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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 volunteer 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.

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 Río, 2004). Cocoa-derived products have been shown to improve markers of cardiovascular function, displaying an amelioration of endothelial function (Schroeter 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, & Schömig, 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 fulfil 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

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($FeSO_4 \cdot 7H_2O$), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), sodium phosphate dibasic dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$), 5,5′-dithiobis (2-nitro-benzoic acid) (DTNB), glutathione (GSH) and ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA-$Na_4 \cdot 2H_2O$) were purchased from Sigma (St. Louis, MO USA). Potassium dihydrogen phosphate (KH_2PO_4) 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 enroled on the basis of the following criteria: non-smoking, BMI between 18 and 25 kg/m^2, 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 (3000*g* 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

Table 1
Nutritional composition and Ferric Reducing Antioxidant Potential (FRAP) of high antioxidants dark chocolate (HADC) and dark chocolate (DC).

	HADC[a]	DC[a]
Energy value (kcal)	521	526
Dry cocoa solids (%)	66.0	55.0
Fat-free dry cocoa solids (%)	28.0	20.0
Total proteins (g)	8.07	5.54
Sugars (g)	37.4	47.2
Dietary fibre (g)	10.5	7.64
Total fat (g)	37.7	35.0
Polyunsaturated (*cis*) (g)	1.64	1.65
Monounsaturated (*cis*) (g)	13.3	12.4
Saturated (g)	22.8	21.1
Total vitamins (mg)	2.75	2.81
Vitamin E (mg)	1.98	2.07
FRAP (mmol Fe^2/kg)	240	159

[a]100 g of product.

Table 2
Physical characteristics, plasma and urine base-line levels of selected biomarkers of the subjects ($n = 15$) (mean ± S.D.).

Biomarker	Mean	SD
Age (years)	30	5
BMI (kg/m^2)	21.8	2.3
Plasma		
Triacylglycerols (mg/dL)	64	19
Total cholesterol (mg/dL)	169	26
α-Tocopherol (μmol/mmol cholesterol)	4.7	0.4
Thiol groups (μM)	624	72
FRAP (μmol Fe^{2+}/L)	964	156
Urine		
FRAP (μmol Fe^{2+}/mg creatinine)	6.9	1.8