Citrus fruit supplementation reduces lipoprotein oxidation in young men ingesting a diet high in saturated fat: presumptive evidence for an interaction between vitamins C and E in vivo¹⁻³

Dror Harats, Shlomit Chevion, Menahem Nahir, Yehudit Norman, Oded Sagee, and Elliot M Berry

ABSTRACT  To determine the effects of vitamin C on cardiovascular risk factors, we studied dietary vitamin C enrichment in 36 healthy male students consuming a diet high in saturated fatty acids. After a 1-mo run-in period during which the subjects consumed 50 mg ascorbic acid/d (low-C diet), half of the subjects were randomly assigned to receive 500 mg ascorbic acid/d for an additional 2 mo (high-C diet). Plasma ascorbic acid increased from 13.5 μmol/L with the low-C diet to 51.7 μmol/L with the high-C diet. Plasma cholesterol increased slightly with the high-C diet, but not above baseline concentrations. This increase was offset by an increase in the lag period of in vitro LDL oxidation, which correlated with plasma ascorbic acid concentrations (r = 0.735, P = 0.0012). Lipoprotein vitamin E concentrations were unchanged with the two diets. There were no effects on concentrations of fibrinogen or factor VII. The fact that ascorbic acid reduced the in vitro susceptibility of lipoproteins to oxidation provides presumptive evidence for an interaction between aqueous and lipophilic antioxidants (vitamins C and E) in maintaining the integrity of LDL particles. Am J Clin Nutr 1998;67:240–5.

KEY WORDS  Citrus fruit, lipoprotein oxidation, fibrinogen, vitamin E, ascorbic acid, antioxidant, low-density lipoprotein, vitamin C, cardiovascular risk, men

INTRODUCTION

Vitamin C, or L-ascorbic acid, is water soluble and is present in its deprotonated state under most physiologic conditions. It is considered to be the most important antioxidant in extracellular fluids (1) and also has many roles as an intracellular antioxidant (2). As an antioxidant, vitamin C donates electrons in both intracellular and extracellular reactions; it is well suited for this role because its free radical intermediate, semidehydroascorbic acid, is nonreactive compared with other free radicals and thus chain reactions are prevented (3). Vitamin C has both enzymatic and chemical functions. It is a cofactor for several enzyme reactions involved in collagen, catecholamine, carnitine, and amino acid metabolism, in which it acts as an electron donor. In many cases vitamin C transfers single electrons to transitional metals such as copper.

Epidemiologic studies have shown a significant association between vitamin C intake and protection against cardiovascular mortality. On univariate analysis of different cardiovascular risk factors, including vitamins, and age-specific ischemic heart disease mortality in 12 countries with cholesterol concentrations, the \( r^2 \) values found for vitamin C and vitamin E (lipid standardized) were 0.41 (\( P = 0.03 \)) and 0.73 (\( P = 0.0004 \)), respectively (4). In this study these factors were stronger predictors of mortality than cholesterol, smoking, or blood pressure, whereas other antioxidants such as vitamin A, carotene, and selenium were not found to be significant. In a 20-y follow-up study, analysis of mortality in patients in the United Kingdom aged > 65 y showed that vitamin C status, whether measured by diet or plasma concentrations, was strongly inversely related to subsequent risk of death from stroke, but not from coronary artery disease (5). A different study in Switzerland found protection against stroke as well (6). However, in another epidemiologic study the inverse relation between angina and plasma vitamin C concentrations was substantially reduced after adjustment for smoking (7). Furthermore, there are some indications that vitamin C concentrations are inversely correlated with the hemostatic factors fibrinogen and factor VIIc (8). Thus, the interrelations between vitamin C and the different manifestations of arteriosclerotic cardiovascular disease are neither clear nor predictable.

The issue of antioxidant supplementation in the modification of cardiovascular risk factors is controversial (9, 10). In particular, it is unclear whether enrichment should be from natural foods or from supplementation with tablets in pharmacologic doses. The dosages of vitamin E found to be protective for heart disease could not be achieved by diet alone (11). Short-term supplementation with vitamin C (1 g/d) for 2 wk had a protective effect on in vivo oxidation of LDL after smoking as measured by the content of thiobarbituric acid-reactive substances in isolated LDL (12). Less is known about the effects of supplementation for longer periods on the kinetics of LDL oxidation (13).

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Citrus fruits, together with olive oil, are an integral part of the Mediterranean diet. We have already investigated the biological effects of monounsaturated fatty acids on lipoprotein concentrations and their susceptibility to oxidation (14, 15). Ascorbic acid has been shown to protect against LDL oxidation in vitro (16, 17), but few if any studies have investigated its effects in vivo after supplementation. Such studies could help elucidate the biological interactions among the different antioxidants. The in vitro work suggests that vitamin C, although absent from the LDL molecule, may nevertheless protect it from oxidation by recycling tocopherols, in accord with the work of Packer et al (18). To test this hypothesis, we studied the effects of citrus fruit supplementation on blood lipid and hemostatic indexes in a group of normolipidemic subjects consuming a diet high in saturated fatty acids, which mimicked the composition of the diet of northern Europe.

