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In vitro effects of a flavonoid-rich extract on LDL oxidation

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Abstract

Flavonoids are phenolic compounds of vegetable origin with antioxidant effects. The present study aimed to determine their properties as LDL antioxidants. LDL were incubated with increasing concentrations of flavonoids (0–16 μg/ml) and LDL oxidation was started by adding $CuCl_2$ (2 μM) to the media. When flavonoids were present in the media, vitamin E consumption, the lag phase of conjugated diene formation, LDL electrophoretic mobility in agarose gels and the appearance of thiobarbituric acid reacting substances (TBARS) were delayed in a concentration-dependent manner. To determine whether flavonoids could terminate LDL oxidation once initiated, two sets of experiments were performed. In the first, LDL oxidation was initiated as described above. At 2 or 4 h of incubation, flavonoids were added (4 μg/ml) and their effect compared to samples where butylate hydroxytoluene or EDTA were added. At 5 h, in the LDL samples where flavonoids were added, the electrophoretic mobility and TBARS production were the same as those present in LDL samples incubated for the whole period in the absence of flavonoids. However, when either butylate hydroxytoluene or EDTA was added, as would be expected, the LDL oxidation process was completely arrested as shown by a reduction in the appearance of TBARS and a lower LDL electrophoretic mobility. In the second experiment, LDL oxidation was initiated as described above and at 0, 10 and 20 min, flavonoids were added (4 μg/ml). When vitamin E was still present in the LDL solution, the flavonoids were able to both increase the lag phase in the formation of conjugated dienes and to delay the consumption of vitamin E. The present results show that in vitro, flavonoids prevent LDL oxidation in a concentration-dependent manner, delaying the consumption of vitamin E, but they cannot terminate or delay LDL oxidation once vitamin E in LDL is consumed.

Keywords: Oxidized LDL; Flavonoids; Cardiovascular disease

1. Introduction

It is thought that LDL oxidation plays a central role in the atherosclerotic process [1–3]. The in vitro effects of oxidized LDL could explain most of the alterations observed in the atherosclerotic plaque, including lipid loaded macrophages [4,5], recruitment and retention of monocytes in the arterial wall [6,7] and the cytotoxicity of endothelial cells [8,9].

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Supplementation with either Vitamin E or C are known to increase LDL resistance to oxidation both in vitro and in vivo [10–12]. Furthermore, several epidemiological studies have shown a negative correlation between plasma concentration of vitamin E and C and the risk of cardiovascular disease [13,14]. Higher intake of vitamin E has also been associated with a decreased incidence of cardiovascular diseases [15,16]. Therefore, antioxidants from the diet could play a relevant role in the prevention of cardiovascular diseases.

Flavonoids, phenolic compounds of vegetable origin commonly included in the diet, have antioxidant properties [17,18] and therefore, may be suitable for decreasing LDL susceptibility to oxidation and for preventing cardiovascular diseases. In fact, the 'French Paradox' has been attributed to the regular consumption of red wine [19], and more specifically, to the high phenolic compound content of the wine [20]. Furthermore, a high dietary intake of flavonoids has been associated with a decreased risk of developing cardiovascular diseases [21]. Finally, in vitro, flavonoids delay ^{125}I-LDL degradation in macrophage cultures because they prevent its oxidation [22].

The objectives of the present study were to determine the effects of a mixture of flavonoids on $CuCl_2$-mediated LDL oxidation and to establish whether they could terminate LDL oxidation once initiated.

2. Materials and methods

2.1. Flavonoids

The flavonoid-rich extract used was the anthocyanoside extract from *Vaccinium myrtillus*, supplied as a gift by Sigma-Tau España (Alcala de Henares, Madrid, Spain), whose main components are: anthocyan, catechin, chalcone, aurone, dimeric procyanidols type B, benzoic acid and tetrahydroy-xanthylium.

