Free Rad. Res., Vol. 33, pp. 419–426
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Flavonoids Protect against Oxidative Damage to LDL *In Vitro*: Use in Selection of a Flavonoid Rich Diet and Relevance to LDL Oxidation Resistance *Ex Vivo*?

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Accepted by Prof. B. Halliwell

(Received 28 January 2000; In revised form 16 February 2000)

The ability of a range of dietary flavonoids to inhibit low-density lipoprotein (LDL) oxidation *in vitro* was tested using a number of different methods to assess oxidative damage to LDL. Overall quercetin was the most effective inhibitor of oxidative damage to LDL *in vitro*. On this basis, a diet enriched with onions and black tea was selected for a dietary intervention study that compared the effect on the Cu^{2+} ion-stimulated lag-time of LDL oxidation *ex vivo* in healthy human subjects of a high flavonoid diet compared with a low flavonoid diet. No significant difference was found in the Cu^{2+} ion-stimulated lag-time of LDL oxidation *ex vivo* between the high flavonoid and low flavonoid dietary treatments (48 ± 1.6 min compared to 49 ± 2.1 min).

Keywords: Flavonoids, quercetin, low-density lipoprotein oxidation, onions, black tea

INTRODUCTION

Flavonoids are polyphenolic compounds found in numerous foods of plant origin and include quercetin, myricetin and kaempferol, found in onions and tea, and catechin, epicatechin, epigallocatechin and epigallocatechin gallate, found in tea.[1–7] The Zutphen Elderly Study,[8] which examined the Dutch diet in relation to risk of coronary heart disease indicated that consumption of flavonoid rich foods such as tea, onions and apples appeared to provide some protection from coronary heart disease. Furthermore, a recent study has shown tea intake to be associated with a lower risk of myocardial infarction.[9]

The protective effect of flavonoid consumption against coronary heart disease observed in some studies[8,9] has been suggested to derive from the antioxidant action of the constituent flavonoids. Indeed, many flavonoids have been shown to display antioxidant action *in vitro*[10,11] including protection of low-density lipoproteins (LDL) *in vitro*[10,11] against the oxidative damage implicated in atherogenesis.[12–14]

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The effect of antioxidant consumption on peroxidation of LDL, which is subsequently isolated from the blood of volunteers is a popular method of assessing their effectiveness *in vivo*, e.g. vitamin E supplementation increases the lag period before peroxidation accelerates when LDL subsequently isolated is incubated with Cu^{2+} ions *ex vivo*.[15] Consumption of flavonoids in tea (750 ml/d) has been reported to increase LDL oxidation resistance (increase in Cu^{2+} ion-stimulated LDL lag-time) *ex vivo*.[16] However, these results have not been reproduced in all studies of plasma oxidation resistance *ex vivo*[17] and LDL oxidation resistance *ex vivo*[18,19] following tea consumption. The consumption of phenolics in wine did increase LDL oxidation resistance, but again only in some studies.[20–22] The usefulness of the human LDL *in vitro* test system in predicting the likely protection of flavonoids against LDL oxidation *in vivo*; as measured by lag-time of LDL oxidation *ex vivo*, following consumption of foods rich in the flavonoids tested *in vitro*, is clearly an important question when selecting diets for dietary intervention studies on the basis of the results obtained from the *in vitro* test system.

The aim of the present study was to assess the influence of dietary flavonoids on Cu^{2+} ion-stimulated oxidative damage to human LDL *in vitro* and to use the results to formulate a diet rich in the flavonoids that were most effective as inhibitors of LDL oxidation *in vitro*, for use in a randomised crossover dietary intervention study. The dietary flavonoids tested *in vitro* included the flavonols; quercetin, rutin (quercetin rutinoside), myricetin, kaempferol, the flavones; luteolin, apigenin, the catechins (flavanols); (+/−)-catechin, (+)-epicatechin, (−)-epicatechin, (−)-epigallocatechin gallate and the anthocyanin cyanidin chloride. In addition, the classical chain breaking food antioxidant butylated hydroxytoluene (BHT) was tested for comparative purposes. A number of different methods were used to assess oxidative damage to LDL *in vitro*; formation of thiobarbituric acid reactive substances (TBARS), continuous monitoring of the formation of conjugated dienes and lag-time of LDL oxidation, formation of lipid peroxides and relative electrophoretic mobility (REM) on agarose gels.

