Caffeic Acid Inhibits Oxidative Stress and Reduces Hypercholesterolemia Induced by Iron Overload in Rats

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Received for publication: July 30, 2004; Accepted for publication: November 4, 2004

Abstract: The effects of caffeic acid, a major phenolic compound of the diet, on oxidative stress and cholesterolemia are studied in rats submitted to oxidative stress by iron overload. Male Wistar rats were fed semi-synthetic diets containing regular (50 mg/kg diet) or high (2000 mg/kg) doses of iron with and without caffeic acid (6460 mg/kg) for 4 weeks. The high doses of iron induced an increase of lipid oxidation in the liver, as measured by thiobarbituric acid-reactive substances (TBARS), and an increase of cholesterolemia. Caffeic acid fully prevented the pro-oxidant effects of high iron doses ($p < 0.001$). It also reduced lipid peroxidation in rats fed the low iron dose ($p < 0.05$). Caffeic acid also increased vitamin E levels in plasma (2.74 µmol/L to 4.09 µmol/L for normal diet; $p < 0.001$; 2.78 µmol/L to 4.94 µmol/L for iron supplemented diet $p < 0.001$). Iron-induced hypercholesterolemia was inhibited by caffeic acid (1.07 g/L to 0.82 g/L; $p < 0.001$). These results demonstrate the antioxidative capacity of caffeic acid, a highly bioavailable polyphenol, in an *in vivo* model of oxidative stress.

Key words: Caffeic acid, antioxidant effects, coffee, iron overload, cholesterolemia, rat

Introduction

Polyphenols are common constituents of foods of plant origin and are major antioxidants in our diet. Several hundreds of different polyphenols have been identified, the large majority being either flavonoids or phenolic acids [1–3]. Phenolic acids have received much less attention than flavonoids despite their abundance in the diet. They are present in various food products such as fruit, vegetables, coffee, or cereals. Their daily intake can reach 500 mg in some persons, particularly regular coffee drinkers [4, 5]. The main phenolic acids in the diet are hydroxycinnamic acids, with caffeic acid as its main representative. Caffeic acid is largely present as chlorogenic acid, an ester with quinic acid. A cup of coffee contains 70 to 300 mg chlorogenic acid, equivalent to 35–150 mg caffeic acid [4].

As antioxidants, hydroxycinnamic acids may protect cell constituents against oxidative damage and therefore limit the risk of various degenerative diseases associated with oxidative stress. Different polyphenols were shown to increase the antioxidant capacity of plasma and tissues and to reduce the susceptibility of low-density lipoprotein (LDL) and lipids to peroxidation, a key mechanism in the atherosclerosis physiopathology [6]. Hydroxycinnamic acids such as caffeic, ferulic, or *p*-coumaric acids

DOI 10.1024/0300-9831.75.2.119

are able to inhibit the *in vitro* oxidation of LDL isolated from human plasma. Caffeic acid was the most active antioxidant as compared to the other phenolic acids. [7, 8]. Caffeic acid was also shown to spare vitamin E in rat plasma oxidized *in vitro* by a radical initiator [9]. *In vivo,* caffeic acid, when administered in the diet, participated in the antioxidant defense system, by sparing vitamin E [10].

In the present work, we study the antioxidant effects of caffeic acid in iron-overloaded (IO) rats, used as a model of oxidative stress. Iron overload is associated with significant changes of the antioxidant status and causes lipid metabolism disturbances by increasing plasmatic cholesterol levels [11, 12]. Using this model, we demonstrate that feeding caffeic acid improves some markers of oxidative stress and reduces hypercholesterolemia induced by iron overload.

Materials and Methods

Chemicals: Ferrous iron, caffeic acid, ferulic acid, and isoferulic acid were purchased from Sigma (Saint-Louis, MO, USA).The triglycerides and cholesterol measurement kits were obtained from Biotrol (Paris, France) and Biomérieux (Charbonnière-les-Bains, France) respectively.The plasmatic iron and total iron binding capacity measurement kits were obtained from Biomérieux (Charbonnière-les-Bains, France).