2.2. Isolation of lipoproteins

LDL were isolated from EDTA-treated plasma obtained from different donors after 12-h fasting by ultracentrifugation in a vertical rotor at 50 000 rev./min for 2.5 h as described by Chung et al. [23]. The isolated LDL were filtered through a 0.22-μm filter (Millipore, Molsheim, France) and stored at 4°C under N_2 in the dark. The susceptibility to oxidation was determined within 24 h.

2.3. Determination of LDL susceptibility to oxidation

Conjugated diene formation was determined as described by Esterbauer et al. [24]. In short, following LDL isolation, LDL were passed through a Sephacryl-400 column (Pharmacia LKB, Biotechnology, Inc., Madrid, Spain) to remove the EDTA. The LDL protein concentration was determined immediately using the Lowry procedure [25]. Then, 0.1 mg/ml of LDL protein was incubated with increasing concentrations of flavonoid extracts (0–16 μg/ml) and 2 μM copper chloride in a 1-ml quartz cuvette at 37°C. Light absorbance was read at 234 nm in a Beckman DU-640 spectrophotometer every 10 min for a maximum of 8 h or until the rapid phase of LDL oxidation reached a plateau. The lag phase was determined as the incubation time corresponding to the intersection of two lines drawn from the changes in optical density; one through the initial, slowly rising curve which corresponds to the utilization of endogenous antioxidants in the LDL and the other, a subsequent, rapidly rising curve which corresponds to the rapid LDL oxidation following the exhaustion of endogenous antioxidants [24]. The lag phase is expressed as minutes after the addition of $CuCl_2$. In some experiments the degree of LDL oxidation was determined following two different methods: (1) mobility on agarose gel electrophoresis and (2) the thiobarbituric acid reacting substances (TBARS) which were measured using malondialdehyde (MDA) formed from 1,1,3,3-tetramethoxypropane as a standard [26]. In parallel experiments, LDL was incubated under the same conditions described above and butylated hydroxytoluene (BHT) and

EDTA (final concentration 0.1 mM) were added to aliquots obtained at different times to stop the ongoing oxidation. These aliquots were used to test the concentration of vitamin E in the LDL solution. Vitamin E was determined by HPLC following the method previously described [27] with the sole modification of using a fluorescent detector to increase the sensitivity of the method (intraassay and interassay variability was 4.9 and 7.2, respectively).

To determine if flavonoids terminate ongoing LDL oxidation, two sets of experiments were performed: (a) LDL were incubated as above but in the absence of flavonoid. At different incubation times, when vitamin E was already consumed (2 and 4 h), the flavonoid-rich extract, BHT, a free radical scavenger or EDTA, a metal chelator were added to the LDL solution to give a final concentration of 4 μg/ml, 1 mM or 1 mM, respectively. BHT and EDTA were used because it is well established that both stop the $CuCl_2$-mediated LDL oxidation. TBARS and LDL electrophoretic mobility were determined; (b) LDL was incubated as above, but the flavonoid-rich extract was added at 0, 10 and 20 min after the addition of $CuCl_2$, when vitamin E could still be present. In these experiments the formation of diene conjugates was followed and the lag phase calculated. At different times of incubation aliquots were also obtained for vitamin E determination.

2.4. Statistical analysis

The means $\pm$ S.E.M. are given. The significance of the difference between the means of two groups was obtained with the one-way analysis of variance and Tukey HSD test for multiple comparisons, using the Systat program (Systat, Inc., Evanston, IL).

3. Results

To evaluate the antioxidant effects of flavonoids on LDL oxidation, LDL were incubated with increasing concentrations of flavonoids and submitted to oxidation with $CuCl_2$. As shown in Figs. 1 and 2 and Fig. 3, the presence of flavonoids in the media increased, in a concentration-dependent manner, both the half-life of vitamin E consumption and the lag phase of conjugated diene formation. The electrophoretic mobility of LDL on agarose gels and the production of TBARS at different times of incubation were also determined as indices of LDL oxida-