We now report the results obtained for the effects of the flavonoids on oxidative damage to LDL *in vitro* and their use in the selection of a flavonoid rich diet (enriched with onions and black tea) and compare these results with the effect on the lag-time of Cu^{2+} ion-stimulated LDL oxidation *ex vivo* of the consumption of a high flavonoid (HF) diet enriched (on the basis of the results obtained) compared with a low flavonoid (LF) diet.

MATERIALS AND METHODS

Materials

All chemicals were of the highest purity available. They were all obtained from the Sigma Chemical Company (Poole, Dorset, UK), with the exception of cyanidin chloride and (−)-epigallocatechin gallate, which were purchased from Apin Chemicals (Oxford, UK).

Preparation of Human LDL

LDL was isolated from plasma using a density gradient and a single differential ultracentrifugation step.[23] For the human LDL *in vitro* test system fresh plasma (not more than 1 day old) was obtained from a blood bank. In the human dietary intervention study venous blood samples were collected on day 14 of each treatment. Following an overnight fast blood samples were collected into 10 ml vacutainers containing EDTA. The blood samples were centrifuged at 1500*g* for 10 min at 4°C and plasma was separated. Plasma for LDL isolation was stored at −70°C with 10% sucrose as described previously.[24] LDL was isolated from plasma by differential ultracentrifugation using a Beckman SW40Ti rotor followed by dialysis under nitrogen for 18 h against 400

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volumes of 10 mM phosphate buffered saline pH 7.4, as described previously.[23] LDL protein was measured according to the modified Lowry Procedure method of Markwell *et al.*[25] LDL oxidation was stimulated by the addition of copper sulphate to a final copper ion concentration of 5 μM. LDL protein concentration was 0.1 mg/ml.

Measurement of LDL Oxidation

Measurement of LDL TBARS was by the thiobarbituric acid (TBA) test, as described previously.[26] Control experiments showed that none of the test compounds interfered with the TBA assay, in that no inhibition was observed when the compounds were added with the TBA reagents instead of at the beginning of the incubations. The LDL lipid peroxide content was determined as described previously.[27] The relative electrophoretic mobility of the LDL was determined as described previously.[26] Continuous monitoring of LDL conjugated diene formation was performed as described previously.[28] Where used, test compounds were added in ethanol (5 μl; final ethanol concentration of 1%); 1% ethanol was used in controls, as appropriate.

Measurement of LDL α-Tocopherol and Fatty Acids

LDL α-tocopherol concentrations were measured by HPLC, utilizing tocopherol acetate as the internal standard as described previously.[29] The fatty acid composition of the LDL was determined by GC using flame ionisation detection, as described previously.[30]

Subjects

Thirty-two healthy, non-smoking, non-supplement taking (including antioxidant supplements) male and female subjects were recruited from staff and students at King's College London and completed both dietary treatments. All subjects had normal haematology and liver function. The physical characteristics of these 32 subjects were, mean age 30.4 ± 7.3 years, mean BMI 23.6 ± 2.7 kg m^{-2}, mean plasma cholesterol 5.2 ± 2.1 mM. Subjects were asked to make no changes to their diet or lifestyle other than those necessary for compliance with the study. At the end of each treatment phase, fasting venous blood samples were collected and the heights and weights of the subjects were recorded. Written informed consent of subjects and their doctors was obtained. The subjects received a modest financial payment for their participation in the study. The study protocol was reviewed and approved by the King's College London Research Ethics Committee.

RESULTS

Effects of Flavonoids on LDL Oxidation *In Vitro*

The flavonoids tested all inhibited the formation of TBARS, in LDL stimulated with Cu^{2+}-ions to undergo oxidation, in a concentration-dependent manner over the concentration range 0.2–30 μM. The IC_{50} values for the inhibition of the formation of TBARS by these flavonoids are shown in Table I

TABLE I Effect of flavonoids on TBARS formation in Cu^{2+} ion-stimulated LDL *in vitro*