Animals, diets: Forty weaning male Wistar rats (Institut National de la Recherche Agronomique), 3 weeks old, weighing 64 g ± 0.4 g (mean ± SEM), were housed in temperature-controlled rooms (22 °C), with a dark period from 20.00 h to 08.00 h and access to the semi-purified diet from 16.00 h to 08.00 h. The rats were fed a standard semi-purified diet (Table I) for one week and were then randomly divided into four groups. Each group received a single different experimental meal during the four-week intervention period, as follows: (1) the standard diet (control), (2) the standard diet supplemented with 0.65% caffeic acid (caffeic diet), (3) the standard diet supplemented with 0.2% ferrous iron (iron-supplemented diet), and (4) the standard diet supplemented with 0.2% ferrous iron and 0.65% caffeic acid (iron and caffeic supplemented diet). Caffeic acid and iron concentrations were equimolar in the last diet (35.8 mmol/kg). The basal vitamin E supply was limited to one-third of the recommended value and a fat source (corn oil) that was relatively prone to oxidation was used to increase the pro-oxidant response. The last week of the experiment, rats were housed in metabolic cages.

Table I: Compositions of experimental diets (g/kg)[1]

	Control	Caffeic acid	Iron	Caffeic acid and iron
Casein	200	200	200	200
Corn oil	50	50	50	50
Saccharose	325	325	325	325
Wheat starch	325	318	315	309
Alphacel	50	50	50	50
Mineral mix[2]	35	35	35	35
Vitamin mix[3]	10	10	10	10
DL- Methionine	3	3	3	3
Choline bitartrate	2	2	2	2
Iron ($FeSO_4{*}7H_2O$)	0.25	0.25	9.9	9.9
Caffeic acid	/	6.5	/	6.5

[1] Sources of ingredients: casein and wheat starch (Louis François,Saint-Maur, France), corn oil, DL-methionine, iron ($FeSO_4$, $7H_2O$), caffeic acid (Sigma Chemical, St. Louis, MO), vitamin AIN-76A mix and choline bitartrate (ICN Biomedicals, Orsay, France).

[2] All diets contained (per kg diet): 4 g Ca, 1.4 g Na, 3.8 g K, 5 mg Cu, and 38 mg Zn. Only iron was not balanced between the diets.

[3] Providing 33% of tocopherol recommended supply, prepared using 1 part AIN-76A vitamin mix plus 2 parts tocopherol-free AIN76A mix.

Animals were maintained and handled according to the recommendations of the Institute Ethics Committee (INRA), in accordance to the decree N° 87-848.

Sampling procedure: Urine (24-hour) was collected during four days of the last week, acidified with 10M HCl (10 µL/mL) and stored at –20 °C.

For plasma sampling, rats were anesthetized with pentobarbital (40 mg/kg body, intraperitoneally) in the postprandial period and maintained on a plate at 37 °C. Blood was drawn from the abdominal aorta into heparinized tubes and centrifuged (10,000 × g, 2 minutes) to obtain plasma. Aliquots of plasma were kept at –20 °C until analysis. Liver and heart were excised and approximately 3 g of liver and the entire heart were immediately freeze-clamped and stored at –80 °C.

Analytical methods: Plasma triglycerides and cholesterol levels were determined using commercial kits (Biotrol, Paris, France and Biomérieux, Charbonnière-les-Bains, France respectively); a polyvalent control serum (33-plus, Biotrol, Paris, France) was treated in parallel to samples and served as control for the accuracy of results in the triglycerides and cholesterol analyses.

Plasma iron and total iron binding capacity levels were determined using commercial kits.

Plasma vitamin E was assayed by reversed-phase high performance liquid chromatography (HPLC) (HPLC apparatus, Kontron series 400, Kontron, Saint-Quentin en

Yvelines, France) using a hexane extract. Briefly, α-tocopherol acetate (Sigma) was added to samples as internal standard. Samples were extracted twice with hexane, after ethanol precipitation of the proteins. The extract was evaporated to dryness under N_2, dissolved in ethanol-methylene chloride (65:35, v/v) and injected into a C18 column (nucleosil; 250 mm, i.d 46 mm, 5 µm particles). Pure methanol, at a flow-rate of 2 mL/minute eluted α-tocopherol in 5.0 minutes and tocopherol acetate in 6.3 minutes. The compounds were detected by ultraviolet (UV) (292 nm), then quantified by internal and external calibration using standard solutions.

For lipid peroxidation studies of heart and liver tissues, homogenates were prepared on ice in a ratio of 1 g wet tissue to 9 mL 150 mmol/L KCl using a Polytron homogenizer. Thio-barbituric acid-reactive substances (TBARS) were measured using a spectrophotometer (Uvikon 941 plus series, Kontron Instruments, Saint Quentin en Yvelines), in tissue homogenates after lipid peroxidation induced by $FeSO_4$ (2 µmol/L)-ascorbate (50 µmol/L) for 30 minutes in a 37 °C water bath in an oxygen-free medium using a standard of 1,1,3,3-tetraethoxypropane as previously described [13].

