

## Original research

### Beneficial effects of oral pure caffeine on oxidative stress



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#### ARTICLE INFO

##### Article history:

Received 14 July 2017

Received in revised form 24 September 2017

Accepted 3 October 2017

##### Keyword:

Oxidative stress  
Coffee  
Caffeine  
Lipid peroxidation  
Glutathione  
Malondialdehyde

#### ABSTRACT

Ingestion of coffee (which is a mixture of over 1000 hydrosoluble substances) is known to protect from type-2 diabetes mellitus and its complications, and other chronic disorders associated with increased oxidative damage in blood and tissues. This protection is generally attributed to polyphenols and melanoidins. Very few studies were conducted on the amelioration of classic blood markers of oxidative stress induced after a few days of caffeine administration, but results vary.

To assess whether caffeine *per se* could account for antioxidant properties of coffee in the short-term, we tested the ability of pure caffeine ingestion (5 mg/kg body weight/day in two daily doses for seven consecutive days) to improve plasma levels of six biochemical indices in healthy male volunteers ( $n = 15$ ). These indices were total antioxidant capacity (TAC), glutathione (GSH), oxidized glutathione (GSSG), GSH to GSSG ratio, lipid hydroperoxides (LOOH) and malondialdehyde (MDA).

We found that all indices changed significantly ( $P < .05$  or  $< .01$ ) in a favourable manner, ranging from  $-41\%$  for GSSG to  $-70\%$  for LHP levels, and  $+106\%$  for GSH levels to  $+249\%$  for the GSG/GSSG ratio. Changes of any given index were uniform across subjects, with no outliers.

We conclude that caffeine has unequivocal, consistent antioxidant properties.

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## Introduction

Oxidative stress is involved in ageing [1–7] and in various diseases, including diabetes mellitus [8–10], atherosclerosis [11,12], rheumatoid arthritis [13–16], Alzheimer's disease [17–19], Parkinson's disease [20–22] and cancer [23–31]. Coffee has an antioxidant power three to five-fold greater than that red wine and tea [32,33]. Accordingly, coffee consumption is associated with a decrease in incidence of the above disease, a beneficial effect that is generally attributed to polyphenols and melanoidins [34,35].

Concerning endocrine and metabolic disorders, coffee exerts a protective effect on type-2 diabetes mellitus [36], decreasing the prevalence of newly detected hyperglycemia [37]. The antioxidants contained in coffee also protect from lipid peroxidation [38,39]. Studies in rats showed that green tea and coffee both inhibited intestinal cholesterol absorption due to their content in epigallocatechin gallate and caffeine [40]. Coffee has recently aroused

interest also because supplementation studies have shown that the consumption of coffee increased the concentration of plasma total homocysteine (tHcy) in humans [39,41]. Elevated plasma tHcy concentrations have been associated with increased lipid peroxidation [42] and it is also suggested to be an independent risk factor for cardiovascular disease [43].

Tools for obesity management including caffeine and green tea have been proposed as strategies for weight loss and weight maintenance. A green tea-caffeine mixture improves weight maintenance, through thermogenesis, fat oxidation, and sparing fat free mass. [41]. Coffee is a complex mixture of potential "nutraceuticals." Indeed, coffee contains about 1500 different substances, approximately half of which are soluble [36]. In order of abundance, typical values for the water-soluble constituents are phenolic polymers (pulp) 8%, polysaccharides 6%, chlorogenic acids 4%, minerals 3%, caffeine 1%, organic acids 0.5%, sugars 0.3%, lipids 0.2%, and aroma 0.1%. The water-soluble constituents of coffee impair the intestinal absorption of L-thyroxine, most likely as a result of physical sequestration of the hormone [36].

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The aim of the study is to assess in human volunteers whether the short term administration of caffeine would be beneficial on lipid peroxidation and a number of indices of oxidative stress.

## Materials and methods

### Study group

Male volunteers had to meet the following criteria in addition to signing the consent form: being of age 18–25 years, nonsmokers, nondrinkers, having normal body mass index (BMI), having a diet that met the dietary reference values indicated by the Società Italiana di Nutrizione Umana (Italian Society for Human Nutrition) [46]. Fifteen volunteers, regular coffee drinkers, were recruited.

