Research report

Effect of consumption of dark chocolate on oxidative stress in lipoproteins and platelets in women and in men

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Introduction

Epidemiological studies suggest that regular consumption of foods and beverages rich in flavonoids is associated with a decreased risk of cardiovascular mortality, including coronary artery disease and stroke. This beneficial cardiovascular effect of flavonoids has been attributed to their natural antioxidant properties and their role in conserving proper tocopherol content in biological membranes (Middleton, Kandaswami, & Theoharides, 2000). The beneficial effects of chocolate on healthy humans have been widely addressed by a large number of studies in recent years. Because it concerns the cardiovascular apparatus, a particular group of flavonoids, namely, the flavan-3-ols (flavanols), has been recently investigated. Plant-derived foods and beverages are particularly flavanol-rich, such as wine, tea, and various fruits and berries, as well as cocoa and cocoa products, where flavanols can be present as either monomers (epicatechin and (-)-catechin or oligomers of epicatechin and/or catechin (procyanidins) (Keen, Holt, Oteiza, Fraga, & Schmitz, 2005). As chocolate is a significant source of flavonoid antioxidants (Nanetti et al., 2008), several short-term clinical trials have shown beneficial cardiovascular effects of chocolate or cocoa consumption, including improvement of endothelial function (Flammer, Hermann, & Sudano, 2007; Heiss, Finis, & Kleinbogard, 2007), inhibition of platelet aggregation, and LDL oxidation.

NO is a gaseous free radical produced intracellularly by NO synthases (NOSs) during the enzymatic conversion of L-arginine to L-citrulline (Nanetti et al., 2007). Having an unpaired electron, NO is a highly reactive free radical that damages proteins, carbohydrates, nucleotides and lipids, and, together with other inflammatory mediators, results in cell and tissue damage, low-grade, sterile inflammation and cellular adhesions (Nanetti et al., 2007). NO potently relaxes arterial and venous smooth muscle and, less strongly, inhibits platelet aggregation and platelet adhesion. NO overproduction may combine with superoxide anion (O2-•) to produce peroxynitrite (ONOOC•), which is involved in cellular dysfunction. Peroxynitrite is formed when the two free radicals react in a near diffusion-limited reaction (Nanetti et al., 2007).
Oxidative stress is defined as an imbalance between the oxidant and antioxidant systems of the body, in favor of the oxidant ones. Oxidative stress plays a role in a few human pathological states. Researchers have investigated the relationships between stress and many different medical disorders, such as cardiovascular diseases (Ohman, Nyberg, Bergdahl, & Nilsson, 2007), diabetes (Habhab, Sheldon, & Loeb, 2009), and cholesterol levels (Mutus, Antolovic, & Cachat, 2001). It is well known that chocolate contains a variety of different compounds such as saturated fat, polyphenols, sterols, di- and tri-terpenes, aliphatic alcohols, and methylxanthines (Lamuela-Raventos, Andres-Lacueva, Permanyer, & Izquierdo-Pulido, 2001).

Specific cardioprotective effects recently ascribed to the cocoa flavonoids include: decreased susceptibility to low density lipoprotein (LDL) oxidation and sparing of alpha tocopherol in vitro and ex vivo, and inhibition of platelet activation and aggregation (Rein et al., 2000). Furthermore, an increase in plasma antioxidant capacity and a decrease in plasma oxidation products are associated with elevated epicatechin concentrations (Wan et al., 2001). Moreover, oxidative modification of LDL has been shown to play a key role in the initiation of atherogenesis (Wan et al., 2001). Studies have shown that flavonoids prevent LDL oxidation in vitro by scavenging radical species or sequestering metal ions (Morel, Lescoat, Cillard, & Cillard, 1994). The oxidative modification of LDL plays an important role in atherogenesis and agents that are able to prevent LDL oxidation in the arterial wall might delay the onset of atherosclerosis (Fuhrman & Aviram, 2001). It is known that cocoa flavonoids can improve endothelial function and/or influence oxidative stress in human subjects but, to our knowledge, no studies are present on gender differences in consumption of dark chocolate. So the aim of this research has been to investigate the effects of 3 weeks consumption of 50 g daily of flavonoid-rich dark chocolate on platelets and lipoprotein oxidative stress in women compared to men. In particular, NO and ONOO⁻ levels were determined in platelets before and after supplementation with dark chocolate for 3 weeks, and levels of thiobarbituric acid-reactive substances (TBARS), conjugated dienes, and hydroperoxide levels in HDL and LDL before and after consumption of flavonoid-rich dark chocolate were determined. The serum lipid profile was also studied.