Analysis of urine metabolites of caffeic acid was done by HPLC with a UV detector. Urine samples (diluted in 0.1 mol/L sodium acetate buffer, pH5, 175 µL) were acidified to pH 4.9 with 20 µL of 0.58 mol/L acetic acid and incubated at 37 °C for 45 minutes in the presence of 1100 U β-glucuronidase and 42 U sulfatase (*Helix pomatia* extract, Sigma chemicals). Samples were extracted by adding 500 µL of methanol/water/200 mM HCl (75:25) and centrifuged for 4 minutes at 14,000 revolutions per minute (rpm). For analysis, 30 µL of each preparation was injected onto a 150 × 4.6 m Hypersil BDS C18-5 µm column (Life Sciences International, Cergy, France). The mobile phase consisted of 5% acetonitrile (phase A) and 30% acetonitrile (phase B), each containing 0.5 % H_3PO_4 (aq). The UV detector was set at 320 nm.

Data analysis: Data were entered into the Instat statistical analysis program (Instat, San Diego, CA). Values are means ± SEM, the differences between values were determined by one-way ANOVA coupled with Tukey's Multiple Comparisons Test or by the Kruskal-Wallis test (non-parametric ANOVA) coupled with Dunn's Multiple Comparison Test. Differences with $p < 0.05$ were considered significant.

Results

Body weight, food intake: Iron and caffeic acid in the diet did not affect food intake and body weight (Table II).

Total plasma iron and total iron binding capacity (TIBC): A significant rise of plasma iron concentration was observed in rats fed with the iron-supplemented diet compared with the control group (3.73 ± 0.21 vs. 1.56 ± 0.23 µmol/L respectively; $p < 0.001$). Caffeic acid did not affect the plasma iron level when it was added to the normal diet (1.56 ± 0.23 vs. 1.69 ± 0.11 µmol/L) or when it was added in the iron diet (3.73 ± 0.21 vs. 4.00 ± 0.19 µmol/L) (Fig. 1A). Total iron-binding capacity was significantly decreased in rats fed with iron-supplemented diet compared with the control group. Caffeic acid in the diets did not significantly modify this capacity (Fig. 1B).

Lipid peroxidation: After exposure of liver homogenate to iron/ascorbate-induced lipid peroxidation, high liver TBARS values were found in rats fed the iron-supplemented diet compared with the control group (166.5 ± 22.2 vs. 303.7 ± 22.8 nmol/g wet weight; $p < 0.001$) (Fig. 2). Caffeic acid restored basal values of TBARS in the iron-supplemented group (151.1 ± 16.9 vs. 115.5 ± 15.9 nmol/g

Table III: Urinary excretion of phenolic acids in rats consuming diets supplemented with caffeic acid or caffeic acid and iron (% ingested dose, mol/mol).

	Diet	
	Caffeic acid	Caffeic acid + Iron
Caffeic acid	13.8 ± 0.7	10.4 ± 0.6***
Ferulic acid	5.3 ± 0.2	3.6 ± 0.1***

Values are means ± SEM (n = 10).
*** $p < 0.001$ vs caffeic acid diet.

Table II: Body weight, food intake, iron, and caffeic acid intake[1]

	Control	Iron	Caffeic acid	Caffeic acid and Iron	ANOVA
Body weight (g)	304.1 +/–5.5	295.4 +/–5.46	317.6 +/–7.93	295.6 +/–8.41	NS
Food intake (g/day)	19.2 +/–0.5	20.0 +/–0.4	19.7 +/–0.5	19.6 +/–0.5	NS
Iron intake (µmol/day)	17.1 +/–0.5	715 +/–13	17.6 +/–0.5	701 +/–17	NS
Caffeic acid intake (µmol/day)	/	/	706 +/–19	704 +/–17	NS

[1] Values are means +/– SEM (n = 10).
[a] NS, not significant, $p > 0.05$.

