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Effects of some flavonoids on the susceptibility of low-density lipoprotein to oxidative modification

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The effects of six flavonoids viz., apigenin, genistein, morin, naringin, pelargonidin and quercetin on the susceptibility of low-density lipoprotein (LDL) to oxidative modification were investigated. Flavonoids were added to plasma and incubated for 3 hr at 37°C, and the LDL fraction was separated by ultracentrifugation. Oxidizability of LDL was estimated by measuring conjugated diene (CD), lipid peroxides and thiobarbituric acid-reactive substances (TBARS), after cupric sulfate solution was added. Quercetin and morin significantly ($P<0.01$ by ANOVA) prolonged the lag time before initiation of oxidation reaction in dose-dependent manner. They also suppressed the formation of lipid peroxides and TBARS more markedly than other flavonoids. The ability to prolong lag time and suppression of lipid peroxides and TBARS formation was in the following order: quercetin >morin >pelargonidin >genistein >naringin >apigenin. LDL exposed to flavonoids reduced oxidizability. These findings suggest that flavonoids may have a role in ameliorating atherosclerosis.

Keywords: Flavonoids, oxidative modification, low-density lipoprotein (LDL)

Oxidation of low-density lipoprotein (LDL) is suggested as a key factor in human atherosclerosis[1]. Oxidatively modified LDL is a potent ligand for scavenger receptors on macrophages and thus contributes to generation of macrophage-derived foam cells, the hall-mark of early atherosclerotic fatty streak lesions[2,3]. Oxidative modification hypothesis of atherosclerosis is supported by findings, such as the presence of epitopes of oxidatively modified LDL in atherosclerotic lesions[3,4] and elevated titers of circulating auto-antibodies against oxidized LDL in patients with carotid atherosclerosis[5]. Antioxidant compounds provide resistance to this process and have been suggested to lower atherogenicity[6]. Considerable epidemiologic[7,8], biochemical[9], and clinical[10] evidences support this hypothesis. LDL contains different lipophilic antioxidants, the most abundant being α-tocopherol, the major form of vitamin E. Other antioxidants, such as γ-tocopherol, carotenoids, and ubiquinol-10 are present in lesser amounts. Vitamin E is considered to be the major non-enzymatic antioxidant present in the lipid structures of cells and lipoproteins. It is a reductant antioxidant, which increases LDL resistance against the oxidative modification. In lipid solutions and dispersions, it inhibits radical formation linearly with time, until consumed in the process[11].

Flavonoids are a group of polyphenolic antioxidants found in vegetables, fruits, beverages and tea. Their dietary intake is reported to be inversely associated with mortality from coronary artery disease[12,13]. Earlier, it was reported that phenolic substances in red wine exert a stronger inhibitory effect than vitamin E, on copper-induced oxidation of LDL[14]. However, De-Rijke *et al*[15] reported that red wine consumption did not affect the oxidizability of LDL in *in vivo* study. Flavonoids have been shown to inhibit oxidative modification of LDL, when added before initiation of oxidation[16,17]. However, the mechanism by which they inhibit LDL oxidation is not clearly understood. Flavonoids due to their amphipathic nature, may act within the LDL particle, in a manner similar to vitamin E, or may act comparable with ascorbic acid in the extraparticle environment of LDL. In this study, we investigated the effects of plasma pre-incubation with six flavonoids on the susceptibility of LDL to oxidative modification.

Flavonoids viz, apigenin, genistein, morin, naringin, pelargonidin and quercetin, and other reagents were purchased from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany).

Blood was collected from a normolipidemic healthy male volunteer in an ethylenediamidine-tetraacetic acid (EDTA)-containing (1 g/L) tube, after an overnight fast. Plasma was separated by low-speed centrifugation at 1000 × *g* at 4°C for 15 min. For enriching LDL with flavonoids, each compound at various concentrations of 50, 100 and 200 μmol/L

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dissolved in 10% dimethyl sulfoxide (DMSO) in phosphate buffer saline (PBS, *p*H 7.4) (20 ml 10% DMSO/L plasma) was added to plasma and then incubated at 37°C for 3 hr.

LDL (density 1.019-1.063 g/ml) was isolated by a rapid isolation technique[18] and dialyzed for 12 hr at 4°C with four 1-L changes in EDTA-free PBS, that had been degassed by using a vacuum procedure. Control LDL was prepared by the same technique, after adding only vehicle (20 ml 10% DMSO/L plasma) to plasma and incubating for 3 hr at 37°C. Earlier, in a preliminary study[19], we reported that DMSO at this concentration did not affect the oxidizability of LDL. The LDL suspension was stored at 4°C under argon in the dark.