Compound	Mean IC_{50} concentration (μmol/L)
(−)-Epigallocatechin gallate	0.49 ± 0.04^a
Quercetin	0.52 ± 0.03^a
BHT	0.61 ± 0.05^a
Luteolin	0.62 ± 0.03^a
(−)-Epicatechin	0.62 ± 0.04^a
Rutin	0.64 ± 0.05^a
(+/−)-Catechin	0.76 ± 0.03^a
(+)-Epicatechin	0.78 ± 0.03^a
Myricetin	1.27 ± 0.07^b
Cyanidin chloride	1.10 ± 0.05^b
Kaempferol	2.43 ± 0.13^c
Apigenin	15.7 ± 1.15^d

Values are mean ± SD derived from 3–5 separate assays. The IC_{50} value is the concentration of compound required to inhibit the formation of LDL TBARS by 50%. Values with different superscripts are significantly different from each other; $P < 0.05$ (ANOVAR).

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TABLE II Effect of flavonoids on mean lipid peroxide content and on mean relative electrophoretic mobility of Cu^{2+} ion-stimulated LDL *in vitro*

Compound	Lipid peroxide content (nmol/mg LDL)		REM	
	2 h	4 h	2 h	4 h
Control	554 ± 47	490 ± 24	2.66 ± 0.30	3.81 ± 0.64
Quercetin	30 ± 29**	108 ± 32**	1.17 ± 0.10**	1.47 ± 0.64**
Luteolin	29 ± 30**	167 ± 25**	1.18 ± 0.20**	1.76 ± 0.33**
Myricetin	134 ± 91**	490 ± 60	1.70 ± 0.42**	3.43 ± 0.50
Kaempferol	431 ± 99*	474 ± 34	2.32 ± 0.53	3.72 ± 0.52
Apigenin	510 ± 31	507 ± 35	2.58 ± 0.44	3.79 ± 0.55

For the determination of mean lipid peroxide content, values are mean ± SD derived from 4–5 separate assays. For the determination of mean REM, values are mean ± SD derived from 7–8 separate measurements. All compounds were added to give a final concentration of 1.25 μM. LDL was present at a final concentration of 0.1 mg/ml. *$P < 0.05$, **$P < 0.001$ compared to the control (unpaired *t*-test).

ranked in the order of their effectiveness. Quercetin was the most effective of the flavonols tested as an inhibitor of the formation of LDL TBARS, while luteolin was the most effective of the flavones tested and all the catechins tested were of similar effectiveness.

Of the selected flavonols and flavones tested (all at a concentration of 1.25 μM; see Table II) for ability to inhibit lipid peroxide formation in LDL stimulated by Cu^{2+}-ions to undergo oxidation, quercetin was the most effective flavonol after 2 and 4 h. In addition, after 2 h luteolin and quercetin were of similar effectiveness as inhibitors of lipid peroxide formation but after 4 h, quercetin was considerably more effective.

Of the selected flavonols and flavones tested (all at a concentration of 1.25 μmol/L; see Table II) for ability to decrease the REM on agarose gels of LDL stimulated by Cu^{2+} ions to undergo oxidation, compared to the control, quercetin was the most effective flavonol after 2 and 4 h. In addition, after 2 h luteolin and quercetin were of similar effectiveness in terms of ability to decrease REM but after 4 h quercetin was considerably more effective.

Of the flavonols tested (all at a concentration of 1.25 μM; see Table III) for ability to increase the lag-time of Cu^{2+} ion-stimulated LDL oxidation, quercetin was the most effective of the flavonols tested and increased the LDL lag-time by 5.6-fold compared to the control, while luteolin was the most effective of the flavones tested and (−)-epigallocatechin gallate was the most effective catechin tested.

TABLE III Effect of flavonoids on Cu^{2+} ion-stimulated LDL lag-time *in vitro*

Compound	Concentration (μmol/L)	Lag-time (min)	Increase above control (min)
Quercetin	0	45 ± 5.3	
	1.25	258 ± 40.2	213[a]
Luteolin	0	44 ± 5.8	
	1.25	237 ± 11.1	193[b]
(−)-Epigallocatechin	0	49 ± 7.8	
gallate	1.25	226 ± 40.5	181[b]
BHT	0	45 ± 7.5	
	1.25	205 ± 23.1	160[c]
(−)-Epicatechin	0	46 ± 8.4	
	1.25	155 ± 17.5	109[c]
Cyanidin chloride	0	49 ± 8.3	
	1.25	158 ± 22.6	109[d]
Rutin	0	46 ± 7.4	
	1.25	152 ± 28.5	106[d]
(+/−)-Catechin	0	46 ± 6.3	
	1.25	138 ± 7.1	92[e]
(+)-Epicatechin	0	46 ± 8.4	
	1.25	128 ± 7.8	82[e]
Myricetin	0	46 ± 4.5	
	1.25	112 ± 17.4	66[f]
Kaempferol	0	52 ± 7.2	
	1.25	84 ± 9.6	32[g]
Apigenin	0	54 ± 6.2	
	1.25	61 ± 9	7[h]