Subjects and methods

Subjects and study setting

Fifty healthy Italian–Caucasian volunteers (25 men, 25 women; age range 28–45 years) were recruited without restriction as to age and socioeconomic status. The subjects were enrolled between March and May 2008.

The BMI was between 21 and 26 kg/m² (calculated with formula: kg/hcm²), waist circumference according to ATP (Adult Treatment Panel) III guidelines (Third Report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults) (Executive Summary published in JAMA, 2001), and the subjects included men and premenopausal women. Subjects were recruited if they fulfilled the following criteria: (i) BMI < 30 kg/m²; (ii) no regular use of any drugs or supplements with antioxidant (β-carotene, vitamins C or E) or lipid-lowering properties; (iii) no chronic diseases such as diabetes, CHD, or other major illnesses; (iv) complete normal blood count; no alcohol intake, and (v) willingness to consume 50 g of the studied chocolate daily for 3 weeks without consuming other cocoa products in this period, (vi) no smoking habits. Moreover, postmenopausal women were excluded because estrogen replacement therapy decreases LDL oxidation and preserves endothelial function. In addition, the subjects were asked to consume a low flavonoid diet throughout the study period, starting it 1 week prior to the study. Instructions for food records were given, checked with the subjects, and analyzed by a nutritionist to avoid weight increase.

The subjects were advised to avoid taking any supplements and all other cocoa products (also tea, red wine and drinks rich in natural antioxidants) and to lead their usual lifestyle throughout the study (i.e., Mediterranean diet).

Subjects, including control group, were studied at baseline (T0) and after consumption of cocoa (50 g/day of dark chocolate bar) for 3 weeks (T1). Dark chocolate was provided by the confectionary company Picchio in Loreto. The composition of dark chocolate is shown in Table 1 including data of chocolate composition provided by the confectionary company.

The study protocol was approved by the Institutional Research Ethics Committee of Marche Polytechnic University and was conducted in accordance with the principles of the Declaration of Helsinki as revised in 2001. All subjects gave written informed consent.

The clinical characteristics of subjects involved in this study at T0 and T1 were obtained by clinical analysis and are shown in Table 2. Blood was drawn in the fasting state for glycemia determination.

Platelet isolation

Peripheral venous blood was drawn after overnight fasting (8–12 h), and immediately mixed with Anticoagulant Citrate Dextrose (ACD) (36 ml citric acid, 5 mM KCl, 90 mM NaCl, 5 mM glucose, 10 mM EDTA, pH 6.8). Platelets were isolated by differential centrifugation in anti-aggregation buffer (Tris–HCl 10 mm; NaCl 150 mm; EDTA 1 mm; glucose 5 mm; pH 7.4) according to Vignini et al. (2008). The method involved a preliminary centrifugation step (200 x g for 10 min) to obtain platelet-rich plasma (PRP). The platelets were then washed three times in anti-aggregation buffer and centrifuged as above in order to remove any residual erythrocytes. A final centrifugation at 2000 x g for 20 min was performed to isolate the platelets. The platelet pellet was washed twice in phosphate buffered saline PBS (containing NaCl 135 mM, KCl 5 mM, EDTA 10 mM, NaPO₄ 8 mM, NaH₂PO₄ H₂O 2 mM, pH 7.2) and immediately used for the experiments or stored at –80 °C.

NO production

NO platelet production was evaluated by Assay Designs™ Total Nitric Oxide Assay Kit (Catalog No. 917-020; 192 Determination Kit); this commercial kit is a complete kit for the quantitative determination of total NO in biological fluids. The kit involves the enzymatic conversion of nitrate to nitrite, by the enzyme Nitrate Reductase, followed by the colorimetric detection of nitrite as a colored azo dye product of the Griess reaction (Griess, Sun, Zhang, Broderick, & Fein, 2003) that absorbs visible light at 540 nm. The modified protocol which employs deproteinization and reduction of nitrates to nitrites in the presence of NADPH-sensitive reductase, prior to addition of the Griess reagent, allows