Oxidizability of LDL was estimated by measuring three indexes, conjugated dienes (CD), lipid peroxides and thiobarbituric acid-reactive substances (TBARS). Freshly prepared LDL (50 mg protein/L) was incubated with $CuSO_4$ (final concentration 10 μmol/L) at 37°C in a Hitachi Spectrophotometer (Hitachi Sangyo Co., Tokyo) fitted with a peltier heater. Absorbance at 234 nm was automatically recorded at 10 min intervals. Lag time before the initiation of oxidation and the propagation rate was determined, as described earlier[20]. After incubation for 90 or 180 min with 10 μmol $CuSO_4$/L , the oxidation reaction was stopped by adding EDTA (100 μmol/L final concentration).

The content of lipid peroxides in LDL was measured colorimetrically with a commercially available kit. For measuring lipid peroxides content in LDL, we used an assay based on the oxidative activity of LOOH, which converts iodide to iodine. Iodine further forms triiodide ion, which absorbs at 365 nm. We used the microtiter plate procedure using a commercially available reagent (CHOD-iodide, Merck, Darmstadt, Germany). LDL oxidation was carried out in polystyrene microtiter plates. CHOD iodide reagent (190 μL) was added to plates and incubated for 60 min at 37°C. The absorbance at 365 nm was measured in a microplate reader (Labsystems, Muluskan MCC/340, Uppsala, Sweden). The concentration of LOOH was calculated from the molar absorption coefficient of 246,000 mol/cm for the triiodide ion and a path length in the microtiter plate of 1 cm for the final volume of 330 μL. The concentration of TBARS was measured as described[21]. The concentration of malondialdehyde (MDA) was calculated using the extinction coefficient for MDA (165,000 mol/cm).

All results are expressed as mean ± S.D. (n=5). Data between groups were compared by analysis of variance (ANOVA). Fisher's test was used, whenever, a statistically significant difference between the two groups was shown by ANOVA.

Addition of flavonoids to plasma for 3 hr at 37°C resulted in lipoprotein antioxidants enrichment; the efficiency of enrichment depended upon the compound concentration in medium. Incubation of Cu^{+2} with the LDL suspension caused extensive oxidation of the lipoprotein, as judged by accumulation of CD, lipid peroxides and TBARS in LDL sample. Enriching LDL with antioxidant agents made it more resistant to copper-induced oxidation, in comparison with a native. This effect was demonstrated with all the indices of oxidation used and appeared to be most pronounced within the first hours of oxidation. Table 1 shows the effects of six flavonoids, on the susceptibility of LDL to Cu^{+2}-induced oxidation. Quercetin, morin and pelargonidin significantly increased lag time before the onset of CD formation ($P<0.01$ by ANOVA) in a dose-dependent manner. Continuous registration of absorbance of LDL sample at 234 nm showed that quercetin (200 μmol/L) exerted the strongest effect, prolonging lag time to more than 3 times, compared to control, while morin prolonged lag time to twice. The lag time was significantly increased in LDL separated from pre-treated plasma with 50-200 μmol/L quercetin, morin and pelargonidin ($P<0.01$ and $P<0.05$ by Fisher's test). The capacity to prolong the lag time was in the following order: quercetin >morin >pelargonidin >genistein >naringin >apigenin.

Suppression of TBARS and lipid peroxides formation was also observed in LDL samples, to

Table 1 — Effect of addition of flavonoids to plasma on the susceptibility of LDL to Cu^{+2}-induced oxidation

[Values are given as mean ± SD of three separate experiments]

	Lag time (min)		
	50 μmol/L	100 μmol/L	200 μmol/L
Apigenin	61.2±7.3	78.3±7.9^a	80.1±3.9^a
Genistein	61.3±3.5	76.1±10.8^a	100.1±12.5^a
Morin	69.1±6.6^a	90.0±8.8^a	131.9±14.0^b
Naringin	60.9±4.9	77.7±9.0^a	98.2±10.1^a
Pelargonidin	66.7±4.5^a	90.0±8.1^a	120.3±11.45^a
Quercetin	69.9±4.9^a	91.2±5.8^a	176.0±9.8^b

Control: 58.1 ± 3.0(min); $P<0.01$(ANOVA).

