Effect of ingestion of dark chocolates with similar lipid composition and different cocoa content on antioxidant and lipid status in healthy humans

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Abstract

The association between in vitro antioxidant capacity of dark chocolates with different cocoa percentage and the in vivo response on antioxidant status was investigated. In a randomized crossover design, 15 healthy volunteers consumed 100 g of high antioxidants dark chocolate (HADC) or dark chocolate (DC). In vitro, HADC displayed a higher Total Antioxidant Capacity (TAC) than DC. In vivo, plasma TAC significantly peaked 2 h after ingestion of both chocolates. TAC levels went back to zero 5 h after DC ingestion whilst levels remained significantly higher for HADC. HADC induced a significantly higher urinary TAC in the 5–12 h interval time than DC. No change was detected in urinary excretion of F2-isoprostanes. Plasma thiols and triacylglycerol (TG) levels significantly increased for both chocolate with a peak at 2 h remaining significantly higher for DC after 5 h respect to HADC. Results provide evidence of a direct association between antioxidant content of chocolate and the extent of in vivo response on plasma antioxidant capacity.

Keywords: Dark chocolate, Total Antioxidant Capacity, Triacylglycerols, Thiols

1. Introduction

Epidemiological evidence suggests that diets rich in plant foods protect from the risk of developing degenerative diseases characterised by high oxidative stress conditions (Valko et al., 2007). This beneficial effect has been hypothesised to be linked to the high content on bioactive molecules as flavonoids and vitamins, equipped with a wide variety of actions, including antioxidant mechanisms (Razquin et al., 2009). Human intervention studies have shown that the ingestion of plant foods such as lettuce, blueberries, wine, tea and chocolate is able to modulate in vivo endogenous non-enzymatic antioxidant network, measured by Total Antioxidant Capacity (TAC) (Serafini & Del Rio, 2004). Cocoa-derived products have been shown to improve markers of cardiovascular function, displaying an amelioration of endothelial function (Schoeter et al., 2006), an inhibition of platelet adhesion and a decrease in blood pressure (Flammer et al., 2007; Murphy et al., 2003; Taubert, Roesen, Lehmann, Jung, & Schömg, 2007). Chocolate displays very high levels of in vitro TAC compared to other foods of vegetable origin (Pellegrini et al., 2003). It has been shown to increase plasma TAC (Serafini et al., 2003) and LDL resistance to oxidation after ingestion in acute and short term intervention studies in healthy (Mursu et al., 2004; Osakabe et al., 2001) and hypercholesterolaemic individuals (Baba et al., 2007).

However, the information provided by in vitro TAC can be translated only partially into in vivo systems due to, among other factors, the low absorption of flavonoids (about 5% of the ingested dose) and the extensive metabolism they undergo (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). Physiological concentrations of phenolic compounds in body fluids are in the order of nanomol/L whilst the extent of increases observed on in vivo antioxidant capacity after ingestion of polyphenol-rich foods refers to μmol/L, making difficult to establish causal relationships (Lotito & Frei, 2006; Manach et al., 2005). On the other hand, it has been suggested that the beneficial effects of flavonoids in humans may be linked to a synergistic interaction with the endogenous redox network (Liu, 2004; Serafini, 2006). Hence, the extrapolation of in vitro TAC to in vivo effects on plasma endogenous antioxidant network is not obvious and data on their associations are lacking (Wang et al., 2000).

In order to fulfill this gap we investigated the association between in vitro antioxidant capacity of dark chocolates with different cocoa percentage but similar lipid content, and the in vivo response on antioxidant and lipid status in healthy humans.