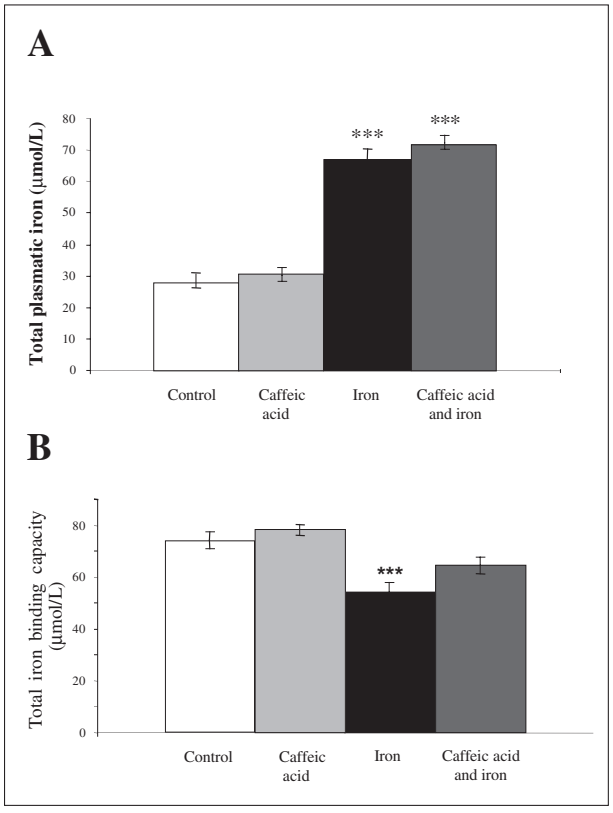

Figure 1: Total plasmatic iron (A) and total iron binding capacity (B) in rats fed the different experimental diets.
Values are means ± SEM (n = 10), *** significantly different from control, $p < 0.001$.

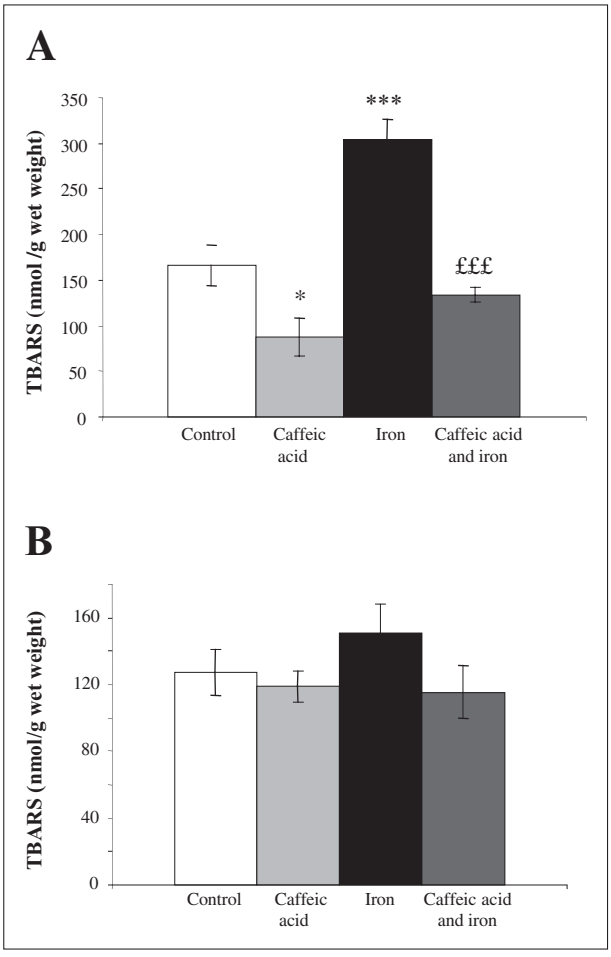

Figure 2: Liver (A) and heart (B) susceptibility to lipid peroxidation in rats consuming experimental diets (TBARS).
Values are means ± SEM (n = 10), * $p < 0.05$; *** $p < 0.001$ vs control; £££ $p < 0.001$ vs 2g/kg iron.

wet weight; $p < 0.001$). Moreover, this phenolic acid decreased the TBARS concentration in caffeic diet compared with control diet (127.2 ± 13.9 vs. 119.1 ± 15.3 nmol/g wet weight).

Heart susceptibility to lipid peroxidation was not changed in all groups.

Plasma cholesterol and triglycerides (TG): The iron-supplemented diet caused hypercholesterolemia compared with the control and caffeic groups (1.065 ± 0.078 g/L vs. 0.648 ± 0.062 and 0.601 ± 0.081 respectively; $p < 0.001$). Caffeic addition restored plasma cholesterol level in iron-supplemented diet (Fig. 3).

Plasma triglycerides were similar for each group (results not shown).

Plasma vitamin E: Alpha-tocopherol concentration did not differ in the control and iron-supplemented groups. Iron did not influence plasma vitamin E concentration in this study (Fig. 4).