The water solution of caffeine given to these volunteers was a galenic formulation prepared by a local pharmacy. This caffeine solution was administered orally, at room temperature, at the dose of 5 mg/kg body weight/day in two daily doses (2.5 mg/kg in the morning and 2.5 mg/kg after lunch) for seven days. The daily dose was equivalent to five cups of coffee. We evaluated the biochemical oxidative markers specified below. Oxidative stress markers were analyzed in plasma before and after the intake of caffeine. Blood for the two time points (baseline and end of the study) was drawn in the morning, with the baseline sample taken prior to the first dose of caffeine and the final sample taken on the morning on day 8.

The markers of oxidative stress measured were (i) total antioxidant capacity (TAC); (ii) Glutathione (GSH); (iii) oxidized glutathione (GSSG); (iv) GSH to GSSG ratio; (v) lipid hydroperoxides (LOOH); (vi) malondialdehyde (MDA). As well known, decreased oxidative stress is associated with an increase in TAC, GSH, GSH to GSSG ratio, and a decrease in the remaining three indices.

### Assays

Lipid peroxidation, was quantified by assessing the oxidative state of the plasma through determination of the levels of lipid hydroperoxides (LOOH,  $\mu\text{mol/l}$ ) by means of spectrophotometric technique analysis, and malondialdehyde (MDA) levels by high-performance liquid chromatography (HPLC). For LOOH, we used the Oxis Bioxytech<sup>®</sup> LPO-560<sup>TM</sup> Assay (Oxis International, Inc., Portland, OR, USA). This assay is based on the oxidation of ferrous ions ( $\text{Fe}^{2+}$ ) to ferric ions ( $\text{Fe}^{3+}$ ) by hydroperoxides under acidic conditions. Ferric ions then bind with the indicator dye, xylenol orange, and form a colored complex. The absorbance of the complex was measured at 560 nm. For MDA measurement, 250  $\mu\text{l}$  serum was added to 50  $\mu\text{l}$  NaOH 6 M and then incubated at 60 °C in water bath for 30 min. Afterwards, proteins were precipitated with 125  $\mu\text{l}$  35% perchloric acid (v/v), with subsequent centrifugation and the mixture was centrifuged at 2800 rpm for 10 min. Next, 250  $\mu\text{l}$  of the supernatant were transferred into an Eppendorf tube and mixed with 25  $\mu\text{l}$  DNPH, which had been prepared as 5 mM solution in 2 M hydrochloric acid. This mixture was incubated for 30 min at room temperature in the dark and 50  $\mu\text{l}$  were analyzed by HPLC [47].

The total antioxidant power (TAC,  $\mu\text{mol/l}$ ) was determined by a colorimetric technique, using a commercial kit (DIACRON (Grosseto, Italy)).

The modulation of antioxidant defenses was determined by analyzing plasma levels of reduced glutathione (GSH,  $\mu\text{mol/ml}$ ), oxidized glutathione (GSSG,  $\mu\text{mol/ml}$ ) and GSH/GSSG ratio. GSH and GSSG were measured by means of HPLC. This extraction procedure requires that blood samples are collected in vacutainer tubes containing K<sub>3</sub>-EDTA. After collection, 100  $\mu\text{l}$  fresh blood were mixed with 12  $\mu\text{l}$  phosphate buffer 10 mmol/l, pH 7.2 (for free

GSH), or 12  $\mu\text{l}$  phosphate buffer 10 mmol/l, pH 7.2, containing 10 mM N-ethylmaleimide (for oxidized GSH). One hundred  $\mu\text{l}$  of this mixture were hemolyzed by adding 900  $\mu\text{l}$  distilled water and immediately deproteinized by adding 200  $\mu\text{l}$  sulfosalicylic acid (12% volume). The content of GSH was assessed in the acid-soluble fraction [48].

### Statistics

For each group, the arithmetic mean of the values found and the relative standard deviation (SD) were calculated. The significance of differences between groups was evaluated by the analysis of variance (ANOVA); P values < .05 were considered statistically significant.

## Results

Data are illustrated individually in Fig. 1, and summarized in Table 1.

All indices changed in a favourable manner, ranging from -41% for GSSG to -70% for LOOH levels, and +106% for GSH levels to +249% for the GSH/GSSG ratio. We did not have any side effects, except for a slight, non-statistically significant, increase in heart rate.