2. Methods and materials

2.1. Chemicals

The 2,4,6-tripyridyl-s-triazine (TPTZ) was purchased from Fluka (Italy). Sodium acetate trihydrate, ferrous sulphate heptahydrate...
(FeSO₄·7H₂O), ferric chloride hexahydrate (FeCl₃·6H₂O), sodium phosphate dibasic dodecahydrate (Na₂HPO₄·12H₂O), 5,5'-dithiobis(2-nitro-benzoic acid) (DTNB), glutathione (GSH) and ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA-Na₂·2H₂O) were purchased from Sigma (St. Louis, MO USA). Potassium dihydrogen phosphate (KH₂PO₄) was purchased from Carlo Erba (Milan, Italy). Glacial acetic acid used was HPLC-grade and purchased from Carlo Erba (Milan, Italy). High-purity water was obtained in the laboratory by using an Alpha-Q system (Millipore Co.).

2.2. Food analyses

Test meal consisted of high antioxidants dark chocolate (HADC) with a 66% of dry cocoa solids (Patent WO 2008/02684) and dark chocolate (DC) with a 55% of dry cocoa solids and were provided by Puratos Group (Belgium). None of them contained milk components. Nutritional composition of both types of chocolate is detailed in Table 1. Nutritional values have been determined by Puratos Group (Belgium) using standardized protocols that follow OICCC Legislation (Office International du Cacao, du Chocolat et de la Confiserie, Brussels).

An extraction process has been performed on both chocolates in order to assess their in vitro antioxidant capacity (Pellegrini et al., 2006). Chocolate (0.5 g) was defatted with 5 mL n-hexane for 5 min in an ultrasonic bath at 30 °C and subsequently centrifuged for 10 min at 1000 g at 4 °C. Antioxidants were then extracted with 5 mL of a mixture of acetone/water (70:30 v/v) under agitation for 10 min at 30 °C in the ultrasonic bath, centrifuged at 1000 g for 10 min at 4 °C and the supernatant was collected. The extraction was repeated with 2 mL of the same mixture and the supernatants were combined for FRAP analysis.

2.3. In vivo study design

Approval for the study has been obtained from the Ethics Committee for Non-Clinical Research of San Raffaele Hospital and all procedures involving human subjects complied with the Declaration of Helsinki as revised in 2000. All participants in the study have given their written consent. We followed a restrictive exclusion/inclusion criteria for the volunteer participating in the study, to maximise homogeneity of the group and minimise confounding factors. After power analysis for determination of the sample size, fifteen healthy volunteer (6 men and 9 women) were enrolled on the basis of the following criteria: non-smoking, BMI between 18 and 25 kg/m², normo-lipidaemic, taking neither antioxidant supplements nor medication for at least one month before the intervention/inclusion criteria for the volunteer participating in the study, to maximise homogeneity of the group and minimise confounding factors. After power analysis for determination of the sample size, fifteen healthy volunteer (6 men and 9 women) were enrolled on the basis of the following criteria: non-smoking, BMI between 18 and 25 kg/m², normo-lipidaemic, taking neither antioxidant supplements nor medication for at least one month before the intervention. A questionnaire on dietary and lifestyle habits was provided to each volunteer. Physical characteristics of the participants and baseline levels of the different biomarkers are described in Table 2. In order to improve homogeneity of baseline conditions before the intervention day, for two days prior to each feeding study and throughout the day of study (0–24 h) the subjects have followed a low antioxidant diet by avoiding foods known to be high in antioxidants (all fresh fruit and vegetables and their products, as chocolate, tea, coffee, fruit juices and wine) and dietary antioxidant supplements. Dietary record has been kept to monitor compliance of the diet.

The study has followed a randomized, cross-over, double-blind design to minimize external variations. The acute ingestion model represents an optimal study design for reducing interindividual variability, because the experimental window utilised is free from any potential confounder (diet, physical activity, circadian variations, etc.) and allows to clearly identifying the effect of the food ingestion on the selected markers. On the day of intervention, after an overnight fast, subjects have been randomised in 2 groups and received 100 g of high antioxidants dark chocolate (HADC) or 100 g of dark chocolate (DC). Venous blood samples have been collected before chocolate ingestion (T₀) and at different time points (0.5, 1, 2, 4 and 5 h) for biomarker measurement. During the sampling time (0–5 h), all eligible participants have been asked to consume a maximum of 500 ml of water.