Caffeic acid preserved vitamin E in the caffeic group (4.09 ± 0.2 vs. 2.74 ± 0.25 µmol/L for control group; $p < 0.05$) and in caffeic acid and iron-supplemented group (4.94 ± 0.45 vs. 2.78 ± 0.11 µmol/L for the iron-supplemented group; $p < 0.001$) (Fig. 4).

The α-tocopherol/(TG + cholesterol) ratio was not significantly different in all groups (Fig. 5).

24h hour- caffeic and ferulic acids urinary excretion per ingested dose: Relative urinary excretion of caffeic acid was significantly decreased in the caffeic acid + iron group compared to the caffeic acid group ($p < 0.001$). At the same time, relative urinary excretion of ferulic acid (*O*-methyl caffeic acid) was decreased in the caffeic acid + iron group compared to the caffeic acid group ($p < 0.001$). (Table III)

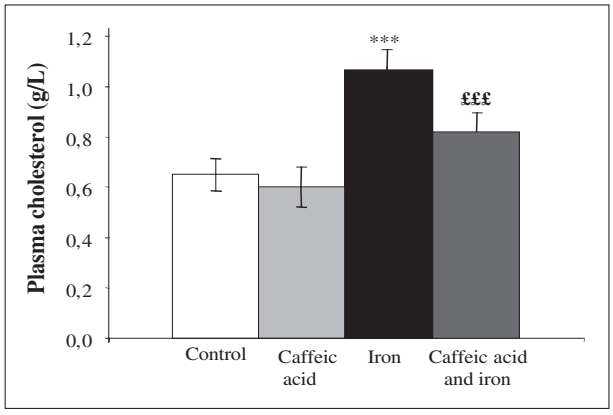

Figure 3: Plasma cholesterol concentration in rats consuming experimental diets.
Values are means ± SEM (n = 10), *** $p < 0.001$ vs control; £££ $p < 0.001$ vs iron.

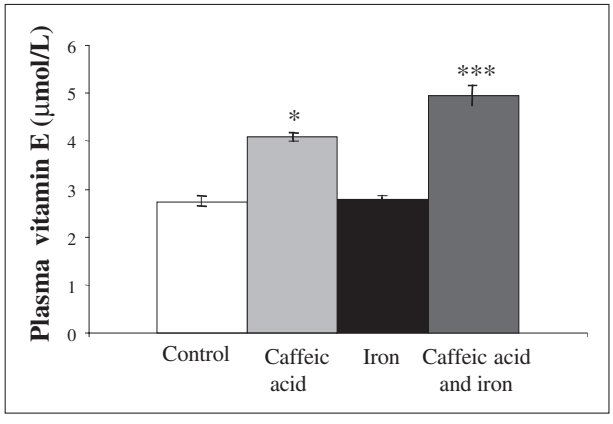

Figure 4: Plasma vitamin E concentration in rats consuming experimental diets.
Values are means ± SEM (n = 10), * $p < 0.05$; *** $p < 0.001$ vs control and iron.

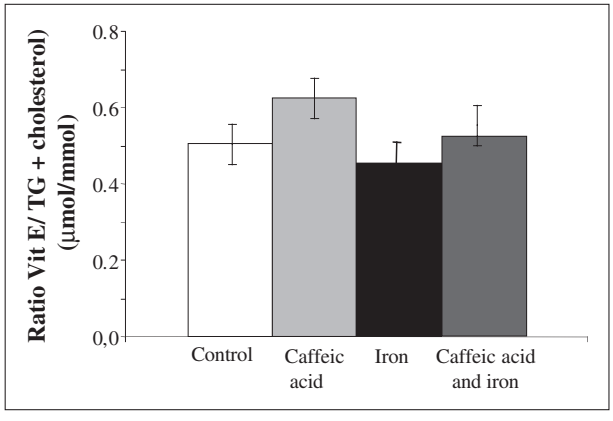

Figure 5: Plasma vitamin E / (TG + cholesterol) ratio in rats consuming experimental diets.
Values are means ± SEM (n = 10).

Discussion

Many different models have been used to study oxidative stress and the effect of antioxidants to prevent oxidative damage, in the context of disease prevention and ageing. In the present study, oxidative stress was induced by dietary supplementation with iron, well known for its pro-oxidant effects at high concentrations. Three doses were tested in a preliminary experiment: 50 mg/kg diet (0.005%); 500 mg/kg (0.05%), and 2 g/kg (0.2%). The last dose was finally selected as the lowest one inducing changes on oxidative stress biomarkers (not shown). In IO conditions, accumulation of iron is observed in parenchymal cells of a variety of tissues, particularly in the liver. This mechanism is analogous to that seen in hereditary hemochromatosis [14, 15]. The modification of iron status induced by dietary IO was clearly indicated by a significant increase of plasma iron and in a reduction of TIBC. The change of iron status resulted in an increase of oxidative stress. Liver TBARS were increased significantly in rats fed the iron diet as compared with the control group. The same trend was observed for heart TBARS, although not significantly. Accumulation of iron in the periportal region of the liver lobules explains more a pronounced effect of IO in this tissue [15].

