

# **OXIDIZED LDL LEVELS DECREASES AFTER THE CONSUMPTION OF READY-TO-EAT MEALS SUPPLEMENTED WITH COCOA EXTRACT WITHIN A HYPOCALORIC DIET**

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## **ABBREVIATIONS:**

BMI: Body mass index

BP: Blood pressure

DBP: Diastolic blood pressure

HDL-c: High-density lipoprotein-cholesterol

LDL-c: Low-density lipoprotein-cholesterol

MPO: Myeloperoxidase

NO: Nitric oxide

oxLDL: Oxidized low-density lipoprotein-cholesterol

SBP: Systolic blood pressure

sICAM-1: soluble Intercellular Adhesion Molecule-1

sVCAM-1: soluble Vascular Cell Adhesion Molecule-1

TG: Triglycerides

## **ABSTRACT**

**Background and aims:** Cocoa flavanols are recognized by their favourable antioxidant and vascular effects. This study investigates the influence on health of the daily consumption of ready-to-eat meals supplemented with cocoa extract within a hypocaloric diet, on middle-aged overweight/obese subjects.

**Methods and results:** Fifty healthy male and female middle-aged volunteers [57.26±5.24 years and body mass index (BMI) 30.59±2.33 kg/m<sup>2</sup>] were recruited to participate in a 4 week randomised, parallel and double-blind study. After following 3 days on a low-polyphenol diet, 25 volunteers received meals supplemented with 1.4g of cocoa extract (645.3mg of polyphenols) and the other 25 participants received control meals, within a 15% energy restriction diet. On the 4th week of intervention individuals in both dietary groups improved (p<0.05) anthropometric, body composition, blood pressure and blood biochemical measurements. Oxidized LDL cholesterol (oxLDL), showed a higher reduction (p=0.030) in the cocoa group. Moreover, myeloperoxidase (MPO) levels decreased only in the cocoa supplemented group (p=0.007). Intercellular Adhesion Molecule-1 (sICAM-1) decreased significantly in both groups, while Vascular Cell Adhesion Molecule-1 (sVCAM-1) did not present differences after the 4 weeks of intervention. Interestingly, cocoa intake showed a different effect by gender, presenting more beneficial effects in men.

**Conclusions:** The consumption of cocoa extract as part of ready-to-eat meals and within a hypocaloric diet improved oxidative status (oxLDL) in middle-aged subjects, being most remarkable in males.

**Registration number:** Registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT01596309).

## INTRODUCTION

Obesity and overweight status are associated with the risk of suffering from chronic diseases such as cardiovascular disease or atherosclerosis (1). Middle-aged and elderly population is growing up in developed countries due to the increase of life expectancy. However, the prevalence of obesity and chronic diseases is high in these age groups (2). Moreover, changes in cardiovascular physiology with aging and the presence of comorbidities, make atherosclerotic complications the leading cause of death in these countries (2).

Oxidative stress is a process directly implicated in atherosclerosis, and therefore associated with the development of cardiovascular diseases (3). Despite the presence of an antioxidant system in the human organism, the imbalance created when antioxidant system is weak and the production of reactive oxygen species is increased, favours the establishment of a harmful oxidative stress situation (4). Moreover, antioxidant status tends to be unfavourable with age (5), and it seems that the antioxidant protection is lower in men than in women (6).

Some foods consumed in human diet, contain natural bioactive compounds with antioxidant properties, such as plant derived polyphenols whose consumption provides protection against oxidative stress and apparently prevents the incidence of cardiovascular events (7).

In this context, cocoa bean is one of the richest dietary source of flavanols, a type of polyphenols with recognized potential health effects (8). Several studies have observed a strong link between their consumption and a decrease of blood pressure (BP) levels (9). Moreover, cocoa flavanols can influence blood lipid levels by decreasing low-density lipoprotein-cholesterol (LDL-c), increasing high-density lipoprotein-cholesterol (HDL-c) and inhibiting oxidized LDL-c production (oxLDL), which is one of the most important triggers implicated in atherosclerosis (10, 11). Furthermore, cocoa polyphenol intake has a positive effect on inflammation, insulin resistance and endothelial function (12, 13).