Sampling time has been decided on the basis of previous studies performed in our laboratory and it is in keeping with the changes previously observed on antioxidant profile (Serafini et al., 2002). Blood has been collected in EDTA and Heparin-tubes and immediately centrifuged (3000g at 4 °C for 15 min), after which the plasma has been separated and stored at −80 °C prior to analysis. Urine has been collected and stored at −80 °C at different time points (0–5, 5–12, 12–24 h) in order to follow changes of selected biomarkers over the 24 h period. In order to preserve the concentration of free isoprostanes in urine for their measurement, indomethacin was added to the samples at a concentration of 10 µg/mL. After one week of wash out, dietary intervention has been repeated, swapping the treatments, until all subjects have received both types of chocolate.

2.4. FRAP assay

The Ferric Reducing Antioxidant Potential (FRAP) assay measures the reducing power of biological fluids and food items (Benzie & Strain, 1996). It is a spectrophotometric method based on redox reactions where antioxidants act as reducing molecules. It tests the ability of a sample to reduce the colourless complex of ferric-tripyridyltriazine to its ferrous coloured form that develops an intense blue colour. Changes in absorbance at 595 nm are quantified with a standard curve and the values obtained are presented in Table 2.
2.5. Plasma thiol groups measurement

Determination of sulphhydryl groups has been performed using an aromatic disulphide, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), water soluble at pH 8. The product is highly coloured at 412 nm and can be used to quantify the concentration of thiol groups in plasma (Ellman, 1959).

2.6. Lipid oxidative damage

Urinary isoprostane 8-iso-Prostaglandin F_2α has been measured with Assay Designs Enzyme Immunoassay (EIA) Kit, based on a competitive sandwich enzyme immunoassay technique, according to the manufacturer’s instructions (TEMA Ricerca S.r.l., Bologna, Italy). Values obtained have been standardized by urinary creatinine content (Abbott laboratories, USA).

2.7. Biochemical analyses

Total cholesterol (TC) and triglycerides (TG) have been measured by commercial kits (Abbott laboratories, USA).

2.8. Plasma vitamin E analysis

Plasma α-tocopherol concentrations were determined by high-performance liquid chromatography as described by Maiani et al. (1995). Plasma samples (200 μL) were deproteinized by the addition of ethanol (1:1 v/v) and then extracted twice with 2 mL of n-hexane containing butylated hydroxytoluene (0.01% w/v). The combined hexane layers were vacuum dried and residues reconstituted in 200 μL of mobile phase (methanol/acetonitrile/tetrahydrofuran, 50:45:5 v/v/v) to bring samples to original volume; 20 μL were injected by an autosampler into a reverse-phase C18 column and eluted isocratically at a flow rate of 1 mL/min. The peak of α-tocopherol was detected and quantified by the use of an internal standard of α-tocopherol acetate (<1 mg/L) at 292 nm with a multil wavelength spectrophotometer detector (Perkin–Elmer L.C. 95; Norwalk, CT, USA) connected to a data acquisition system (PE Nelson 1020, Perkin–Elmer).

2.9. Statistics

Data are expressed as absolute values and delta of changes respect to baseline (mean ± standard error) following test-meals. Statistical analysis of the absolute changes were carried out with Two Way Repeated Measures (Two Factor Repetition) analysis of variance ANOVA, with chocolate and time as within-subject factors. Bonferroni post hoc analysis of variance was used to isolate differences between treatments. The area under the curve (AUC) of plasma concentration–time (0–5 h) was estimated by using the linear trapezoidal rule. P-Pearson correlation coefficients were established between the AUC of plasma thiol groups and AUC of plasma triacylglycerols, as well as in the different time points.

3. Results

3.1. In vitro Total Antioxidant Capacity (TAC) of dark chocolates

HADC displayed a remarkably 51% higher in vitro reducing power FRAP than DC (Table 1), reflecting its higher cocoa content. The dose response curves showed a linear correlation between FRAP values and the amount of both chocolates tested (r = 0.995 for HADC and r = 0.996 for DC; data not shown).