Table 1

<table>
<thead>
<tr>
<th>Nutrient component</th>
<th>Per 100 g</th>
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<tbody>
<tr>
<td>Energy (kcal)</td>
<td>515</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>6.0</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>46.0</td>
</tr>
<tr>
<td>Total fat (g)</td>
<td>33.0</td>
</tr>
<tr>
<td>Saturated acid (g)</td>
<td>7.0</td>
</tr>
<tr>
<td>Oleic acid (g)</td>
<td>7.6</td>
</tr>
<tr>
<td>Linoleic acid (g)</td>
<td>0.9</td>
</tr>
<tr>
<td>Linolenic acid (g)</td>
<td>0.1</td>
</tr>
<tr>
<td>Catechins (mg)</td>
<td>25.2</td>
</tr>
<tr>
<td>Epicatechins (mg)</td>
<td>151.5</td>
</tr>
<tr>
<td>Total procyanidin (mg)</td>
<td>108.1</td>
</tr>
</tbody>
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measurement of combined nitrite (NO\(_2^-\)), and nitrate (NO\(_3^-\)) (recently named together as NO\(_x\)) and can be successfully applied for measurement of NO levels in human body fluids (Guevara et al., 1998). Since all products of NO and its derived species ultimately give only NO\(_2\) and NO\(_3\), this modified Griess reaction allows a quantitative tally of all NO produced during a given period (Weissman & Gross, 2001). In this assay equal amounts of 1% sulphanyllic acid and 0.1% N-(1-naphthyl) ethylene diamine were added to the samples and the resulting absorbance was measured at 543 nm. Blank (background) was determined in each experiment utilizing medium incubated without a sample. The amount of NO\(_x\) in each sample was determined using a standard curve generated with known concentrations of NO\(_x\) and expressed as nmol NO\(_x\)/mg protein. Protein concentration was determined by Bradford BioRad protein assay, using serum albumin as a standard to normalize the NO\(_x\) concentration data (Bradford, 1976).

This assay can be performed in a conventional spectrophotometer using 96-well microtiter plates, (provided with the kit) which require less sample volume and offer increased speed, throughput, and precision (due to simultaneous measurement of all standards and unknowns).

### Peroxynitrite production

Peroxynitrite production in platelets was evaluated using the fluorescence probe 2,7-dichlorofluorescein diacetate (DCFDA) as previously described (Tannous et al., 1999; Vignini et al., 2007). DCFDA free base was prepared daily, by mixing 0.05 mL of 10 mmol/L DCFDA with 2 mL of 0.01 N NaOH, at room temperature for 30 min. The mixture was neutralized with 18.0 mL of 25 mmol/L phosphate-buffered saline (PBS) pH 7.4; this solution was maintained on ice in the dark until use (Nanetti et al., 2008).

Briefly, platelets were incubated for 15 min with 5 mM DCFDA-free base at 37 °C. Then the DCFDA treated platelets were divided into two sets: one set was incubated with the addition of a mixture of l-arginine 100 mM and NG-nitroarginine (l-NMMA) 100 mM for 15 min at 37 °C in the dark while the other set was incubated with the above mentioned mixture but only with PBS. After washing platelets twice in PBS (25 mmol/l) pH 7.4, samples were sonicated to lyse platelet pellets. The mixture was then centrifuged at 200×g for 5 min and the fluorescence was measured in the supernatant in a Perkin–Elmer LS-50B spectrofluorometer, at an excitation wavelength of 475 nm and emission wavelength of 520 nm. Blank samples contained all reagents except platelets. The fluorescence results obtained after l-NMMA incubation were subtracted from those without l-NMMA. ONOO\(^-\) production was corrected by protein concentration and it was expressed in arbitrary fluorescence numbers/mg prot.

### Preparation of low-density lipoproteins

Blood samples were obtained at 08:00 h after an overnight fast (8–12 h) and were collected into heparin-containing Vacutainer tubes. Plasma was prepared by centrifugation at 1300×g for 15 min and used for the preparation of lipoproteins. LDL, VLDL and HDL were isolated from the plasma by density gradient ultracentrifugation using a TV-865 B vertical rotor according to the method of Vignini et al. (2004). The HDL and VLDL obtained by ultracentrifugation were dialyzed against 0.9% NaCl at 4 °C for 24 h and stored at −80 °C. The protein concentration of HDL and LDL was determined by the method of Bradford (Bradford, 1976).

### Plasma Lipid hydroperoxides

The levels of lipid hydroperoxides in lipoprotein (100 μg) were evaluated using the FOX assay as described by Ferretti et al. (2004). Samples were incubated with FOX reagent, then centrifuged at 4,500 rpm for 20 min. The absorbance of the supernatant was determined at 560 nm and hydroperoxide levels were calculated using the molar coefficient (4.3 × 104 M\(^-1\) cm\(^-1\)) (Ferretti et al., 2004).

