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Short Term Citrus Flavonoid Supplementation of Type II Diabetic Women: No Effect on Lipoprotein Oxidation Tendencies

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Flavonoids, which are dietary components and have possible drug uses, inhibit lipoprotein oxidation *in vitro*. The present study considered whether flavonoid supplementation in humans could influence lipoprotein vulnerability to oxidation. Citrus flavonoid supplementation (about 1 g/day, 3 weeks), or placebo, was given to 40 Type II diabetic women, a population prone to oxidative stress. Absorbance spectra of plasma from 4 subjects revealed that some flavonoid absorption occurred. When tested *in vitro*, a supplement extract, with spectrum peak height similar to that of plasma samples, completely inhibited copper-induced oxidation of very low plus low density lipoproteins. In contrast, neither flavonoid supplementation nor placebo influenced lipoprotein susceptibility to copper-stimulated oxidation *in vitro* (lag time or propagation rate). Thus, this study demonstrated that increased flavonoid consumption *by humans* does not necessarily alter lipoprotein susceptibility to oxidation *assessed in vitro*.

Keywords: Flavonoids, diabetes, lipoprotein oxidation, quercetin

INTRODUCTION

Flavonoids are polyphenolic compounds which appear in human diets and which have been considered for drug use.[1] Many benefits are speculated to result from flavonoid consumption including inhibition of lipoprotein oxidation,[2–4] a process thought to lead to atherosclerosis.[5] This inhibition clearly occurs *in vitro*.[2–4] However, no relationship has yet been established between human flavonoid intake and lipoprotein susceptibility to oxidation. Such a relationship could help explain a Dutch epidemiology study inversely correlating flavonoid intake with low rates of coronary heart disease.[6]

This study tested the hypothesis that high flavonoid ingestion influences lipoprotein vulnerability to oxidation. Although the goal was to learn about events occurring *in vivo*, measurements were made of lipoprotein oxidation

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initiated *in vitro*. These measurements are assumed to reflect donor variations in lipoprotein vulnerability to oxidation.[7,8] One of these variations seems to be preformed lipid peroxides.[8]

This study examined very low plus low density lipoproteins (VLDL + LDL), both of which oxidize.[9] This approach deletes long ultracentrifugation and dialysis steps often used to prepare just LDL. Conceivably, both steps can affect oxidation results.[9,10] This study examined Type II diabetic women since such subjects often show high lipoprotein vulnerability to oxidation.[11]

MATERIALS AND METHODS

Subjects and Treatment

Forty postmenopausal, Type II diabetic women (age ± SD = 60 ± 12 years; body weight = 90 ± 25 kg), were recruited from Internal Medicine, The Ohio State University. Each took sulfonylurea or insulin by prescription. None used flavonoid supplements. Study protocol was approved by the Human Subjects Review Committee of The Ohio State University. Subjects gave informed written consent and were randomly assigned to receive placebo (malto dextran) or a citrus flavonoid supplement (Citrus Flavonoid Complex, Amni, Hayward, CA) for 21 days. According to the manufacturer, each tablet contained about 0.5 g of flavonoids (1 g of complex). Two tablets were taken daily at different meals for a dose assumed to be about 1 g flavonoids/day. Each subject was given 45 tablets and returned the unused portion. Return of five or more tablets would have been considered noncompliance.

On Days 0 and 22, about 10 ml of blood was drawn into tubes with EDTA. Subjects were told not to take supplements on Day 22. For a second study phase, 4 subjects were rerecruited for a crude assessment of flavonoid uptake. These subjects had ceased phase one treatment for at least 6 mo. The subjects underwent the 21 day flavonoid treatment with slight modification. Blood was drawn on day 21, 7 h after ingestion of just one tablet for that day.

Analytical Methods

The presence of flavonoid in the supplement and in plasma was verified using spectrophotometric wavelength scan.[3] Flavonoids were extracted from the supplement by three steps: crushing the tablet to a powder with mortar and pestle; mixing with 15 ml of 95% ethanol per tablet; and removing undissolved material by centrifugation for 5 min at 16,500 × *g*.