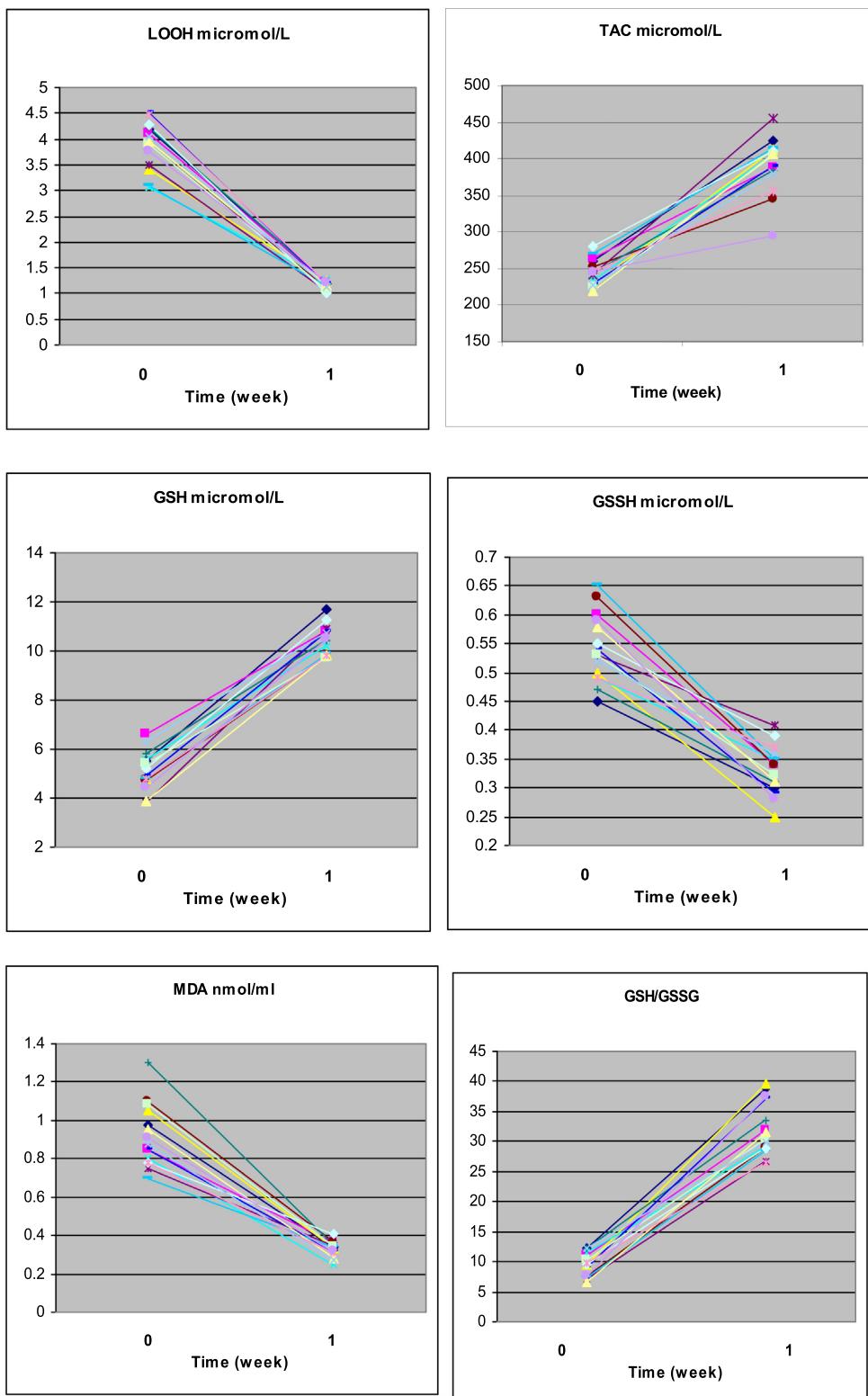
Fig. 1 shows that changes of any given index were uniform across subjects, with no outliers.

### Discussion

As summarized in Table 2, the indices of oxidative stress we have studied in the present paper are of relevance, including the diabetes mellitus setting. Concerning the object of our study, viz. caffeine, data from the literature show beneficial effects on TAC and lipid peroxidation [44,45,49,50] with important additional actions of DNA protection from on oxidative breakage by hydroxyl radicals [51] and of decreased platelet aggregation [52,53].