Given the increasing availability of ready-to-eat meals and the potential beneficial effects of cocoa flavanols on human health, the objective of this trial was to assess the influence on oxidative status blood glucose and lipid profile of the daily consumption of

ready-to-eat meals supplemented with cocoa extract within a hypocaloric diet on middle-aged overweight/obese subjects. Moreover, we analysed whether the effect of cocoa consumption on oxidative status depends on gender.

## **METHODS**

### **Study population**

The volunteers, 50 healthy Caucasian (23 men and 27 women) with  $57.26 \pm 5.24$  year and with BMI of  $30.59 \pm 2.33$  kg/m<sup>2</sup> were recruited between March and May of 2012, by advertisements in local newspapers. The study was carried out in the Metabolic Unit of the University of Navarra (Spain). The participants gave written informed consent to participate in the trial. The inclusion criteria were age (50-80 years), BMI (27.0-35.5 kg/m<sup>2</sup>) and to maintain a stable weight (<5% of variation) the previous three months to the intervention. The exclusion criteria were history of metabolic disorders, gastrointestinal diseases, diabetes, cancer, inflammatory diseases (such as rheumatoid arthritis), food allergies, cognitive alterations, current sliming, hormone replacement, anti-inflammatory or BP lowering treatments, medication that could influence appetite or nutrient absorption, inability to perform the follow-up and being smoker. The study was approved by the Research Ethics Committee of the University of Navarra (ref. no 006/2012) and followed the Helsinki Declaration guidelines. The trial was registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT01596309) on 9<sup>th</sup> may 2012.

### **Study design and diet monitoring**

The study was designed as a 4 week double-blind, randomised, placebo-controlled parallel nutritional intervention. One week before the beginning of the study the volunteers had to exclude cocoa and cocoa containing products from their habitual diet and three days prior to the start of the trial were asked to consume a low-polyphenol diet without energy restriction. After these previous preparatory days, volunteers were provided every week with ready-to-eat meals, which were weekly supplied by Tutti Pasta S.A (Navarra, Spain). From 50 allocated volunteers, 25 subjects received meals supplemented with 1.4 g/d cocoa extract and 25 volunteers received control meals (the composition of cocoa extract is shown in Table 1). The randomisation was performed using the “random between 1 and 2” function in the Microsoft Office Excel (Microsoft Iberica, Spain). Boxes in which the meals were provided had the same appearance and

differed only on the code label, ensuring the double-blind. Those meals were consumed within a hypocaloric diet with an energy restriction of 15%. Resting metabolic rate was calculated by the Harris-Benedict equation applying the corresponding individualized physical activity factor, which was calculated as average daily exercise (14). The macronutrient distribution of the diet was 45% of total caloric value from carbohydrates, <30% from lipids and 22-25% from proteins. The volunteers were asked to exclude cocoa containing foods and polyphenol rich foods maintaining their habitual physical activity during the intervention. At the beginning and end of the study, a 3-day validated food-recall questionnaire was used to assess nutrient intake, which was analysed using the DIAL software (Alce Ingenieria S.L, Madrid, Spain). To evaluate the adherence to meal consumption, volunteers had to fill a notebook with the name of the dish and dessert that they consumed daily.

### **Total polyphenol content and characterization of the cocoa extract**

Cocoa extract and analytical characterization were provided by Nutrafur S.A (Murcia, Spain). Total polyphenol content was determined by the Folin-Ciocalteu colorimetric method. Briefly, 50 mg of cocoa extract was mixed with 100 mL of water for soluble extracts or 4 mL of ethanol+96ml of water for insoluble extracts. Then they were shaken during 15 minutes and filtered. Absorbance was measured at 765 nm. The total polyphenol content was calculated from the calibration curve (1ppm-8ppm) using catechin hydrate (Sigma-Aldrich C1788) as a standard value and expressed as milligrams of catechin per 1.4 g.