For lipoprotein oxidation measurements, plasma LDL + VLDL was prepared by precipitation with $MgCl_2$ and dextran sulfate.[9] The VLDL + LDL cholesterol concentration, measured with a kit from Sigma Chemical Company (St. Louis, MO), was adjusted to 75 μg/ml and oxidation was initiated by 8 μM $CuSO_4$. Oxidation was monitored by conjugated diene formation as described by Esterbauer.[7] Lag phase was determined by drawing a line tangent to the linear propagation phase of the curve, and then extrapolating this line through the horizontal axis (time in min). The time interval between copper addition and the extrapolation point was defined as the lag time.

RESULTS

Supplement extracts produced an absorbance spectrum pattern (Figure 1) characteristic of citrus flavonoids such as quercetin.[3] As in previous work,[3] mixing extract with plasma shifted the absorbance peak to the right (Figure 2). It is assumed that this shift is primarily due to flavonoid-albumin binding, which also increases absorbance intensity.[3] Spectrophotometric analysis was also done for plasma from each of 4 flavonoid supplemented subjects treated as described in Materials and Methods. The absorbance peak location (Figure 3) was similar to that of control plasma with flavonoid addition *in vitro* (Figure 2).

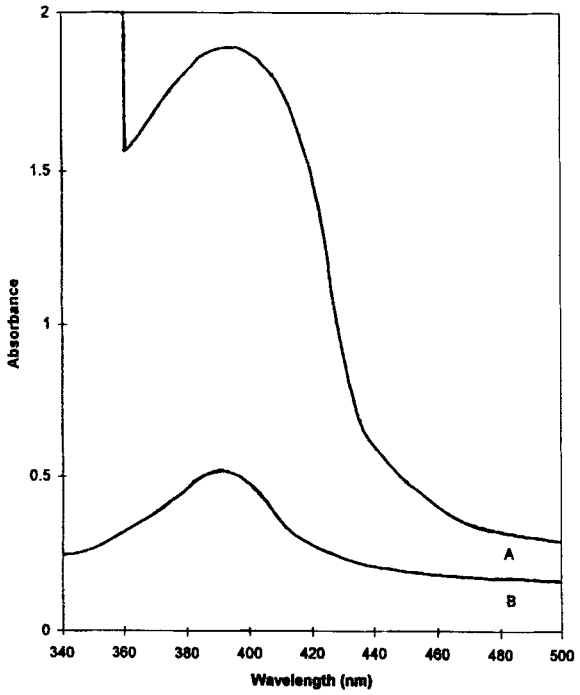

FIGURE 1 Absorption spectrum of citrus flavonoid supplement extract. Extract was prepared as described in text. A = Extract diluted 1 : 40 (v : v) in water; B = Extract diluted 1 : 500 (v : v) in water.

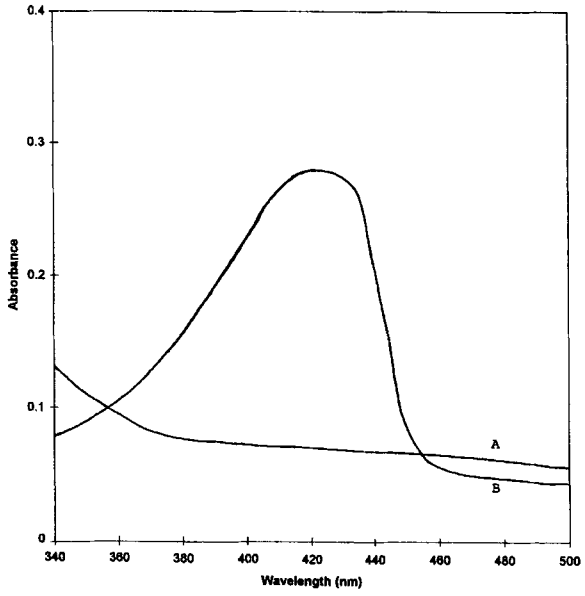

FIGURE 2 Absorption spectrum of plasma (1 : 10 dilution, v : v in phosphate buffered saline). A = control plasma (no flavonoid supplement); B = control plasma plus flavonoid added *in vitro* (1 : 100 (v : v) final dilution).