### Susceptibility to oxidative stress in vitro

Lipoproteins were incubated at 37 °C for 18 h with a freshly prepared CuSO\(_4\) solution added to the final concentration of 10 μM to a solution containing 1 mg/ml of LDL or HDL protein. The degree of lipoprotein oxidation was determined by measurement of the lipoperoxide level and TBARS before and after peroxidative stress (Rabin et al., 1994). TBARS were determined using the method of Yagi (1994). Results are expressed as nanomoles malondialdehyde per 100 μg protein. Proteins were measured by the method of Bradford (1976). Oxidizability was determined as the difference in TBARS content before and after incubation with CuSO\(_4\).

### Determination of conjugated dienes

Conjugated dienes were assayed by monitoring the increase in absorbance at 234 nm as described previously (Esterbauer, Gebicki, Puhl, & Jürgens, 1992). This is a single point assay.

### Statistical analysis

The results for each subject are expressed as means ± standard deviations. The differences between baseline and end-point values within the pooled groups were evaluated using ANOVA and Bonferroni’s test. Statistical analysis was performed using the SAS statistical package (Statistical Analysis System Institute, Cary, NC). Differences were considered significant with p < 0.05.

### Results

All subjects completed the study and maintained their weight and waist circumference. The clinical characteristics of the study group before and after consumption of chocolate are shown in Table 2. Overall, compliance with the nutritional instructions and restriction was good, as none of the subjects reported consumption of the restricted foods (tea, red wine, cocoa, and chocolate products other than the studied chocolate). There was a nonsignificant change in BMI, fasting plasma glucose levels (glycemia) and fasting plasma cholesterol levels. Moreover there was a significant in-
crease of HDL cholesterol in T1 compared to T0 ($p < 0.001$), more evident in women than in men. Moreover LDL cholesterol decrease significantly ($p < 0.001$) in women in T1 compared to T0, but this value decrease not significantly in men in T1 in respect to T0. Moreover there was a significant increase of triglyceride in T1 in respect to T0 ($p < 0.001$), more evident in women than in men.

Platelet NO concentrations significantly increased in T1 compared to T0, ($p < 0.05$) in women and in men (Fig. 1). In fact NO increased after supplementation by 15.7% compared to basal determination in women, and by 32.2% in men. The basal levels of NO were higher in women than in men. In our samples, ONOO$^-$ levels significantly decreased in T1 compared to T0, and the levels were significantly lower in women than in men ($p < 0.05$) (Fig. 2). After supplementation peroxynitrite values decreased in women by 24% and in men by 18.6%.

These decreases were higher in women than in men and basal levels were lower in women than in men. TBARS concentration, lipid hydroperoxides, and conjugate diene formation in HDL significantly ($p < 0.05$) decreased after 3 weeks of dark chocolate consumption compared to baseline and was more evident in women than in men (Figs. 3–5). TBARS concentration in women’s HDL decreased by 26.7% while in men’s HDL by 23.4%; lipid hydroperoxides decreased in women’s HDL by 62.8% while in men’s HDL they decreased by 21.1%. Furthermore, in both the LDL and HDL, baseline hydroperoxides levels were significantly ($p < 0.05$) higher in women than in men. Moreover conjugate diene formation decreased in women’s HDL by 55.9%, while in men’s HDL it decreased by 48.2%. Furthermore, in both the LDL and HDL, baseline levels of hydroperoxides were significantly ($p < 0.05$) higher in women than in men; TBARS concentration, lipid hydroperoxides, and conjugated diene formation in LDL also significantly decreased after 3 weeks of dark chocolate consumption compared to baseline. Moreover these modifications were significantly different in women than in men. In fact TBARS concentration decreased in women’s LDL by 26.7% after supplementation and in men’s LDL by 21.6%; lipid hydroperoxides decreased in women’s LDL by 83.6% while in men’s LDL they decreased by 64.7%. Furthermore conjugate diene formation decreased in women’s LDL by 48.2%, while in men’s LDL it decreased by 21.6%.

**Discussion**

The protective effect of dietary flavonoids, a class of semi-essential food components (Havsteen, 2002), against many diseases, in particular cardiovascular disease and cancer, as supported by a myriad of studies examining potential sites and modes of action (Waterhouse, Shirley, & Donovan, 1996) has been well described.

Cocoa and chocolate are a rich source of polyphenols. In recent years cocoa, coffee and tea have been reevaluated for their antiox-
and inhibit the oxidation of LDL ex vivo (Mathur et al., 2002). The concentration of serum HDL cholesterol and the oxidative modification of LDL play important roles in the pathogenesis of atherosclerosis (Safeer & Cornell, 2000) and previous studies suggested that consumption of cocoa or chocolate may have beneficial effects on both. In fact consumption of cocoa and dark chocolate have been demonstrated to increase the concentration of HDL cholesterol (Rein et al., 2000) and plasma antioxidant capacity, decrease the formation of lipid oxidation products (TBARS) and conjugated dienes. Several studies have highlighted that the oxidative pathway differs between genders, being more pronounced in women as a result of a delicate balance between estrogen production and increased oxidative stress produced during the entire reproductive period (Agarwal, Gupta, & Sharma, 2005 Nov; Dopsaj, Martinovic, Dopsaj, Stevuljevic, & Bogavac-Stanojevic, 2011 Jan; Wiener-Megnazi et al., 2011).