SUBJECTS AND METHODS

The experimental design of the study was similar to that described previously (14, 15). A metabolic kitchen was set up on a yeshiva campus and provided meals under the instruction of a trained dietitian. Thirty-six male student volunteers who were not suffering from any endocrine diseases or taking medication were recruited; none of the subjects had hyperlipidemia. More than 90% were nonsmokers and alcohol consumption was minimal (less than three units per week per person). No subject changed his exercise habits during the 3-mo study period. The research protocol was approved by the local Helsinki committee and informed consent was obtained from each participant.

Ten different basic menu plans were prepared, with a range of nutrient composition as shown in Table 1. All subjects ate this diet, which contained ≈50 mg vitamin C, for 1 mo (run-in period). Thereafter, they were randomly assigned to two groups according to body mass index and vitamin C and cholesterol concentrations, as shown in Table 2. One group (low-C) continued consuming the same diet whereas the other (high-C) received additional vitamin C from the juice of freshly squeezed (automatic squeezer) oranges drunk within 10 min of preparation. The estimated vitamin C content of the high-C group diet was 500 mg/d. The study was concluded after an additional 2 mo. Weight was recorded every 2 wk and in 85% of the subjects weight was not altered by >1 kg. Blood samples were drawn periodically throughout the experimental period for measurement of plasma lipid and ascorbate concentrations. At the end of the study, blood samples from each group (seven from the high-C and six from the low-C group) were selected randomly for measurement of lipoprotein oxidation. Concentrations of fibrinogen and factor VII were also measured in the two groups.

Laboratory measurements

Plasma cholesterol and triacylglycerol were determined by an enzymatic procedure in a batch analyzer (Vitalab; Vital Scientific, Diesen, Netherlands). HDL cholesterol was determined after precipitation of lipoproteins containing apolipoprotein B with phosphotungstic acid. LDL concentrations were obtained by subtraction with use of the equation by Friedewald et al (19).

Oxidation was induced after an overnight dialysis of lipoproteins against phosphate-buffered saline. The lipoprotein content of conjugated dienes was measured at 234 nm without lipid extraction by using a modification of the procedure described by Esterbauer et al (20). LDL oxidation was measured in the absence of any ascorbic acid. The lag time and the kinetics of LDL oxidation were determined by monitoring the changes in absorbance measured at 234 nm, observed at room temperature (23 °C), of freshly isolated LDL (50 mg protein/L). LDL–treated groups after incubation of LDL with 15 μmol CuSO₄/L. Absorbance was recorded every 10 min for 3 h.

Vitamin E concentrations were determined by HPLC with a C₁₈, 10-m reversed-phase column, according to a modification of the method of Harats et al (21). To 0.2 mL serum or 50 μg LDL was added 2 μg α-tocopherol acetate (internal standard) and 2 mL ethanol containing 50 mmol butylated hydroxytoluene/L and 4 mL hexane and the mixture vortexed vigorously. Then 2 mL H₂O was added and the mixture was vortexed again and centrifuged (1000 g for 5 min at 5 °C) for phase separation. The upper phase was aspirated and evaporated under N₂. The samples were redissolved in methanol:water (99:1, by vol), which also served as the mobile phase.

Ascorbate was quantified by HPLC with electrochemical detection after the method of Motchnik et al (22). Fibrinogen and factor VII were measured by using laboratory kits from the Instrumentation Laboratory (Milan, Italy) (23) and Baxter Diagnostics (Deerfield, IL) (24).

Statistical analysis

<table>
<thead>
<tr>
<th>TABLE 1 Composition of the 10 basic diets and snack supplements used in the experimental protocol</th>
</tr>
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<tbody>
<tr>
<td>Basic diet</td>
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<tr>
<td>-----------------------------------------------</td>
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<tr>
<td>Energy (kJ)</td>
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<tr>
<td>Protein (% of energy)</td>
</tr>
<tr>
<td>Carbohydrates (% of energy)</td>
</tr>
<tr>
<td>Fat (% of energy)</td>
</tr>
<tr>
<td>Saturated fatty acids (% of energy)</td>
</tr>
<tr>
<td>Monounsaturated fatty acids (% of energy)</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids (% of energy)</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
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</table>

<sup>a</sup>Range.
were no significant changes from baseline values. However, at the end of the experiment, cholesterol and LDL values were higher in the high-C group. In both groups, cholesterol and LDL concentrations decreased after the 1-mo run-in period of the high–saturated fatty acid diet (1 mo time point compared with baseline), perhaps because of the decrease in cholesterol content of the habitual diet and the effect of entering into the discipline of the experimental regimen. There were no changes in triacylglycerol or HDL concentrations throughout the study.