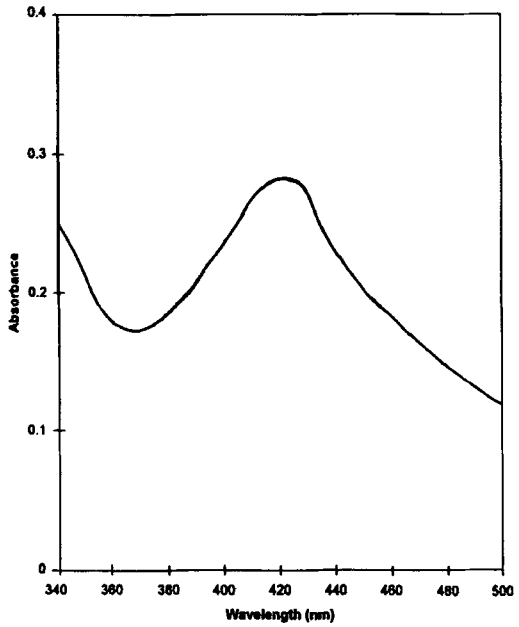

FIGURE 3 Absorption wavelength scan of plasma (1 : 10 dilution, v : v in phosphate buffered saline) from diabetic subject after flavonoid supplementation. This pattern was typical of that obtained from 4 different subjects. Subjects were treated as described in text.

A supplement extract, diluted to give an absorbance spectrum peak height similar to that of Figure 3, totally inhibited oxidation *in vitro* of VLDL + LDL derived from 2 ml of plasma (Figure 4). In contrast, neither flavonoid supplementation nor placebo had significant effects on VLDL + LDL oxidation lag time nor propagation rate (Table I). Readings changed little for each subject after either placebo (mean absolute percent change $< 1\%$), or flavonoid (mean absolute percent change of $4 \pm 2\%$).

When VLDL + LDL was prepared from plasma spiked with flavonoid as in Figure 2B, the absorption spectrum showed no flavonoid peak; adding flavonoid directly to the lipoproteins gave a pattern similar to that of Figure 1B (data not shown). This implied that little, if any, flavonoid carried over from the plasma to the lipoprotein oxidation assays.

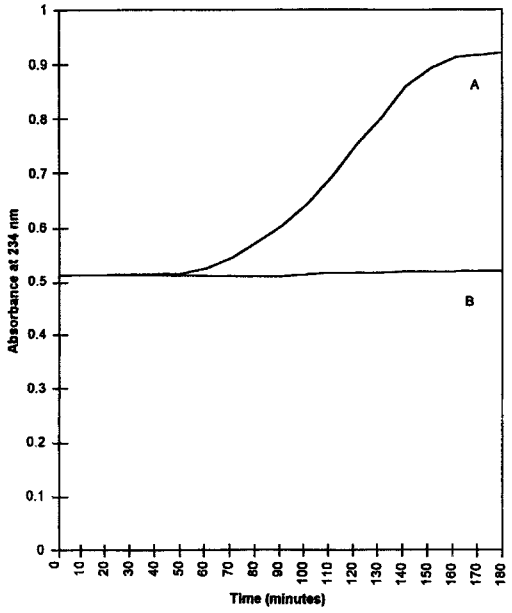

FIGURE 4 Oxidation time course for VLDL + LDL plus and minus flavonoids added *in vitro*. A = VLDL + LDL alone; B = VLDL + LDL plus flavonoid extract (1 : 1000 (v : v) final dilution). Reaction monitoring was terminated at 180 min.

TABLE I Effects of citrus flavonoid supplementation on VLDL

	VLDL + LDL oxidation *in vitro*	
	Lag time (min)	Propagation rate (pMoles/min/mg cholesterol)
Pre-placebo	70 ± 22	1.5 ± 0.4
Post-placebo	74 ± 13	2.0 ± 0.4
Pre-flavonoid	68 ± 15	1.8 ± 0.3
Post-flavonoid	73 ± 16	1.7 ± 0.4

Values are means ± SD for 20 subjects. Pre and post supplement values were not significantly different for either the placebo or flavonoid group (paired Student's *t*-test).