As recently reviewed [54] prior to us others [39,43,55–57] have evaluated the short-term effects of drinking caffeine on the oxidative stress. While in 4/5 such studies, the number of subjects is lower than ours, only a few have evaluated all the six markers of blood oxidative stress we did. Effects on DNA protection are demonstrable as early as two hours after coffee ingestion [43], confirming previous intervention studies that provided evidence for long-term coffee consumption correlating with reduced DNA background damage in healthy volunteers. Continued coffee intake was associated with further decrements in background DNA damage within the 8 h intervention. Mean tail intensities (TIs%) decreased from 0.33 TI% (baseline, 0 h) to 0.22 TI% (within 8-h coffee consumption). The authors concluded that repeated coffee consumption was associated with reduced background DNA strand breakage, clearly measurable as early as 2 h after first intake resulting in a cumulative overall reduction by about one-third of the baseline value [43].

As reviewed elsewhere, the total antioxidant capacity of plasma is the primary measure and marker to evaluate the status and potential of oxidative stress in the body [58]. Lipid hydroperoxides and MDA have been documented as a primary biomarker of free radical mediated lipid damage and oxidative stress [58]. GSH, the most abundant nonprotein thiol that defends against oxidative stress, is considered as a biomarker of redox imbalance at cellular level [58]. In contrast, GSSG is unable to perform antioxidant functions. GSSG can be reduced back to GSH (and the GSH:GSSG ratio maintained high) by glutathione reductase and associated oxidation of NADPH to NAD<sup>+</sup>, unless such enzymatic activity is overwhelmed by excessive amounts of reactive oxygen species (59).



**Fig. 1.** Individual data in the 15 volunteers for each of the six indices measured. Abbreviations are: GSH = glutathione; GSSG = oxidized glutathione; LOOH = lipid hydroperoxides; MDA = malondialdehyde; TAC = Total antioxidant capacity.

The effect of coffee consumption on the modulation of plasma antioxidant capacity was evaluated in 10 studies [54]. Eight studies (seven chronic interventions and one acute trial) also investigated the role of coffee in the modulation of blood GSH levels as a substrate of GPx [glutathione peroxidase] and GST [glutathione

S-transferases] enzymes. Four out of seven chronic intervention studies documented an increase in GSH levels [42,58,28], while two long-term studies [12,22] and one study performing both an acute and a chronic intervention [55] did not show any significant effect. Coffee ineffectiveness was attributed to the degradation and

**Table 1**

Changes in the indicated indices of oxidative stress observed in 15 healthy male volunteers after one-week administration of 5 mg/kg body weight/day in two daily doses.

Index	Caffeine administration		Statistics (P)
	Before	After	
Lipid hydroperoxides (LOOH), $\mu\text{mol/L}$	3.88 $\pm$ 1.85	1.16 $\pm$ 0.35 (-70%)	<.05
Malondialdehyde (MDA), $\text{nmol/ml}$	0.9 $\pm$ 0.3	0.3 $\pm$ 0.1 (-67%)	<.01
Oxidized glutathione (GSSG), $\mu\text{mol/L}$	0.56 $\pm$ 0.3	0.33 $\pm$ 0.4 (-41%)	<.01
Glutathione (GSH), $\mu\text{mol/L}$	5.1 $\pm$ 1.5	10.5 $\pm$ 2.7 (+106%)	<.01
GSH to GSSG ratio	9.11 $\pm$ 2.8	31.8 $\pm$ 3.4 (+249%)	<.01
Total antioxidant capacity (TAC), $\mu\text{mol/L}$	244.5 $\pm$ 40.3	398.2 $\pm$ 37.0 (+163%)	<.05

\*The beneficial outcome after caffeine administration is a decrease for the first three indices and an increase for the last three indices.

Data are mean  $\pm$  SD. Differences between means  $\pm$  SD by ANOVA.

metabolic conversion of different coffee constituents in the body or to the short duration of the intervention [55].

The effect of coffee consumption on markers of lipid oxidation has been investigated [11,12,18,19,21–23,31,56]. Five out of 12 studies investigated only the acute effect of coffee consumption [16,19,21,23,56,61], five were chronic intervention studies [12,18,19,21,22,61,62], while two studies investigated both acute and chronic effects [23,64]. In these studies, isoprostanes (IsoPs) and malondialdehyde (MDA) were the most frequently considered markers of lipid damage. Besides 8-IsoPGF2 and MDA, further markers of lipid damage and/or protection considered in the present review were oxidized LDL, resistance to LDL oxidation, serum LDL-conjugated dienes and hydroxyl fatty acids. The analysis of the