3.2. Effect of dark chocolate ingestion on plasma FRAP and single antioxidants

The effect of dark chocolate ingestion on plasma levels of FRAP is displayed in Fig. 1: both chocolates were able to significantly increase plasma reducing power, with different efficiency depending on their content on antioxidants. HADC and DC significantly increased plasma FRAP 30 min post-ingestion (+4.1%; p < 0.05 and +4.5%; p < 0.01), 1 h (+4.4%; p < 0.05 and +4.0%; p < 0.05) and reached a peak of increase after 2 h (+7.2%; p < 0.001 and +6.2%; p < 0.001). After 4 h FRAP levels for DC started to decline (+2.5%), going back to baseline levels at 5 h (0%). On the contrary, plasma levels of reducing power for HADC remained significantly higher respect to baseline also after 4 h (+4.1%; p < 0.05) and 5 h (+6.8%; p < 0.001) and were significantly different respect to DC at 5 h (p < 0.001). HADC and DC had a similar impact on plasma FRAP until 2 h (+6 μmol/L), but after the 4th hour, the strongest antioxidant effect of HADC started to become evident (+13 μmol/L) reaching a clear significant effect at 5 h (+60 μmol/L; p < 0.001).

As displayed in Fig. 2, HADC and DC significantly increased urinary FRAP in the 0–5 h interval time post-ingestion respect to 12–24 h interval time (A3 μmol Fe_2+mg creatinine; p < 0.05 and A3 μmol Fe_2+mg creatinine; p < 0.05, respectively for HADC and DC). Urinary FRAP level went back to baseline 5 h post-ingestion of DC. Instead, it remained significantly higher in the 5–12 h interval time post-ingestion of HADC (A3 μmol Fe_2+mg creatinine; p < 0.05) and significantly different from DC (p < 0.05). No differences were detected between the treatments in the interval time 12–24 h.

The effect of HADC and DC consumption on plasma concentrations of vitamin E and thiol groups is reported in Table 3. Plasma α-tocopherol levels did not change during the entire period of observation. Plasma thiol groups significantly increased for both types of chocolate reaching a peak at 2 h post-ingestion (+22.7%; p < 0.001 and +27.5%; p < 0.001 for HADC and DC, respectively), started to decline at 4 h (10.1%; p < 0.05 and 12.8%; p < 0.05 for HADC and DC, respectively) and arrived at baseline levels at 5 h. There were no significant differences in the urinary levels of the isoprostanes at the different interval times of urine collection within each treatment and between treatments (data not shown).

3.3. Effect of dark chocolate ingestion on markers of lipid metabolism

Cholesterol plasma levels did not change during the entire period of observation as displayed in Table 3. No statistical changes were detected between treatments. TG plasma levels significantly increased for both type of chocolates at 1 h, reaching a peak at 2 h post-ingestion (+54 mg/dL; p < 0.001 and +50 mg/dL; p < 0.001 for HADC and DC, respectively) and started to decline at 4 h. After 5 h from ingestion, TG concentration remained significantly higher for DC (p < 0.01 vs baseline) and in comparison to HADC (p < 0.01).

The increase of plasma TG concentrations was parallel to the increases of thiol groups for both types of chocolate. Significant P-Pearson correlation coefficients were established between the AUC of plasma thiol groups and AUC of plasma triacylglycerols in the 5 h interval time post-ingestion for both types of chocolate (r = 0.73, p < 0.01 for HADC; r = 0.85, p < 0.01 for DC). The significant correlations were also established in the different single time points, in the case of DC for all time points until 5 h whereas for HADC the correlation was significant until 4 h, in agreement with the different statistical increase of TG (Table 4).
4. Discussion and conclusion