HPLC was used to quantify the flavonoids and theobromine in the cocoa extract. For that, the extract was dissolved in dimethylsulfoxide (DMSO) 5 mg/mL. This solution was filtered through a 0.45 mm nylon membrane. The HPLC equipment used was a Hewlett-Packard Series HP 1100 equipped with a diode array detector. The stationary phase was a C18 LiChrospher 100 analytical column (250 x 4 mm i.d.) with a particle size of 5 mm (Merck, Darmstadt, Germany) thermostated at 30°C. The flow rate was 1 mL/min and the absorbance changes were monitored at 280 nm. The mobile phases for chromatographic analysis were: (A) acetic acid/water (1:99) and (B) acetonitrile. A linear gradient was run from 96 % (A) and 4 % (B) to 90 % (A) during 25 min; changed to 87 % (A) in 5 min (30 min, total time); in 5 min changed to 50 % (A) (35 min, total time), after equilibrate in for 10 min. Phenolic compounds in Cocoa extract were identified by comparison of their retention time with the correspondence standard (Sigma-Aldrich) and by their UV spectra obtained with the diode array detector.

### **Anthropometric, body composition and blood pressure**

At baseline and at the end of the dietary intervention, anthropometric, body composition and blood pressure measurements were performed. The measurements of weight, height, waist circumference and body composition were taken in underwear after an overnight fast as described elsewhere (15). BP was taken 3 times with automatic monitor (Intelli Sense. M6, OMRON Healthcare, Hoofddorp, Netherlands), to use the average value obtained from the last two measurements.

### **Blood biochemical analysis**

Fasting (10 h) blood samples were collected between 8:00-9:30 a.m at baseline and at the end of the intervention using EDTA and CLOT tubes. After, samples were left for 10-15 minutes at room temperature. The, tubes were centrifuged to obtain plasma and serum aliquots (15 minutes, 1 500g, 4°C), were stored at -80°C until analysis. Plasma glucose, total cholesterol, HDL-c, triglycerides (TG) and proteins were measured by colorimetry in an auto-analyser Pentra C200 (Horiba Medical, Montpellier, France). LDL-c was calculated using Friedewald equation. Plasma insulin, oxLDL, MPO (Mercodia, Upssala, Sweden), sVCAM-1 and sICAM-1(R&D Systems, Minneapolis, USA) were quantified with specific ELISA kits in a Triturus auto-analyser (Grifols, Barcelona, Spain).

### **Statistical analysis**

Considering oxLDL as the main variable, the sample size was estimated taking into account a reduction of 14.1 U/L and an interquartile range of 16.3 U/L, according to the study carried out by Khan et al (11). With a bilateral confidence index of 95% ( $\alpha=0.05$ ) and a statistical power of 80% ( $\beta=0.80$ ) the sample size was estimated in 44 subjects. Considering a possible drop-out rate of 15%, the final sample size was established in 50 subjects. Normality of the variables was assessed using Shapiro-Wilk test. Data are expressed as mean (SD) for normally distributed variables or as median and interquartile range for non-normally distributed. Comparisons between baseline and end point, and between studies groups were accordingly analysed by Student paired t-test, Wilcoxon test, independent t-test or U Mann-Whitney, depending on the normality of the variables. Spearman correlation tests were applied to evaluate the relationship between changes ( $\Delta$ ) on oxLDL, total cholesterol, and glucose levels. Multiple linear

regression analysis was used to assess the effect of cocoa supplementation over  $\Delta$  oxLDL adjusting for different models, in which the independent variables were weight, total cholesterol and LDL-c, differently combined.  $p < 0.05$  was considered significant. The software used was SPSS 15.1 for Windows (SPSS Inc, Chicago, USA).