Values are mean ± SD derived from 5–11 separate assays. Values with different superscripts are significantly different from each other; $P < 0.05$ (ANOVAR).

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Selection of a Flavonoid Rich Diet on the Basis of the Effect of the Flavonoids on LDL Oxidation *In Vitro*

On the basis of the above results a flavonoid rich diet was selected for a dietary intervention study aimed at comparing the effect of a HF diet compared with a LF diet. Quercetin was consistently the most effective inhibitor of oxidative damage to LDL *in vitro*, the catechins in particular (−)-epigallocatechin gallate were also effective. Dietary sources of luteolin are very limited e.g. celery and thus although luteolin was generally of similar effectiveness to quercetin as an inhibitor of oxidative damage to LDL *in vitro*, the quantities of celery (one of the richest dietary sources but contains only 22 mg/kg of luteolin[4]) that would have to be consumed were too large to make its inclusion practical. Consequently a diet rich in predominantly quercetin and also catechins was selected.

The dietary treatments selected for the randomised crossover study were two 14 d treatments with a 14 d wash out period between treatments. During the HF diet one 150 g onion cake containing 89.7 mg quercetin and one 300 ml cup of black tea containing 1.4 mg quercetin, without milk were consumed daily by the subjects and during the LF diet the consumption of specific flavonoid rich foods (as specified by Hertog *et al.* 1992[4]) and tea was avoided. During the LF dietary treatment subjects were also asked to consume 6 g/d high oleic sunflower oil (76% C18:1, 14% C18:2n6, Anglia Oils Ltd., England), as contained in the 150 g onion cake. The combined onion cake and black tea dietary supplement provided 131 mg/d of flavonoids (predominantly quercetin), of which 91.1 mg/d was quercetin (tea and onion cake) and 9.3 mg/d was epigallocatechin gallate (tea).

Effect of Consumption of a Flavonoid Enriched Diet on LDL Oxidation Resistance *Ex Vivo*

The lag-time for Cu^{2+} ion-stimulated LDL oxidation *ex vivo* and the concentrations of LDL cholesterol, α-tocopherol and fatty acids are shown in Table IV. The lag-time for the Cu^{2+} ion-stimulated LDL oxidation *ex vivo* did not differ significantly between the HF and LF treatments (48 ± 1.6 min compared to 49 ± 2.1 min). The concentrations of LDL α-tocopherol and fatty acids also did not differ significantly between the HF and LF treatments.

DISCUSSION

Considerable interest has been shown in the potential beneficial effects on health of dietary flavonoids. Their potent *in vitro* antioxidant activity[11] has contributed to this interest. Quercetin in particular displays powerful antioxidant properties *in vitro*; predominantly against oxidative damage to membrane lipids and lipoprotein particles.[10,11] In the current study, the flavonol quercetin was overall the most effective inhibitor of oxidative damage to LDL *in vitro*. The catechin

TABLE IV The lag-time for Cu^{2+} ion-stimulated LDL oxidation *ex vivo* and LDL α-tocopherol and fatty acid concentrations following the low flavonoid and high flavonoid dietary treatment periods

	Low flavonoid diet		High flavonoid diet	
	Mean	SEM	Mean	SEM
Lag-time of LDL oxidation (min)	49.5	2.08	48.1	1.58
α-Tocopherol (μmol/mmol cholesterol)	2.02	0.09	2.11	0.08
Saturated fatty acids (%)	24.1	0.23	23.7	0.17
Polyunsaturated fatty acids (%)	44.3	0.44	44.2	0.47
Monounsaturated fatty acids (%)	18.1	0.30	18.3	0.39