Addition of caffeic acid to the diet significantly inhibited lipid peroxidation in the liver in both iron-supplemented and normal rats. A similar trend was observed in heart tissue. Caffeic acid also increased vitamin E concentration in the plasma. The sparing effect of vitamin E by caffeic acid has been observed in an *in vitro* electron paramagnetic resonance (EPR) study with LDL [16] and in previous *in vivo* study where Sprague-Dawley rats were fed a diet containing 0.2 % caffeic acid [17]. Similar results were also obtained in humans with different polyphenols [18, 19].

When calculated over (TG + cholesterol) concentration, the same tendency for vitamin E levels was observed, although the trend was not statistically significant.

Antioxidant effects of caffeic acid in our study could either be due to a modification of iron status or to a direct action of caffeic acid in inner tissues. Polyphenols such as caffeic acid are known to form stable complexes with ferric ions in the gut, making the iron less available for absorption [20, 21]. However, at the high level of iron supplementation used in the present work, caffeic acid did not affect iron status. Antioxidant effects of caffeic acid are therefore most likely explained by a direct action of caffeic acid on inner tissues. Ten percent and 14% of the caffeic acid-ingested dose was recovered in urine in, respectively, IO rats and normal rats, showing its good bioavailability. The lower excretion in IO rats could be explained by its oxidative degradation catalyzed by iron in the gut

or inner tissues. Nardini *et al*, using a similar protocol on normal rats and similar doses of caffeic acid in the diet, did not observe any effect of caffeic acid on lipid peroxidation (plasma TBARS) [10]. This apparent discrepancy could be explained by the timing of the animals' sacrifice. In the present work, animals were killed while in a post-prandial state, when the tissues were exposed to caffeic acid and its metabolites, whereas Nardini *et al* collected the tissues after an overnight fast, when both iron and caffeic concentrations were low.

Similar antioxidant effects to those observed here for caffeic acid in IO rats have been observed with flavonoids, α-tocopherol, and ascorbic acid. Silybin, a flavonolignan, administered by gastric intubation, decreased malondialdehyde levels in the liver [22] and rutin, the rutinoside of quercetin, injected intraperitoneally suppressed free radical production in liver microsomes [23]. Alpha-tocopherol (200 IU/kg diet) [24, 25] and ascorbic acid (0.3 % in the diet) [26] were also shown to prevent the iron-induced lipid peroxidation in IO rats.

Iron supplementation also induced a 1.8-fold increase of the total cholesterol concentration in plasma, in agreement with previous studies [11, 12]. This increase was partially prevented by caffeic acid supplementation. However, caffeic did not affect cholesterolemia in rats non-supplemented with iron as previously observed in both rats and hamsters [17, 27]. A dependency of cholesterolemia over antioxidant status has been reported previously. In IO rats, the increase of plasma cholesterol level was associated with a dramatic decrease in plasma ascorbic acid [11, 28]. Hypercholesterolemia could possibly be explained by a decrease of cholesterol 7α-hydroxylase activity, an enzyme involved in the catabolism of cholesterol and synthesis of bile acids and which requires ascorbic acid as a cofactor [29]. Caffeic acid could act in synergy with ascorbic acid through mechanisms that remain to be elucidated [16].

These antioxidant and hypocholesterolemic effects of caffeic acid could counteract detrimental effects attributed to some of the constituents of coffee, the major source of caffeic acid in the diet. Caffeic acid likely explains the increase of antioxidant capacity of plasma observed after consumption of coffee [30] in spite of the presence of hydrogen peroxide or 1,2,4-benzenetriol oxidants [31]. Similarly, caffeic acid could also contribute to limit the hypercholesteromic effects of some coffee constituents such as the diterpene cafestol [32]. The dose of caffeic acid used in the present work would correspond for humans to a consumption of 3 g of caffeic acid per day for the same concentration in the rat and human diets. This amount of caffeic acid is three times higher than the estimated daily polyphenol intake [1]. One cup of coffee contains up to 300 mg chlorogenic acid [4]. Three grams of caffeic acid would therefore be equivalent to about 20 cups of coffee. The dose used here is high. Further investigation is therefore still needed to study the effects of lower doses of caffeic acid. Dietary supplementation with phenolic acids may result in much higher exposure level than that achieved with the diet. It is often inferred that such a high intake may result in pro-oxidant effects [33]. However, this was not observed with caffeic acid in the present study.