## RESULTS

### Participants and adherence to diet and meal consumption

From the initial 488 subjects who contacted with the Metabolic Unit, 113 met the inclusion criteria and 50 subjects were randomised. Finally 47 volunteers completed the intervention, 24 subjects in control group and 23 in cocoa group (Figure 1). After 4 weeks of intervention, both groups reported similar adherence to meal consumption (98.4 (2.2) % control and 98.5 (3.3) % cocoa group) and the dietary records showed that there were no significant differences between groups in macronutrient and calorie intake (data not shown). None of the volunteers reported side effects, taste dislike or changes in physical activity during the study. Moreover, there were no statistical differences at baseline in any of the assessed variables between groups.

### Anthropometric, body composition, blood pressure and blood biochemical parameters after 4 weeks of intervention

Subjects in both dietary interventions significantly improved BP, anthropometric and body composition variables. Interestingly, lean mass percentage increased significantly in both groups (Table 2). Also, concentrations of total cholesterol, LDL-c, HDL-c, TG, total proteins and insulin significantly decreased (Table 2). However, no changes were observed in blood glucose levels (Table 2).

### oxLDL, MPO, sVCAM-1 and sICAM-1 after 4 weeks of intervention

After 4 weeks of intervention, oxLDL concentration decreased significantly ( $p < 0.001$ ) in both groups, showing higher reduction in cocoa group ( $p = 0.030$ ) as it is shown in Fig 2. Moreover,  $\Delta$ oxLDL was positively correlated with  $\Delta$ total cholesterol ( $\rho = 0.574$ ;  $p < 0.001$ ) and  $\Delta$ glucose ( $\rho = 0.297$ ;  $p = 0.043$ ). MPO levels decreased significantly only in cocoa supplemented subjects ( $p = 0.007$ ), without observing differences with control group. Concerning sICAM-1, a significant decrease in both arms was observed, while no changes were found in sVCAM-1 (Table 2).

Considering that the literature reports poorer antioxidant status in men than in women, Table 3 shows the effect of cocoa consumption by gender on % $\Delta$ oxLDL. Different multiple linear regression models (model 1, 2 and 3) were obtained. % $\Delta$ weight was included as independent variable because it could have an effect on % $\Delta$ oxLDL, and % $\Delta$ total cholesterol and % $\Delta$ LDL-c were included due to the correlations found between them. The lineal regression analysis showed a significant effect of dietary group on % $\Delta$ oxLDL with model 2 and 3 in men. Moreover, model 1, 2 and 3 were significant only in men. The 2<sup>nd</sup> model was the best to predict the variability of % $\Delta$ oxLDL, which was 50.3% (p= 0.001).

## **DISCUSSION**

In this study, weight, waist circumference, total fat mass and truncal fat mass of the participants improved after following the intervention. The improvements were observed in both groups, indicating that cocoa supplementation did not have additional effect on those variables and attributing the observed changes to the hypocaloric diet followed by the volunteers. Our results are in agreement with two previous studies and one meta-analysis, in which no changes were observed in body size and composition of overweight/obese subjects after the cocoa supplementation (9, 16, 17). Golomb et al observed a negative correlation between the frequency of dark chocolate consumption and BMI (18). However that is an epidemiological study in which the population studied had different characteristics. The increase observed on lean mass is interesting, as it is generally reduced after following a hypocaloric diet, being more notable with ageing (19). Based on previous observations, the 22-25% of protein instead of 15% can be pointed out as the responsible of this positive outcome (19).

On the other hand, no additional benefits on blood lipid and glucose profile after cocoa supplementation were found. Some studies have reported that cocoa flavanols may increase HDL-c and decrease LDL-c levels (10, 11), although these outcomes are controversial and there is not a consensus on these effects (17). A meta-analysis carried out by Shrimpe et al (17), suggests that HDL-c levels increase in longer term trials with low fat consumption, whereas LDL-c and total cholesterol decrease in short-term studies in patients younger than 50 years-old (17). This finding is in agreement with our results. On the other hand, Neufingerl et al (20) have recently observed that the frequent consumption of 850 mg of pure theobromine significantly increased HDL-c levels.