DISCUSSION

Flavonoid supplementation of Type II diabetic women did not change susceptibility of VLDL + LDL to oxidation. This result was not due to the lack of assay sensitivity in our laboratory, nor due to widely fluctuating oxidation values in each individual. The latter conclusion is based on the very low percent changes per individual for the two blood draws. In regard to the former, our laboratory has detected changes in lipoprotein oxidation values in recent studies of humans and rats.[12,13]

The lack of a flavonoid ingestion effect occurred even though the supplement strongly inhibited lipoprotein oxidation *in vitro*. The difference seemed not to result from an inability of the supplemented flavonoids to reach the plasma. In fact, plasma citrus flavonoid concentrations in supplemented subjects rose to values similar to those which completely inhibited VLDL + LDL oxidation *in vitro* (Figures 3 and 4). This rise did not likely reflect a short-lived flavonoid accumulation. Based on a human study of the citrus flavonoid quercetin,[14] near peak plasma flavonoid levels can last for over 10 h after even one episode of flavonoid ingestion. Moreover, levels can stay above baseline for several days.[14] Therefore, in our study, where there were multiple ingestions, a very sustained elevation likely occurred. Still, this elevation seemed not to affect events *in vivo* which can affect lipoprotein oxidation assessed *in vitro*. Any potential effects of the supplemented flavonoids would have to have occurred *in vivo*. There was little, if any, carry over of the plasma citrus flavonoids into the oxidation assay.

Possibly, flavonoid inhibition of lipoprotein oxidation *in vitro*[2–4] (Figure 4) involves flavonoid binding to the oxidizing agent used *in vitro*, such as copper ions. Flavonoids can chelate copper.[15] This action may contribute to flavonoid inhibition of lipoprotein oxidation *in vitro*, but might not be of any help *in vivo*. There, plasma copper is generally not in the free ionic state used to oxidize lipoproteins *in vitro*.[16]

The lack of flavonoid supplementation effect on VLDL + LDL oxidation seen here may or may not have been duplicated if changes were made to the dose, supplementation time period, subject type, or flavonoid type. However, with regard to time period, it could be argued that 3 weeks was sufficient to see changes. Two weeks or less of some treatments reduce lipoprotein susceptibility to oxidation.[17–19] However, this

does not rule out that the particular subject treatment or subject type used here require longer times to obtain changes. As far as flavonoid type, some recent work suggests that the present results may be typical. Chronic intake by rats of the tea flavonoid catechin did not alter lipoprotein oxidation tendencies (DiSilvestro, R.A. and Blostein-Fujii, A., unpublished results).

The flavonoid dose used here (about 1 g/day) should have substantially increased the subjects' flavonoid consumption. By old estimates of dietary flavonoid intake,[20] the supplement could double typical intakes. Moreover, using a recent report on Dutch diets,[6] the supplement dose would far exceed usual dietary intakes (< 50 mg/day). Admittedly, the recent estimate may be low since it was derived from only five flavonoids. However, even if the estimate is raised several fold, the supplement would still have greatly increased flavonoid consumption.

This supposition is evidenced by a lack of a noticeable plasma citrus flavonoid peak in Figure 2A (no supplement) compared to the definite peak in Figure 3 (supplemented). This peak occurred even though some ingested flavonoid was likely metabolized to other compounds. Support for this contention was given by the broad absorption peak for plasma from supplemented subjects compared to a narrower peak for plasma with flavonoid added *in vitro*.

To our knowledge, this is the first report on human consumption of a flavonoid supplement with regard to lipoprotein oxidation. However, in another recent paper,[21] similar results to ours are reported for consumption of tea, a rich source of certain flavonoids. In both the current and former work, there was no effect on lipoprotein oxidation. In both cases, this conclusion was based entirely on events initiated *in vitro*. It is customary to assume that differences observed for these events reflect differences in blood donor vulnerability to lipoprotein oxidation.[7,8] However, it may be noteworthy that in both the present and recent studies,[21] little ingested flavonoid seemed to be associated with the isolated lipoprotein used to assess oxidation *in vitro*. Therefore, it might be speculated that high intake of citrus or tea flavonoids may affect lipoprotein oxidation *in vivo* in a manner not affecting the oxidation measurements taken *in vitro*.

In summary, this study found that increased flavonoid consumption does not necessarily alter susceptibility of lipoproteins to oxidation.

Acknowledgement

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