Caffeic acid in coffee is largely present in an esterified form, chlorogenic acid. These two compounds differ with respect to their bioavailability. Chlorogenic acid is partially hydrolyzed in the gut and its ingestion results in direct exposure of inner tissues to caffeic acid. However, the level of caffeic acid measured in plasma or urine is much lower than that observed after direct feeding of caffeic acid [34, 35]. Therefore, the exact role of caffeic acid in achieving antioxidant protection through coffee consumption needs to be further explored.

Acknowledgements

This work was supported by BERKEM SA, Gardonne, France.

The authors thank C. Lab, B. Lyan, L. Jaffrelo, J. Marcand-Sauvant and S. Thien for the expert technical assistance.

References

1. Scalbert, A. and Williamson, G. (2000) Dietary intake and bioavailability of polyphenols. J. Nutr. 130, 2073 S–2085 S.
2. Manach, C., Scalbert, A., Morand, C., Rémésy, C. and Jimenez, L. (2004) Polyphenols – Food sources and bioavailability. Am. J. Clin. Nutr. 79, 727–747.
3. Shahidi, F. and Naczk, M. (2003) Phenolics in food and nutraceuticals, 2nd Edition, Vol.?, CRC Press, Boca Raton, Florida.
4. Clifford, M.N. (1999) Chlorogenic acids and other cinnamates – Nature, occurrence and dietary burden. J. Sci. Food. Agric. 79, 362–372.
5. Radtke, J., Linseisen, J. and Wolfram, G. (1998) Phenolsäurezufuhr Erwachsener in einem bayerischen Teilkollektiv der Nationalen Verzehrsstudie. Z. Ernährungswiss. 37, 190–197.
6. Scalbert, A., Manach, C., Morand, C., Rémésy, C. and Jiménez, L. (2004) Dietary polyphenols and the prevention of diseases. Crit. Rev. Food Sci. Nutr., in press.
7. Nardini, M., Daquino, M., Tomassi, G., Gentili, V., Difelice, M. and Scaccini, C. (1995) Inhibition of human low-density lipoprotein oxidation by caffeic acid and other hydroxycinnamic acid derivatives. Free Radical Biology and Medicine 19, 541–552.