main findings revealed that most of the interventions failed to demonstrate a significant decrease in markers of lipid damage with exception of results found by Ochiai et al. [57] and Sirota et al. [65]. The former reported a significantly reduced urinary 8-epiPGF2 following consumption of a coffee beverage (providing 600 mg of CGAs) when compared with placebo in healthy men. Results showed that consumption of 200 mL Turkish roasted coffee during a meal based on red-meat cutlets resulted in a significant inhibition of postprandial plasma MDA. No effect between treatments and control/placebo were instead found by other authors [16,23,61]. The investigation by Leelarungrayub et al. [56] deserves a special mention, because it reports a significant higher level of MDA in men consuming caffeinated coffee, when compared to decaffeinated coffee or control, followed by a submaximal exercise test. Authors reported that, similarly to what observed in previous investigations, results demonstrated an increased intramuscular fat oxidation following consumption of caffeine-rich foods. For what concerns the other markers of lipid damage, only Yukawa et al. [66] found a modest reduction of LDL oxidation susceptibility and a decrease of MDA levels following consumption of 3 coffees/day for 1 week. No significant effect was instead found by Mursu et al. [39] on serum LDL-conjugated dienes and plasma hydroxyl fatty acids, or by Teekachunhatean et al. [55] on MDA levels and by Hoelzl et al. [67] on both MDA and oxidized LDL.

In their article [54], Martini et al. conclude that, despite the high inter-study heterogeneity, data suggest that consumption of coffee may increase glutathione levels and reduce the levels of DNA damage. These effects are more evident in chronic interventions than in acute studies.

In summary, we have demonstrated that 7-day administration of pure caffeine induces unequivocally beneficial changes in a number of oxidative-stress biochemical indices, the magnitude of these changes being the greatest for the GSH to GSSG ratio.

**Table 2**

Summary of indices of oxidative stress and diabetes mellitus [ricontrallare referenze].

Index	General	Pertinence for diabetes
Lipid hydroperoxides (LOOH)	Peroxidation of lipids produces highly reactive aldehydes, including MDA, acrolein, 4-hydroxyenonenal, 4-oxonenonal, and isolevuglandins [68]. It has been reported that peroxy radicals can remove hydrogen from lipids, producing hydroperoxides that further propagate the free-radical pathway [69]	Increased lipid peroxidation occurs in both type 1 and type 2 diabetes mellitus [38] LOOH increase particularly in patients with vascular complications [70]. Lipid peroxidation in diabetes induces many secondary chronic complications including atherosclerosis and neural disorders [71,72]
Malondialdehyde (MDA)	MDA is a three carbon, low molecular weight aldehyde representing the main product of polyunsaturated fatty acid peroxidation. It is characterized by a high toxicity due to its ability to react with other molecules like DNA and protein [54–58,28,59,30,60–75]	Increased MDA level in plasma and many tissues was reported in diabetic patients [76,77] Increased levels of MDA in diabetics suggests that peroxidative injury may be involved in the development of diabetic complications
Glutathione (GSH)	MDA is documented as a primary biomarker of free radical mediated lipid damage and oxidative stress [74] GSH is the most abundant nonprotein thiol that defends against oxidative stress [76]. GSH is an efficient antioxidant present in almost all living cells and is also considered as a biomarker of redox imbalance at cellular level [78,79]	Reduced levels of GSH are found in diabetes [79]. Decreased GSH level may be one of the factors in the oxidative DNA damage in type 2 diabetics
Oxidized glutathione (GSSG)	GSSG is reduced back to GSH by the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent catalysis of the flavoenzyme GSH reductase	GSSG levels in plasma from diabetic subjects were higher than those from controls
GSH to GSSG ratio	This ratio is used to evaluate oxidative stress status in biological systems	Plasma GSH/GSSG showed a significant decrease in type 2 diabetes as compared to normal. Hyperlipidemia, inflammation, and altered antioxidant profiles are the usual complications in diabetes mellitus as a result of decreased GSH/GSSG ratio
Total antioxidant capacity (TAC)	TAC is the primary measure and marker to evaluate the status and potential of oxidative stress in the body	TAC is significantly lower in diabetic subjects with poor glycaemic control than healthy subjects, while patients with good glycaemic control had plasma antioxidative values similar to controls [66]. Decrease in TAC of plasma is associated with increased complications of diabetes, which include cardiovascular disease, nerve damage, blindness, and nephropathy TAC is markedly reduced in sciatic nerve homogenates of diabetic animals [61]

The authors declare no conflict of interest.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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