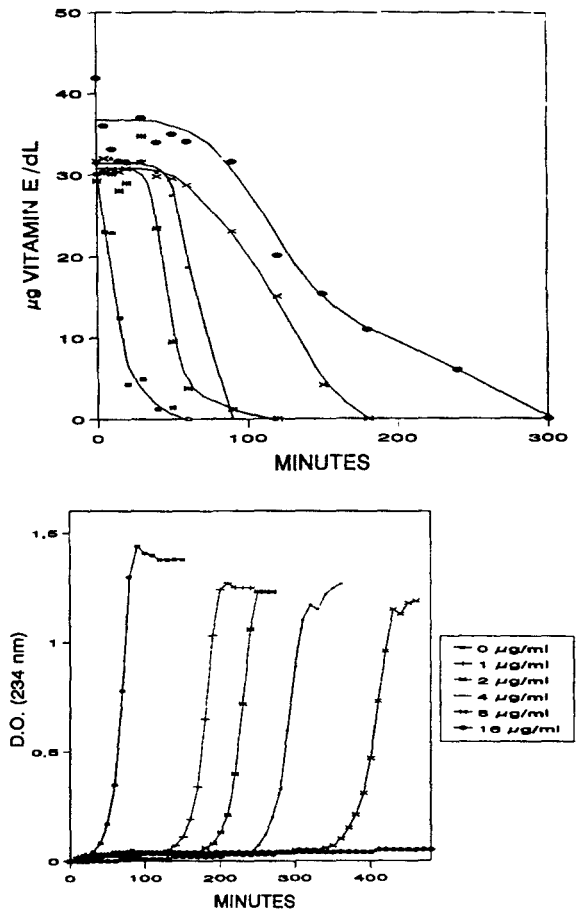

Fig. 1. Effects of flavonoids on the consumption of vitamin E (panel A) and formation of conjugated dienes (panel B) in LDL incubated in vitro. LDL (0.1 mg of LDL protein/ml) was incubated with increasing concentrations of the flavonoid-rich extract (0–16 μg/ml). Oxidation was induced with the addition of $CuCl_2$ (2 μM). At different times, aliquots were obtained and vitamin E determined, as described in Section 2.3. The formation of conjugated dienes was determined by changes in the light absorbance at 234 nm every 10 min. This representative experiment was replicated with LDL from eight different donors, with similar results.

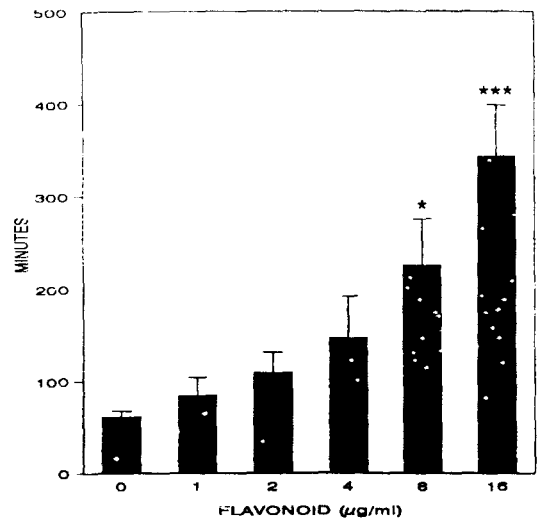

Fig. 2. Effects of flavonoids on the lag phase of conjugated dienes formation in LDL incubated in vitro and submitted to $CuCl_2$ oxidation as described in Fig. 1. In three out of eight samples, LDL did not oxidize when incubated with the highest concentration of flavonoid-rich extract (16 μg/ml) after 480 min, the maximal time of incubation. For statistical purposes in these three samples the lag phase was considered to be 480 min. Results are expressed as mean ± S.E. of eight samples. Mean differences between groups were tested by one-way analysis of variance and the Tukey HSD test for multiple comparisons. *: LDL incubate with vs. without flavonoids. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

tion. As shown in Fig. 4, flavonoids delayed the increase in LDL electrophoretic mobility and, as shown in Table 1, they decreased TBARS formation. These results further emphasize the inhibitory effects of flavonoids on LDL oxidation.