In the present study we have evaluated the in vitro activity and in vivo human response in terms of antioxidant status of two chocolates with different cocoa content. HADC, with the highest cocoa content, displayed a remarkably higher in vitro antioxidant capacity than DC. In vitro FRAP values for HADC and DC were comparable with previous results (Serafini et al., 2003), showing a higher antioxidant capacity of chocolate than other antioxidant-rich foods such as olive oil, blueberries, red wine or green tea (Pellegrini et al., 2006). In vivo, both HADC and DC were able to significantly increase plasma antioxidant defences in healthy humans, measured as reducing power. Maximum peak of increase on plasma FRAP occurred at 2 h, in agreement with previous evidences in humans (Serafini et al., 2003). Ingestion of 100 g HADC and DC provided antioxidant reducing equivalents of 24 mmol Fe²⁺ and 15.9 mmol Fe²⁺, reflected on maximum delta of changes on plasma FRAP of 67.15 µmol Fe²⁺/L and 61.10 µmol Fe²⁺/L for HADC and DC, respectively. This increment on plasma antioxidant capacity corresponded to a 1.11% and 1.54% of the ingested dose of antioxidants. The in vivo antioxidant efficiency mirrored the different cocoa solids content (66% cocoa solids for HADC vs 55% cocoa solids for DC) and antioxidant concentration of the tested chocolates. HADC induced a higher and longer increase in plasma reducing power than DC. The maintenance of high reducing power of plasma over the 5-h analysed period for HADC can be explain by its higher content of cocoa solids.

In addition, urinary reducing power FRAP increased with both types of chocolate, reflecting the excretion of antioxidant metabolites. After chocolate ingestion, compounds that have typically been found in urine correspond to both catechin monomers (methylated and non-methylated forms) as well as low-molecular weight phenolic acids generated from the metabolism of procyandins in the colon by gut microflora and latterly absorbed (Manach et al., 2005; Rios et al., 2003). The increase on urinary FRAP took place in more extent with HADC confirming the higher absorption of antioxidants displayed by plasma values of FRAP.

Our results are in agreement with other human trials that observed improvements on plasma antioxidant capacity after dark chocolate ingestion. Rein and colleagues (Rein et al., 2000) have reported significant increases in plasma antioxidant capacity measured by chemiluminescence and a decrease on plasma lipid...
and after ingestion of high antioxidants dark chocolate (HADC) and dark chocolate (DC) (Wang et al., 2000). However, it is crucial to underline that in ingestion of different doses (27, 53, 80 g) of the same dark chocolate we observed no effects on urinary excretion of isoprostanes after dark chocolate ingestion; it is possible that the inhibition of lipid oxidation by cocoa flavan-3-ols mainly occurs in subjects with an increased oxidative stress; in the present study, volunteer had a mean BMI of 21.8 kg/m² and a mean age of 30 years, with no reported negative lifestyle habits; then they are supposed to have a relative low basal level of lipid peroxidation.

A significant increase of plasma thiol levels was detected after ingestion of both types of chocolate. We hypothesise that it could reflect an antioxidant endogenous response to the lipid load after chocolate ingestion (Burdge & Calder, 2005). Increased levels of different forms of thiols have been observed in conditions of high oxidative stress (Ueland et al., 1996) as in postprandial hypertriglyceridemia. This hypothesis is further supported by the significant correlation observed between the changes in plasma TG and thiols for both treatments.

Oxidation as TBARS at 2 h post-ingestion of 80 g flavonoid-rich dark chocolate in healthy people, with a maximum peak of (−)-epicatechin levels at 2 h that corresponded approx. to the 0.3% of the ingested dose. In contrast to these findings, the same authors have reported a lack of effect on TAC and lipid oxidation after ingestion of different doses (27, 53, 80 g) of the same dark chocolate (Wang et al., 2000). However, it is crucial to underline that in the second study chocolate was eaten in association with a bagel of bread, which might have caused a poor absorption of flavonoids.