Therefore, the higher lipid peroxidation levels observed in women of this study confirm the more pronounced oxidative stress pathway typical of women in fertile age, in line with previous studies (Wiener-Megnazi et al., 2011) and could suggest a better response in the explicit assumption of antioxidant defense mechanisms against oxidative damage, resulting as more marked in women than in men. It has been reported that the formation of conjugated dienes is the first step in the process leading to the formation of oxidized fatty acids (Ferretti, Bacchetti, Menanno, & Curatola, 2004). In the present study, using procedures such as the FOX assay and TBARS, we determined a more extensive pathway of lipid oxidation. These effects after chocolate consumption might be due to flavonoids contained in dark chocolate. Moreover, the decreased LDL peroxidation could depend on fatty acids in chocolate according to previous studies which have shown a higher inhibition of lipid peroxidation by monounsaturated fatty acids compared to polyunsaturated ones (Kubes, Suzuki, & Granger, 1991).

Regarding the results on platelet function, was observed an increase in platelet levels of nitric oxide in T1 compared to T0, with a lesser increase in women than in men (15.6% in women vs. 32.2% in men), but with higher baseline levels in women than in men.

The higher baseline levels in women could serve to counter the oxidative stress pathway typical of the stronger potential, while the smaller NO increase in women than in men after consumption of dark chocolate could indicate that, as the physiological mechanism is inherently more complex and involved along with estrogen in maintaining the fragile balance typical of the oxidative potential, it is affected only minimally by short-term supplementation. In contrast, men, more prone to cardiovascular risk and not physiologically involved in the delicate oxidative balance, had lower basal levels of NO which increased more after supplementation.

On the contrary platelet peroxynitrite levels decreased in T1 compared to T0. Peroxynitrite platelet levels decreased in T1 compared to T0, with a greater decrease in women than in men (23.9% in women vs. 18.6% in men), but with lower baseline levels in women than in men.

The greatest decreases in platelet ONOO· observed in women, in agreement with the greatest decreases observed in the peroxidation in LDL and HDL, indicated that the production of ONOO· could be directly affected by the supplementation of chocolate for the presence of flavonoids, which stabilize the superoxide radical, and likewise the basal levels of ONOO·, lower in women than in men, may also be related to higher basal levels of NO produced by eNOS and physiologically more involved in the oxidative balance of the picture and not in the production of ONOO·.

These results were consistent with a recent review showing that the intake of cocoa and derivatives rule optimal levels of NO and induce a lower production of superoxide anion (Fraga, Galleano, Verstraeten, & Oteiza, 2010). In fact NO has multiple roles in the regulation of the cardiovascular system. Further, endothelium-derived NO also counteracts platelet aggregation to the site of inflammation (Eritsland, 2000) thus exerting a significant anti-inflammatory effect. On a different level, NO is critical in determining the oxidative stress in several conditions by reacting with the superoxide to produce peroxynitrite. Furthermore, the increase of NO after dark chocolate supplementation could have a protective role due to anti-oxidant effects of dark chocolate. In
fact peroxynitrite levels decreased after supplementation, thus reducing the adverse effect of free radicals.

In conclusion, our study showed that a short-term intake of dark chocolate might improve lipoprotein and platelet profile in healthy humans, more in women than in men and might exert a protective effect on the cardiovascular system. This protective role, better in women than in men, would be particularly favorable during menopause, risk period during which, because of physiological disruption of estrogen, women are more susceptible to oxidative damage and thus cardiovascular risk.

Our results, showing no significant changes in plasma glucose and cholesterol imbalances divided into different lipoproteins, suggest a possible future application of the study of patients with diabetes and/or suffering from dyslipidemia. Further studies are in progress to elucidate long-term effects of dark chocolate consumption in improving the antioxidant status.

However, it must be underlined that a potential limitation of the present study is the lack of a control arm treated with a placebo and of a double-blind randomization. Because of these limitations, our results can be considered highly suggestive of an effect of chocolate supplementation on cardiovascular risk, but also indicates the need for further placebo-controlled studies on this issue.

References