Once it was demonstrated that flavonoids could delay $CuCl_2$-mediated LDL oxidation, we wanted to determine whether flavonoids could terminate LDL oxidation once it had been initiated. LDL samples were incubated with $CuCl_2$ in the absence of flavonoids and 2 or 4 h later, flavonoids (4 μg/ml) were added to the medium. The same protocol was followed in parallel but instead of flavonoids, a free radical scavenger, BHT, or a metal chelator, EDTA, was added. For comparison, other aliquots of LDL were incubated under the same conditions but without any addition to the media, therefore allowing maximal oxidative conditions. Incubations were continued up to 5 h, at which time LDL electrophoretic mobility on agarose gels and TBARS production were determined. The addition of flavonoids at 2 and 4 h after initiation of the LDL oxidation process, neither decreased the LDL electrophoretic mobility (Fig. 5) nor the TBARS content of the LDL when compared to LDL aliquots incubated in the absence of any antioxidant (Table 2). However, when BHT or EDTA were added at 2 or 4 h of incubation, both compounds, terminated the LDL oxidation process as indicated by the lower TBARS content (Table 2) and the slower electrophoretic mobility of the samples (Table 2; Fig. 5) when compared to LDL aliquots incubated in the absence of any antioxidant (Table 2; Fig. 5). These results, therefore, show that in contrast to BHT and EDTA, flavonoids are unable to terminate LDL oxidation once initiated.

Previous reports have shown that when LDL is submitted to $CuCl_2$ oxidation, the antioxidants are consumed before the oxidation of fatty acids starts [28,29]. As flavonoids could not terminate LDL oxidation once initiated, it was decided then to determine whether the antioxidant effects of

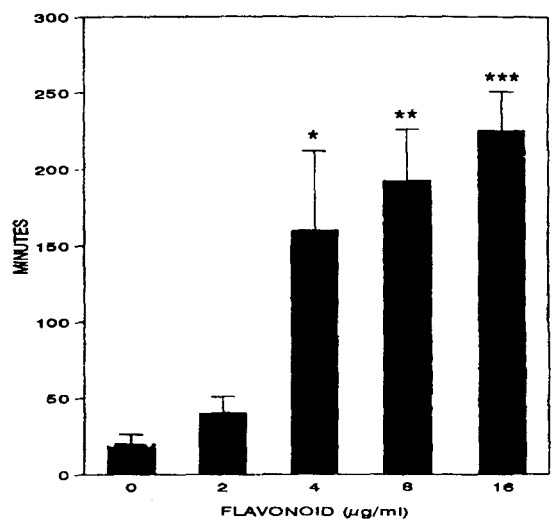

Fig. 3. Effects of flavonoids on vitamin E consumption. The experiments were performed as described in the methods section and the half-life of vitamin E was calculated. When vitamin E was not consumed after 250 min, that was considered the half-life of vitamin E consumption. The statistical analysis was performed as described in Fig. 2.

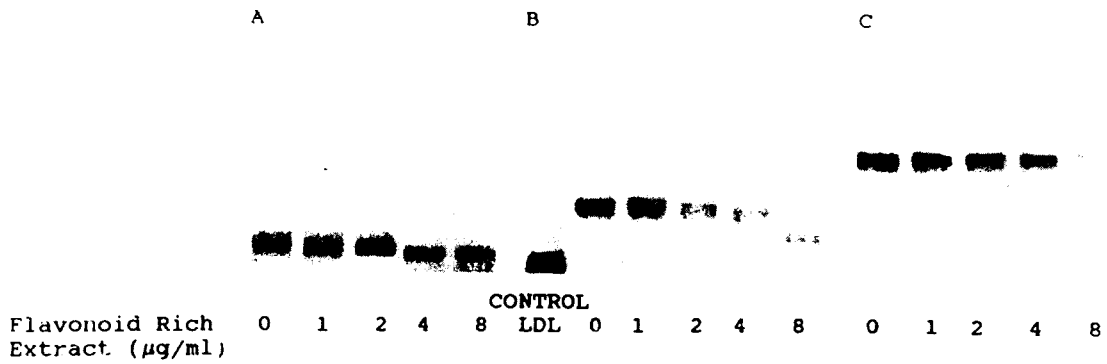

Fig. 4. Effects of flavonoids on LDL electrophoretic mobility. LDL was incubated with flavonoids and submitted to oxidation as previously described. LDL electrophoretic mobility on agarose gels was determined 3, 6 and 20 h (panel A, B and C, respectively) after initiation of LDL oxidation. This representative experiment was reproduced with LDL obtained from four different donors.