They explained that theobromine increases the concentration of apolipoprotein A-I, the major apolipoprotein of HDL-c. However, to consume 850 mg of theobromine, 100 g of dark chocolate are needed, which additionally contains saturated fats and calories. Moreover they did not completely control the physical activity and diet during the intervention, factors that may influence HDL-c levels (20).

No significant changes of TG, total cholesterol and glucose were observed in the meta-analysis by Shrima et al (17). However, Hooper et al (13) suggest a possible beneficial effect on TG and HOMA after the daily consumption of 50-100mg of epicatechins (13), although no effects were evident at higher or lower doses. Our subjects consumed 153.44 mg/d of epicatechins in the cocoa group, which could explain the absence of effects. Moreover, a limitation is the length of the study, which no provides information about long-term effects.

Although BP decreased, no additional effect due to cocoa consumption was noted, which is in agreement with the meta-analysis carried out by Ried et al (21) where it is suggested that flavanol-rich chocolate does not reduce blood pressure below 140/80 mmHg. However, some studies have found a reduction in BP probably associated with the increase of nitric oxide (NO) bioavailability after cocoa flavanols consumption (12).

MPO and oxLDL are molecules involved in the development of oxidative stress and consequently in atherosclerosis (22-23). Particles of oxLDL are generated during lipid peroxidation, and are able to damage endothelial cells. MPO is a leukocyte-derived enzyme that produces reactive intermediate compounds, which plays an oxidative role over LDL-c, contributing to the transformation into oxLDL. Nitrite, the major oxidation product of NO, is the substrate of MPO and favours the production of nitrogen reactive species, which contribute to oxidise LDL-c (23). In our study, the decrease on oxLDL concentration was significantly higher in the cocoa group. This finding could be linked to the significant decrease of MPO levels only in cocoa group, an effect probably related to the capacity of cocoa flavanols to modulate oxidative reactions catalysed by MPO (24). On the other hand, sICAM-1 and sVCAM-1, indicators of endothelial dysfunction, are usually overexpressed in atherosclerotic plaque. Although they are up-regulated by oxLDL (25-26), we have not observed a higher reduction in cocoa group as a consequence of the oxLDL decrease. In this sense, Farouque et al (27) reported that consumption of flavanol-rich chocolate bar and beverage during 6 week did not alter the

concentrations of ICAM-1 and VCAM-1. However, Jung-Suk et al (28), suggested that flavonoids are able to inhibit the expression of sVCAM-1 and s-ICAM-1.

The antioxidant status becomes weak with age (5), with apparent poorer antioxidant status in men than in women (6). In agreement with this, we have found that cocoa consumption, adjusted for different models, has an effect on % $\Delta$ oxLDL of men, while in women this result was not observed. This antioxidant effect is probably related with the radical scavenging capacity of cocoa flavanols, which protects cells against oxidative stress and endothelial dysfunction (11). Other hypothesis could be that men were more oxidised than women, thus, the effect of cocoa flavanols is more effective, given that some studies have not observed additional benefits after intake of antioxidants (29).

## **CONCLUSION**

This study supports that the consumption of cocoa extract as part of a ready-to-eat meals and within a hypocaloric diet, improve oxidative status (oxLDL) in middle-aged subjects, and particularly in men, supporting the antioxidant effect of cocoa flavanols.

However, additional research is needed to better understand the role of cocoa flavanols in cardiovascular diseases. Thus, a long-term trial including a larger sample of men, could contribute to clarify these promising findings.

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## **FIGURE LEGENDES**

**Figure 1** Flow-chart of the intervention

## Figure 2 Oxidized LDL (oxLDL) reduction in control and cocoa group.

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