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epigallocatechin gallate and the flavone luteolin (differs structurally from quercetin only by the loss of a hydroxyl group at position 3 in ring C) were also particularly effective. The ability of the individual flavonoids tested to inhibit LDL oxidation *in vitro* was mostly consistent with the structural features proposed by Bors *et al*.[31] Effective protection of LDL against oxidation was thus associated with flavonoids possessing 4 or more hydroxyl groups, such as quercetin and the catechins, with a decrease in effectiveness for flavonoids containing 3 or less, such as apigenin. Flavonoids have been shown to interact with copper-ions[32] and although metal-ion chelation could have contributed to the concentration-dependent inhibition of TBARS by the flavonoids tested, this is unlikely to be the primary mechanism because IC_{50} values were $< 1\,\mu M$ compared to a copper ion concentration of $5\,\mu M$. Indeed some of the flavonoids tested were effective at concentrations as low as $0.25\,\mu M$. Furthermore, addition of copper-ions at $2.5\,\mu M$ produced a lag-time similar to that observed with $5\,\mu M$ (data not shown). Furthermore, a linear relationship between lag phase and concentration was obtained for the compounds tested (data not shown) providing further evidence that chelation of copper ions was not the primary protective mechanism. A chain breaking antioxidant mechanism has also been proposed for these compounds,[33] which is consistent with the findings of this study: a prolonged concentration-dependent increase in lag-time (data not shown).

Although quercetin, luteolin and (−)-epigallocatechin gallate were consistently the most effective of the flavonols, flavones and catechins tested, the inhibitory effects of the different phenolic compounds tested were not always well-correlated between the different assays for the measurement of LDL peroxidation. These different markers of oxidation thus need to be considered, for instance the levels of both lipid hydroperoxides and TBARS increase early during the oxidation process but while in the case of the lipid hydroperoxides this increase is followed by a peak and then a decrease to low levels, the decrease in TBARS is much slower; levels of conjugated dienes also peak and then decrease.[14] Electrophoretic mobility, by contrast, increases during oxidation and then remains stable but shows little change during the early stages of oxidation.[14] In the current study although quercetin and luteolin after 2 h were of similar effectiveness in terms of ability to decrease REM and inhibit lipid peroxide formation, after 4 h quercetin was considerably more effective. In order to assess the effectiveness of compounds as inhibitors of LDL oxidation *in vitro* it is thus important to measure a number of different markers of LDL oxidation.

On the basis of the results obtained, a diet enriched with onions (rich in quercetin) and black tea (rich in quercetin and catechins) was selected. Although epidemiological evidence has shown a relationship between dietary intake of flavonoids and reduced risk of coronary heart disease,[8,9] following the consumption of 131 mg/d of flavonoids from tea and onions (91 mg/d quercetin), no significant differences were found, in the current study, in LDL oxidation resistance *ex vivo* between the LF and HF dietary treatment periods. The intake of dietary flavonoids in the current study was approximately five times the estimated habitual intake in The Netherlands[5] and Denmark[34] and around three times the mean intake associated with reduced risk of CHD.[8]

Although no significant differences were found in LDL oxidation resistance *ex vivo* between the LF and HF dietary treatment periods in the current study, tea flavonoid supplementation (750 ml/d) has been found to reduce the susceptibility of LDL to oxidative modification as measured by Cu^{2+}-stimulated LDL lag-time[16] but these results have not been reproduced in all studies. Consumption of black tea 1500 ml/d for one week[18] or consumption of 225 g fried onions followed by sampling over a 48 h period[35] were found to have no effect on resistance of LDL to oxidation *ex vivo*. Furthermore consumption of the equivalent of 6 cups of black tea did not

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increase plasma resistance to lipid peroxidation over a 3 h sampling period.[17]

In conclusion, flavonoids (in particular quercetin and the catechins) protect against oxidative damage to LDL *in vitro*. However, selection of foods rich in these flavonoids, such as onions and black tea, and their subsequent consumption does not increase LDL oxidation resistance *ex vivo*. This suggests a re-evaluation may be needed of the usefulness of flavonoid ability to protect against LDL oxidation *in vitro* in the selection of flavonoid rich diets for dietary intervention studies aimed at investigating flavonoid ability to protect against oxidative damage to LDL *in vivo*.

Acknowledgements

The Ministry of Agriculture, Fisheries and Food, UK is thanked for research support.

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