Table 1
Effects of flavonoids on TBARS formation by $CuCl_2$-mediated LDL oxidation (nM of MDA/0.1 mg of LDL protein)

Incubation time (h)	Experiment	Flavonoid concentration (μg/ml)					
		0	1	2	4	8	16
3	1	3.7	2.8	1.9	2.0	1.4	1.0
	2	2.6	2.5	2.4	1.5	0.4	0.3
6	1	8.6	7.7	6.2	4.3	3.4	3.3
	2	4.9	5.3	5.3	4.9	2.2	0.3
20	1	8.6	11.6	9.0	3.5	0	0
	2	5.8	4.1	4.3	4.2	4.3	4.3

flavonoids were dependent upon the presence of vitamin E in the LDL. LDL was submitted to oxidation with $CuCl_2$ and at a short time thereafter, when vitamin E would most likely still be present, the flavonoid-rich extract was added and both the vitamin E consumption and the formation of conjugated dienes was followed. As shown in Fig. 6, the addition of flavonoids at short time points after the initiation of the LDL oxidation process delayed the consumption of vitamin E. In another set of experiments following the same protocol, the formation of conjugated dienes was determined, and, as shown in Fig. 7, the addition of flavonoids at time 0 or 10 min delayed the formation of conjugated dienes, when vitamin E was still present. However, flavonoids did not have any effect on the lag phase if added at 20 min of incubation, when vitamin E was already consumed (Fig. 7).

4. Discussion

The results presented in this study show that flavonoids increased LDL resistance to oxidation in vitro. It was found that when LDL was submitted to $CuCl_2$-mediated oxidation, the addition of flavonoids in the media prolonged the consumption of vitamin E, increased the lag phase for the formation of conjugated dienes and decreased LDL electrophoretic mobility on agarose gels and TBARS production. These effects were concentration-dependent. The present results confirm and expand previous data showing the antioxidative effects of flavonoids on macrophage-mediated LDL oxidation [22].

When flavonoids were added to the LDL solution, once the oxidation process was initiated, their antioxidant effect was related to the presence of vitamin E. When vitamin E was still present in