^{a,b}Significantly differ from control (Fisher's test): $^aP<0.01$ and $^bP<0.05$

which these compounds were added (Table 2). Quercetin suppressed the formation of TBARS and lipid peroxides more markedly than other flavonoids in dose-dependent manner. TBARS and lipid peroxide formation in the medium was significantly reduced in the LDL separated from plasma, incubated with 50-200 μmol/L quercetin, morin and pelargonidin (*P*<0.05 & *P*<0.0001). The suppression ability of TBARS and lipid peroxides formation was in the following order: quercetin >morin >pelargonidin >genistein >naringin >apigenin.

In this study, we showed that Cu^{+2}-catalyzed oxidation of LDL, isolated from plasma pre-incubated with the flavonoids, was significantly inhibited in a dose-dependent manner, as assessed by lag time before the initiation of oxidation and the formation of lipid peroxides and TBARS. To add these compounds to LDL *in vitro*, we incubated EDTA-containing plasma at 37°C for 3 hr with each one of them at the concentrations 50, 100 and 200 μmol/L; LDL was then separated and dialyzed against PBS before the induction of oxidation. Therefore, the compounds not associated with LDL particles were removed along during dialysis. Thus, those on the surface of or within LDL particles were considered to be responsible for inhibiting LDL oxidation.

Table 2 — Effects of addition of flavonoids to plasma on the susceptibility of LDL to Cu^{+2}-induced by measuring of lipid peroxides and thiobarbituric acid-reactive substances (TBARS)

[Values are given as mean ± SD; n=4. One representative experiment of three is shown; the other two experiments gave similar results]

	Lipid peroxides (nmol/mg protein)	TBARS (nmol MDA/mg protein)
Control	214.0 ± 2.0	15.60 ± 1.60
Apigenin		
50 μmol/L	212.02 ± 2.30	13.93 ± 1.06
100 μmol/L	205.62 ± 12.33	11.52 ± 1.14
200 μmol/L	191.69 ± 7.14[a]	8.25 ± 2.02[a]
Genistein		
50 μmol/L	207.96 ± 4.35	12.29 ± 1.52
100 μmol/L	159.73 ± 8.50[a]	9.12 ± 1.01[a]
200 μmol/L	92.24 ± 10.24[b]	6.86 ± 1.18[b]
Morin		
50 μmol/L	197.52 ± 4.80[a]	9.51 ± 0.88[a]
100 μmol/L	129.44 ± 14.54[b]	8.39 ± 0.47[a]
200 μmol/L	59.83 ± 10.62[b]	4.89 ± 1.68[b]
Naringin		
50 μmol/L	208.87 ± 13.20	13.10 ± 0.94
100 μmol/L	179.47 ± 10.92[a]	10.22 ± 0.83[a]
200 μmol/L	111.24 ± 16.45[b]	7.14 ± 1.67[b]
Pelargonidin		
50 μmol/L	209.90 ± 11.55	12.85 ± 1.13
100 μmol/L	139.50 ± 8.40[b]	8.76 ± 0.91[a]
200 μmol/L	87.75 ± 15.36[b]	6.37 ± 0.79[b]
Quercetin		
50 μmol/L	196.01 ± 5.50[a]	9.42 ± 0.57[a]
100 μmol/L	79.33 ± 7.15[b]	6.62 ± 0.93[b]
200 μmol/L	25.90 ± 8.20[b]	3.23 ± 0.29[b]

Levels of lipid peroxide and TBARS in absence of Cu^{+2} were approx. 0. 0; *P*<0.01 (ANOVA)

[a,b]Significantly differ from control: [a]*P*<0.05 and [b]*P*<0.0001

LDL pre-incubated *in vitro* with polyphenols, such as quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation (induced by macrophages) and aggregation[22]. Fuhrman *et al*[23] showed that the formation of TBARS for LDL pre-treated with lycopene or the flavonoid glabridin was decreased, in comparison with native LDL. Miura *et al*[24] reported that the lag time before onset of conjugated diene formation in porcine LDL was prolonged >100 min in the presence of flavonoids, compared with only 21 min in their absence. Antioxidative activity of several flavonoids was compared. The ability to prolong lag time before copper-induced oxidation was reported to be in the following order: quercetin >epigallocatechin gallate >theaflavin >myricetin[25]. Quercetin and morin also inhibit the oxidation of LDL mediated by macrophages[24,26]. Flavonoids have been shown to supress superoxide anion[27], hydroxyl radicals[28], and lipid peroxyl radicals[27]. They are also reported to chelate iron[27], and copper[29] and this may partly explain their antioxidant effects, i.e., reduced formation of free radicals in the macrophage medium and the cell-free system. Another mechanism by which flavonoids may inhibit LDL oxidation is suppression of the generation of release of free radicals from the macrophages themselves.