8. Ohta, T., Nakano, T., Egashira, Y. and Sanada, H. (1997) Antioxidant activity of ferulic acid beta-glucuronide in the LDL oxidation system. Bioscience Biotechnology and Biochemistry 61, 1942–1943.
9. Raneva, V., Shimasaki, H., Ishida, Y., Ueta, N. and Niki, E. (2001) Antioxidative activity of 3,4-dihydroxyphenylacetic acid and caffeic acid in rat plasma. Lipids 36, 1111–1116.
10. Nardini, M., Natella, F., Gentili, V., DiFelice, M. and Scaccini, C. (1997) Effect of caffeic acid dietary supplementation on the antioxidant defense system in rat: An in vivo study. Archives of Biochemistry and Biophysics 342, 157–160.
11. Dabbagh, A. J., Mannion, T., Lynch, S. M. and Frei, B. (1994) The effect of iron overload on rat plasma and liver oxidant status in vivo. Biochem. J. 300, 799–803.
12. Whittaker, P., Wamer, W. G., Chanderbhan, R. F. and Dunkel, V. C. (1996) Effects of alpha-tocopherol and beta-carotene on hepatic lipid peroxidation and blood lipids in rats with dietary iron overload. Nutrition and Cancer – an International Journal 25, 119–128.
13. Rayssiguier, Y., Gueux, E., Bussiere, L. and Mazur, A. (1993) Copper deficiency increases the susceptibility of lipoproteins and tissues to peroxidation in rats. J. Nutr. 123, 1343–1348.
14. Halliday, J. W. and Searle, J. (1996) Hepatic iron deposition in human disease and animal models. Biometals 9, 205–209.
15. Powell, L.W., Bassett, M.L. and Halliday, J.W. (1980) Hemochromatosis: 1980 update. Gastroenterology 78, 374–381.
16. Laranjinha, J. (2001) Redox cycles of caffeic acid with alpha-tocopherol and ascorbate. Methods Enzymol. 335, 282–295.
17. Frank, J., Kamal-Eldin, A., Razdan, A., Lundh, T. and Vessby, B. (2003) The dietary hydroxycinnamate caffeic acid and its conjugate chlorogenic acid increase vitamin E and cholesterol concentrations in Sprague-Dawley rats. J. Agric. Food Chem. 51, 2526–2531.
18. Simonetti, P., Ciappellano, S., Gardana, C., Bramati, L. and Pietta, P. (2002) Procyanidins from Vitis vinifera seeds: in vivo effects on oxidative stress. J. Agric. Food Chem. 50, 6217–6221.
19. Carbonneau, M. A., Leger, C. L., Monnier, L., Bonnet, C., Michel, F., Fouret, G., Dedieu, F. and Descomps, B. (1997) Supplementation with wine phenolic compounds increases the antioxidant capacity of plasma and vitamin E of low-density lipoprotein without changing the lipoprotein Cu(2+)-oxidizability: possible explanation by phenolic location. Eur. J. Clin. Nutr. 51, 682–690.
20. Brune, M., Rossander, L. and Hallberg, L. (1989) Iron absorption and phenolic compounds: importance of different phenolic structures. Eur. J. Clin. Nutr. 43, 547–558.
21. Hurrell, R. F., Reddy, M. and Cook, J. D. (1999) Inhibition of non-haem iron absorption in man by polyphenolic-containing beverages. Brit. J. Nutr. 81, 289–295.
22. Pietrangelo, A., Borella, F., Casalgrandi, G., Montosi, G., Ceccarelli, D., Gallesi, D., Giovannini, F., Gasparetto, A. and Masini, A. (1995) Antioxidant activity of silybin *in vivo* during long-term iron overload in rats. Gastroenterology 109, 1941–1949.
23. Afanas'ev, I. B., Ostrachovitch, E. A., Abramova, N. E. and Korkina, L. G. (1995) Different antioxidant activities of bioflavonoid rutin in normal and iron-overloading rats. Biochemical Pharmacology 50, 627–635.
24. Lucesoli, F. and Fraga, C. G. (1999) Oxidative stress in testes of rats subjected to chronic iron intoxication and alpha-tocopherol supplementation. Toxicology 132, 179–186.
25. Galleano, M. and Puntarulo, S. (1997) Dietary alpha-tocopherol supplementation on antioxidant defenses after in vivo iron overload in rats. Toxicology 124, 73–81.
26. Dillard, C. J., Downey, J. E. and Tappel, A. L. (1984) Effect of antioxidants on lipid peroxidation in iron-loaded rats. Lipids 19, 127–133.
27. Auger, C., Laurent, N., Laurent, C., Besancon, P., Caporiccio, B., Teissedre, P. L. and Rouanet, J. M. (2004) Hydroxycinnamic acids do not prevent aortic atherosclerosis in hypercholesterolemic golden Syrian hamsters. Life Sci. 74, 2365–2377.
28. Odumosu, A. and Wilson, C. W. (1979) Tissue ascorbic acid, fenfluramine, and changes in fat metabolism. Int. J. Obes. 3, 123–131.
29. Turley, S. D., West, C. E. and Horton, B. J. (1976) The role of ascorbic acid in the regulation of cholesterol metabolism and in the pathogenesis of atherosclerosis. Atherosclerosis 24, 1–18.
30. Natella, F., Nardini, M., Giannetti, I., Dattilo, C. and Scaccini, C. (2002) Coffee drinking influences plasma antioxidant capacity in humans. J. Agric. Food Chem. 50, 6211–6216.
31. Hiramoto, K., Kida, T. and Kikugawa, K. (2002) Increased urinary hydrogen peroxide levels caused by coffee drinking. Biol. Pharm. Bull. 25, 1467–1471.
32. de Roos, B. and Katan, M. B. (1999) Possible mechanisms underlying the cholesterol-raising effect of the coffee diterpene cafestol. Curr. Opin. Lipidol. 10, 41–45.
33. Mennen, L. I., Walker, R., Bennetau-Pelissero, C. and Scalbert, A. (2005) Risks and safety of polyphenol consumption. Am. J. Clin. Nutr, in press.
34. Olthof, M. R., Hollman, P. C. H. and Katan, M. B. (2001) Chlorogenic acid and caffeic acid are absorbed in humans. J. Nutr. 131, 66–71.
35. Gonthier, M. P., Verny, M. A., Besson, C., Remesy, C. and Scalbert, A. (2003) Chlorogenic acid bioavailability largely depends on its metabolism by the gut microflora in rats. J. Nutr. 133, 1853–1859.

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