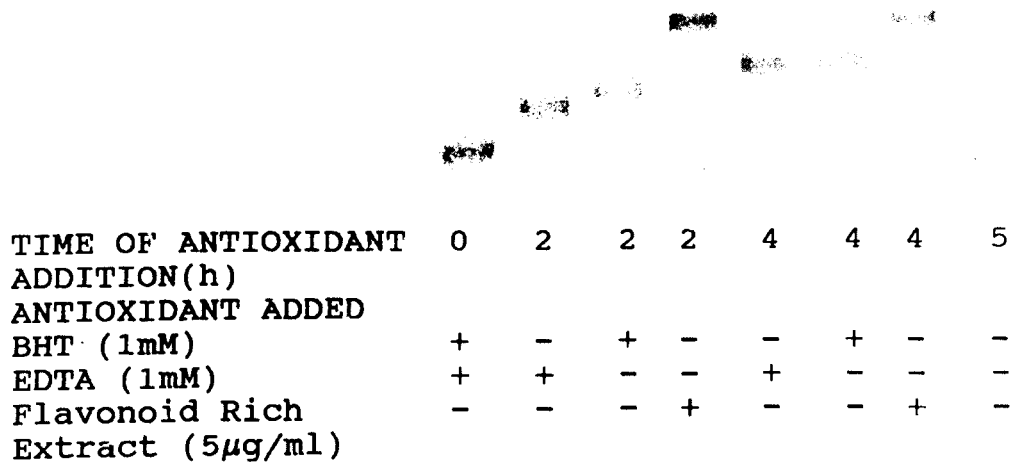

Fig. 5. Effects of flavonoids on oxidized LDL. LDL was oxidized with $CuCl_2$ as described in Section 2.3. At 2 and 4 h after initiation of the oxidation, flavonoids (4 μg/ml), a free radical scavenger, BHT (1 mM) or a metal chelator, EDTA (1 mM) were added and the results compared to a sample without any antioxidant added. LDL electrophoretic mobility was determined after 5 h of incubation. No effects were observed on LDL electrophoretic mobility when flavonoids were added to the medium. In contrast, BHT and EDTA arrested the process. The results from this representative experiment were reproduced with LDL from four different donors.

Table 2
Effects of flavonoids, BHT and EDTA on TBARS formation (nM of MDA/0.1 mg of LDL protein) and electrophoretic mobility (EM) on agarose gels (mm from the origin) by $CuCl_2$-mediated LDL oxidation. LDL aliquots were incubated for 5 h

		Time of antioxidant addition (h)							
		0	2	4	2	4	2	4	5
Antioxidant added									
BHT (1 mM)		+	+	+	−	−	−	−	−
EDTA (1 mM)		+	−	−	+	+	−	−	−
Flavonoid extract (4 μg/ml)		−	−	−	−	−	+	+	−
Experiment									
1	TBARS	0	3.6	11.3	2.7	11.3	14.7	14.7	14.8
	EM	0.5	1.1	1.4	0.7	1.4	1.9	1.9	1.9
2	TBARS	0.2	9.5	19.3	9.9	19.3	18.4	21.7	22.0
	EM	0.6	1.4	1.7	1.2	1.6	2.2	2.2	2.3
3	TBARS	2.1	2.3	1.6	1.2	2.5	4.1	4.2	4.0
	EM	0.6	0.8	0.9	0.8	1.1	1.7	1.4	1.6
4	TBARS	0	0.3	1.2	1.4	1.3	6.5	6.8	6.8
	EM	—	—	—	—	—	—	—	—

the LDL, flavonoids prolonged the lag phase for the formation of conjugated dienes and delayed the consumption of vitamin E, therefore delaying the whole oxidation process. However, when flavonoids were added later on during the oxidation process, when vitamin E was already consumed, their addition neither regenerated the vitamin nor decreased or stopped the oxidation process, as indicated by the lack of effect on the lag phase in the formation of conjugated dienes, the LDL electrophoretic mobility on agarose gels and the TBARS production. These results suggest that the flavonoids decrease LDL oxidation only if they are present in early stages of the oxidation process, when endogenous antioxidants are still present. In fact, similar findings have been shown with vitamin C, another water soluble antioxidant. When vitamin C is added to freshly isolated LDL, it decreases its rate of oxidation, but if the LDL were previously stored for several weeks, and already minimally oxidized, vitamin C not only did not prevent LDL oxidation, but was even pro-oxidant [30].

When LDL is submitted to copper-mediated oxidation, it is thought that free radicals are generated through the Fenton reaction [31,32]. The

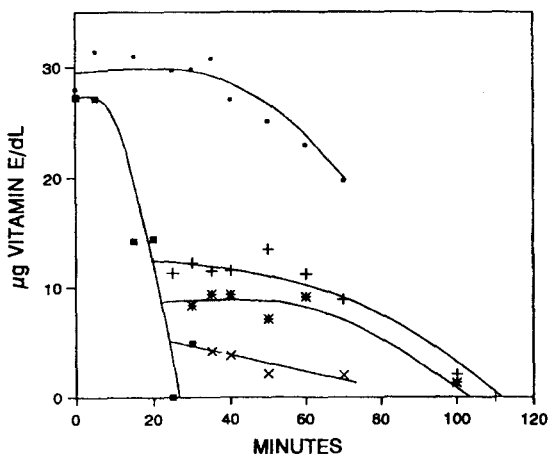

Fig. 6. To determine if flavonoids could arrest vitamin E consumption once the LDL oxidation was initiated. LDL was submitted to oxidation with $CuCl_2$ as previously described (■). At 0 (□), 15 (+), 20 (*) and 25 (×) min of oxidation, flavonoids were added to the medium. Aliquots were obtained at different times, as described in Section 2.3 and vitamin E measured. This representative experiment was reproduced with LDL from three different donors.