Table 3

Table 3 Plasma concentrations of α-tocopherol (μmol/mmol cholesterol), thiol groups (μM) total cholesterol (mg/dL) and triacylglycerols (mg/dL) expressed as mean values ± SEM before and after ingestion of high antioxidants dark chocolate (HADC) and dark chocolate (DC) (n = 15).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>PLASMA</th>
<th>HADC</th>
<th>DC</th>
<th>HADC</th>
<th>DC</th>
<th>HADC</th>
<th>DC</th>
<th>HADC</th>
<th>DC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-Tocopherol (μmol/mmol cholesterol)</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
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<tr>
<td></td>
<td>Thiol groups (μM)</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
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<tr>
<td></td>
<td>Triacylglycerol (mg/dL)</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>T0.5</td>
<td>4.71</td>
<td>0.13</td>
<td>4.60</td>
<td>0.14</td>
<td>626</td>
<td>52</td>
<td>621</td>
<td>47</td>
<td>168</td>
</tr>
<tr>
<td>T1</td>
<td>4.52</td>
<td>0.14</td>
<td>4.58</td>
<td>0.14</td>
<td>620</td>
<td>26</td>
<td>606</td>
<td>23</td>
<td>170</td>
</tr>
<tr>
<td>T2</td>
<td>4.57</td>
<td>0.13</td>
<td>4.50</td>
<td>0.14</td>
<td>619</td>
<td>23</td>
<td>631</td>
<td>20</td>
<td>170</td>
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<td>T4</td>
<td>4.44</td>
<td>0.15</td>
<td>4.53</td>
<td>0.15</td>
<td>725</td>
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<td>729</td>
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<td>4.62</td>
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</table>

Table 4

Table 4 P-Pearson correlation coefficients between deltas of change on plasma thiol groups (SH) and deltas of change on plasma triacylglycerol (TG) after ingestion of high antioxidants dark chocolate (HADC) and dark chocolate (DC) (n = 15).

<table>
<thead>
<tr>
<th>ASH</th>
<th>HADC</th>
<th>DC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T0.5</td>
<td>T1</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>HADC</th>
<th>ΔTG</th>
<th>T0.5</th>
<th>T1</th>
<th>T2</th>
<th>T4</th>
<th>T5</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0.5</td>
<td>r = 0.526</td>
<td>r = 0.748**</td>
<td>r = 0.931***</td>
<td>r = 0.591</td>
<td>r = 0.502</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>r = 0.226</td>
<td>r = 0.899***</td>
<td>r = 0.861***</td>
<td>r = 0.814***</td>
<td>r = 0.724**</td>
<td></td>
</tr>
<tr>
<td>DC</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* p < 0.05 single time point post-ingestion vs T0.
** p < 0.01 single time point post-ingestion vs T0.
*** p < 0.001 single time point post-ingestion vs T0.
suggests a potential link between the antioxidant content of cocoa and the increase of TG plasma levels after ingestion. We observed no changes on total cholesterol levels; foods from plant origin as chocolate are not exogenous sources of cholesterol but of saturated fatty acids that could adversely increase the total cholesterol and LDL levels (Ding, Hutfless, Ding, & Girotra, 2006). However, studies have also demonstrated that saturated fatty acids from cocoa possess a neutral effect on plasma total and low-density lipoprotein cholesterol concentrations, due to the most abundant presence of stearic acid, 18:0, that is rapidly converted in oleic acid 18:1 n-9 by a 9-desaturase on enterocytes (Kris-Etherton, Derr, Mustad, Seligson, & Pearson, 1994). On the other hand, some authors point out that polyphenols derived from cocoa pow- der may contribute to a reduction in LDL cholesterol and an elevation in HDL cholesterol, and then no change on total cholesterol would take place (Baba et al., 2007).

As overall, results clearly show a clear and significant in vivo antioxidant response mirroring in vitro antioxidant and cocoa content of dark chocolate in human. A potential direct association between changes in TG plasma levels and the response of endoge- nous antioxidants has been suggested. Further research is needed in order to identify the bioactive molecules involved in the antiox- idant effect of cocoa-rich products.

Acknowledgments

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References