Our data showed that quercetin and morin had stronger inhibitory activity against LDL oxidation than other flavonoids. As phenolic hydroxyl groups are considered to be necessary for radical scavenging activity in flavonoids and since quercetin and morin have more phenolic hydroxyl groups than other flavonoids, may possibly be one of the reasons for their more marked effects. Their incubation to plasma over a long period of time may enrich the LDL particles sufficiently to make them less susceptible to oxidative reaction. In conclusion, we clearly showed that incubation of plasma with flavonoids protected LDL from Cu^{+2}-induced oxidation reaction. These

compounds significantly decreased the susceptibility of LDL to oxidative modification; therefore, they may have favourable effects in ameliorating atherosclerosis.

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References

1 Steinberg D, Parthasarathy S, Carew T E, Khoo J C & Witztum J L (1989) *New Engl J Med* 320, 915-924
2 Parthasarathy S & Rankin S M (1992) *Proc Lipid Res* 31, 127-143
3 Leake D S (1993) *Brit Heart J* 69, 476-478
4 Witztum J L & Steinberg D (1991) *J Clin Invest* 88, 1785-1792
5 Steinbrecher U P, Zhang G & Lougheed M (1990) *Free Radic Biol Med* 9, 155-168
6 Abbey M, Nestel P L & Baghurst P A (1993) *Am J Clin Nut* 58, 525-532
7 Gey K., Puska P, Jordan P & Moser U K (1991) *Am J Clin Nutr* 53, 326S-329S
8 Rimm E B, Stampfer M J, Ascherio A, Givannucci E & Golditz G A (1993) *New Engl J Med* 328, 1450-1456
9 Todd J, Anderson L & Meredith I (1995) *New Engl J Med* 332, 488-493
10 Regnstrom J, Nilsson J, Tornvall P, Landou C & Hamsten A (1992) *Lancet* 339, 1183-1186
11 Suzukawa M, Ishikawa T, Yoshida H & Nakamura H (1995) *J Am Coll Nutr* 14, 46-52
12 Hetrog M G L, Feskens E J M, Hollman P C H, Katan M B & Kromhout D (1991) *Lancet* 339, 1183-1186.
13 Hetrog M G L, Kromhout D & Aravanis C (1995) *Arch Intern Med* 155, 381-38.
14 Frankel E N, Kanner J, German J B, Parks E & Kinsella J E (1993) *Lancet* 341, 454-457.
15 De-Rijke Y B, Demacker P N M, Assen N A, Sloots L M & Katan M B (1996) *Am J Clin Nutr* 63, 329-334`
16 Cook N C & Samman S (1996) *J Nutr* 7, 66-71
17 Torel J, Cillard J & Cillard P (1986) *Phytochemistry* 25, 383-387
18 Chung B H, Segrest J P & Ray M J (1986) *Methods Enzymol* 128, 181-209
19 Safari M R, Taherkhani H, Ani M, Naderi G A & Asgary S (2002) *Iran Biomed J* 6 (4), 111-115
20 Esterbauer H, Striegl H, Puhl H & Rothender M (1989) *Free Radical Res Commun* 6, 67-75
21 Buege J A & Aust S D (1978) *Methods Enzymol* 52, 302-310
22 Hayek T, Fuhrman B, Vaya J, Rosenblat M, Belinky P & Coleman R (1997) *Arteriosclerosis Thrombosis Vascular Biol* 17, 2744-2752
23 Fuhrman B, Volkova N, Rosenblat M & Aviram M (2000) *Antioxidant Redox Signaling* 2, 491-506
24 Miura S, Watanabe J & Sano M (1995) *Biol Pharmacol Bull* 18, 1-4
25 De-Whalley C V, Rankin S M, Hoult J R S, Jessup W & Leake D S (1990) *Biochem Pharmacol* 39, 1743-1750
26 Wu T W, Fung K P, Yang C C & Weisel R D (1995) *Life Sci* 57, PL51-PL56
27 Afanas'ev I B, Derozhko A L, Brodskii A V, Kostyuk V A & Potapovitch A I (1989) *Biochem Pharmacol* 38, 1763-1769
28 Husian S R, Cillard J & Cillard P (1987*) Phytochemistry* 26, 2489-2491
29 Thompson M, Williams C R & Elliot G E P (1976) *Anal Chem Acta* 85, 375-381