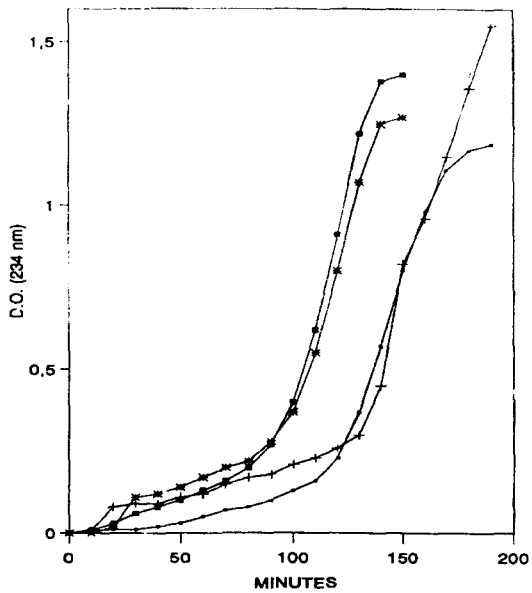

Fig. 7. To establish that flavonoids could arrest LDL oxidation when vitamin E was still present in the LDL. LDL oxidation was initiated as previously described and at 0, 10 and 20 min, the flavonoid-rich extract was added. Prior to this, an aliquot of the LDL solution was obtained for vitamin E determination. The formation of conjugated dienes was followed as described in Fig. 1. LDL incubated without flavonoids (■); LDL incubated with flavonoids at 0 (□), 10 (+) or 20 (*) min after the initiation of the oxidation. The vitamin E concentration was 45.62, 42.63 and 0 µg/dl at 0, 10 and 20 min of incubation, respectively. This experiment was repeated with LDL from four different donors, with similar results.

water soluble free radicals produced through this reaction, would initially be taken up by the LDL antioxidants contained in the water-lipid interface of the LDL, including vitamin E. Although lipid soluble, its phenolic OH group is at the lipid-water interface of the LDL. Once the antioxidants are consumed, the fatty acids and other lipids contained in the core of the LDL would be oxidized. We propose then, that the water soluble flavonoids could prevent LDL oxidation by taking up the water soluble free radicals generated by copper through the Fenton reaction, decreasing the consumption of the LDL antioxidants contained in the lipid-water interface. This interpretation agrees with our finding that flavonoids decrease the rate of vitamin E consumption in LDL submitted to copper oxidation. Similar effects could occur with

other LDL antioxidants. In contrast, when vitamin E and probably other antioxidants contained in the water-lipid interface are no longer present in the LDL particle and the lipids contained in its core are being oxidized, the addition of water soluble flavonoids is unable to terminate the process. This is in accordance with the inability of flavonoids to stop LDL oxidation in more advanced stages of the process. Nevertheless BHT, a lipid soluble free radical scavenger, prevents both LDL oxidation and stops the ongoing process once initiated because it can be incorporated in the core lipid of the LDL.

Overall, the mechanism by which flavonoids prevent LDL oxidation resembles that proposed to explain the effects of vitamin C, a water soluble antioxidant that protects against LDL oxidation [33–35]. It has been shown that vitamin C delays vitamin E consumption in LDL submitted to copper-mediated oxidation, prolonging the lag phase in the formation of conjugated dienes and increasing LDL resistance to oxidation [33–35].

In conclusion, these results show that, in vitro, flavonoids increase LDL resistance to oxidation, decreasing the consumption of vitamin E and this mechanism could contribute to the protective effects against cardiovascular diseases linked to high intake of flavonoids.

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