPME) fused-silica fibers were used, coated with poly (dimethylsiloxane)/divinylbenzene (PDMS/DVB-65 μm), acquired from Supelco (Bellefonte, PA, USA).

2.2. Subjects

Advertisements at the University food service center were used to recruit young adult males from November 2007 to March 2008 at the Universidad Industrial de Santander. Responders ($n = 136$) were selected by assessing blood lipid profile (verified by laboratory testing of total cholesterol, LDL, HDL, VLDL, and triglycerides) using criteria for exclusion and inclusion. The exclusion criteria were: any abnormality in the lipid profile (defined as LDL $\geq$ 120 mg/dL, HDL $\leq$ 40 mg/dL, total cholesterol $\geq$ 200 mg/dL and/or triglycerides $\geq$ 100 mg/dL), diagnosed coronary artery disease, diabetes mellitus, arterial hypertension, use of any prescribed medication, restrictive diets and any migraine or cocoa products allergic antecedents.

Subjects selected for enrollment met the following criteria: non-smokers (at the moment of the trial), normolipemic, aged 20–30, a body mass index (BMI) between 16.0 and 27.4 kg/m^2 (considering standard defined parameters for Colombian population) and those with average intake of fat, sugar and high antioxidant content foods, verified using a validated short dietary survey (Lean: Eating Questionnaire) from Nacional Cancer Institute's Epidemiology and Genomics Research Program of the NIH. Subjects received three meals a day in the university canteen from the University Food Nutritional Service to ensure standardized diet conditions for all subjects throughout the experimental period using an average Colombian diet, of 2287 calories as a total caloric value (protein 15%, fats 35%, and carbohydrates 60%). Physical activity was defined as any conditioning or sports type of exercise planned, structured, and performed more than two days a week. High-stress levels were assessed using student-life inventory scale [13] and defined as any total score $\geq$ 170 points. Study protocols and informed consent procedures were approved by the Fundación Santa Fe de Bogotá Institutional Ethical Committee (Santa Fe de Bogotá D.C., Colombia). Written informed consent was received from all participants who were financially compensated (local market gift card equivalent to 20 USD were given) for participating in the study. The flow chart for subject participation is shown in Fig. 1. This trial was registered at clinical clinicaltrials.gov as NCT01347450.

2.3. Study design and randomization

The study protocol was carried out as a prospective, randomized and placebo-controlled clinical trial with single-blind masking, with subjects blinded to the treatment in a parallel assignment intervention model, comparing cocoa supplementation with placebo. Subjects were allocated using simple randomization techniques (computer-based random number list generator software, allocation ratio 1:1) for 1 of 2 groups to receive food supplementation during 7 days. Randomization was performed by an independent investigator after participants had been selected according to eligibility criteria. All participants (but not all investigators) were kept blinded to the treatment conditions. Once allocation and randomization sequence was generated, either placebo and chocolate products were placed in identical sealed and labeled cardboard boxes, delivered using study random allocation number sequence, and a neutral investigator did this. Another investigator who was blinded to the sequence distributed the intervention to the participants in an ascending and sequential order according to the randomization schedule. Each box had an identification card with the allocation number assigned, each participant filled it out with its name and university code, in order to verify they received the same intervention along the 7 days The experimental group (cocoa group) received a sugary drink prepared with 30 g of cocoa powder and a 65% dark chocolate bar (50 g) each day. The control group (placebo group) received a sugary drink prepared with 30 g of non-polyphenol placebo powder, matching organoleptic properties with similar taste, color, smell, quantity, and caloric content. The dark chocolate, cocoa, and placebo products were produced and furnished by the manufacturer (Casa Luker company) procyanidins concentrations in this products were analyzed by Chromatography Laboratory of Industrial University of Santander, also there were not complete match of all macronutrients and caloric content between placebo and cocoa test products (Table 1). Each drink was prepared by using 300 mL of water and 30 g of sugar and divided into two parts to be administered 150 mL with both breakfast and lunch. Participants were advised to maintain their regular physical activity and to avoid flavonoid-rich foods, vitamin supplements, cigarette and alcoholic drinks for 7-days before and after the beginning of the trial. They were also trained to fast for 12 h overnight before the taking of blood samples, both before and 7-d after the intervention.

2.4. Susceptibility of plasma lipids to oxidation with copper (II) ions

2.4.1. Ex vivo human plasma oxidation

For the conjugated dienes assay, total plasma was diluted 1:50 in saline phosphate solution. For hexanal determination plasma was deproteinized by adding 100 μL of 10% (w/v) TCA to 900 μL of diluted plasma (1:1 in phosphate solution), vortexing for 1 min and centrifuging at 1200 rpm for 15 min at 4 °C [14]. Total plasma or deproteinized plasma were incubated with copper (II) chloride (100 μM) at 37 °C for 8 h. Following this, a methanolic solution of BHT (20 g/L) was added to stop the radical-mediated chain-reaction, and the samples were cooled immediately on ice for 10 min.

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