Ascorbate concentrations changed significantly throughout the experiment in the high-C group, as shown in Figure 1. The oxidation of LDL was measured in 10 subjects from each group at the end of the 2-mo supplementation period. Citrus fruit supplementation significantly increased the lag time of LDL oxidation from 70.3 ± 6.1 min in the low-C group to 102.4 ± 7.4 min in the supplemented group (P = 0.0034). The correlation between vitamin C concentrations and the lag time was 0.735 (P = 0.0012). This is shown in Figure 2. The protection of LDL was independent of changes in vitamin E status, as shown in Table 4. There were no significant differences in concentrations of fibrinogen and factor VII between the two groups.

**DISCUSSION**

Vitamin C is one of the major water-soluble antioxidants. The interesting finding of this study was that dietary enrichment with vitamin C increased the lag time of lipophilic lipoprotein oxidation, suggesting that this protection was through a restoration of tocopherol to the reduced state (6). Fuller et al (25) showed similar results recently in smokers by using ascorbate supplementation by tablet. These studies provide presumptive evidence for a biologically significant interaction between vitamin E and vitamin C in normolipidemic subjects. The fact that the increase in lag time correlated with ascorbic acid concentrations suggests that the effect is dose dependent. To confirm this, it would be necessary to measure

**TABLE 2**
Baseline characteristics of the study population \(^1\)

<table>
<thead>
<tr>
<th></th>
<th>Low-C ((n = 17))</th>
<th>High-C ((n = 19))</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI ((kg/m^2))</td>
<td>22.4 ± 2.21</td>
<td>22.6 ± 2.07</td>
</tr>
<tr>
<td>Cholesterol ((mmol/L))</td>
<td>3.79 ± 0.61</td>
<td>3.75 ± 0.47</td>
</tr>
<tr>
<td>Vitamin C ((\mu mol/L))</td>
<td>21.15 ± 2.5</td>
<td>24.46 ± 3.19</td>
</tr>
</tbody>
</table>

\(^1\) \(\bar{x} \pm \text{SEM}\). Low-C, received \(\approx 50\) mg ascorbic acid/d for the entire study period; high-C, received \(\approx 500\) mg ascorbic acid/d for the entire study period; high-C, received \(\approx 50\) mg ascorbic acid/d for 1 mo and then \(\approx 500\) mg ascorbic acid/d for the next 2 mo.

**RESULTS**

The average age of the participants was 19.8 y (range: 18–23 y). Body weight in the two groups did not change significantly during the experiment. The mean weight of the low-C group was 70.7 ± 8.7 kg at the start compared with 71.2 ± 8.9 kg at the end of the study \((P = 0.125, \text{paired } t \text{ test})\); for the high-C group, mean weight was 73.9 ± 7.4 kg at the start compared with 74.8 ± 7.7 kg at the end of the study \((P = 0.139)\).

Changes in plasma lipoproteins are shown in Table 3. After 3 mo of consuming the diets high in saturated fatty acids, there were no significant changes from baseline values. However, at the end of the experiment, cholesterol and LDL values were higher in the high-C group. In both groups, cholesterol and LDL concentrations decreased after the 1-mo run-in period of the high–saturated fatty acid diet (1 mo time point compared with baseline), perhaps because of the decrease in cholesterol content of the habitual diet and the effect of entering into the discipline of the experimental regimen. There were no changes in triacylglycerol or HDL concentrations throughout the study.

**TABLE 3**
Plasma lipid values and reduced ascorbate in response to vitamin C–enriched diets \(^1\)

<table>
<thead>
<tr>
<th></th>
<th>0 mo (baseline)</th>
<th>1 mo</th>
<th>3 mo</th>
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</thead>
<tbody>
<tr>
<td>Cholesterol ((mmol/L))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-C</td>
<td>4.21 ± 0.60</td>
<td>3.65 ± 0.56</td>
<td>3.74 ± 0.58</td>
</tr>
<tr>
<td>High-C</td>
<td>4.16 ± 0.53</td>
<td>3.71 ± 0.38(^2)</td>
<td>4.14 ± 0.65</td>
</tr>
<tr>
<td>Triacylglycerol ((mmol/L))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-C</td>
<td>1.05 ± 0.36</td>
<td>0.97 ± 0.49</td>
<td>1.08 ± 0.46</td>
</tr>
<tr>
<td>High-C</td>
<td>1.13 ± 0.43</td>
<td>1.14 ± 0.90</td>
<td>1.15 ± 0.46</td>
</tr>
<tr>
<td>LDL ((mmol/L))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-C</td>
<td>2.31 ± 0.52</td>
<td>1.95 ± 0.39</td>
<td>2.13 ± 0.56</td>
</tr>
<tr>
<td>High-C</td>
<td>2.52 ± 0.46</td>
<td>2.02 ± 0.45(^2)</td>
<td>2.61 ± 0.76(^2)</td>
</tr>
<tr>
<td>HDL ((mmol/L))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-C</td>
<td>1.28 ± 0.28</td>
<td>1.19 ± 0.38</td>
<td>1.12 ± 0.28</td>
</tr>
<tr>
<td>High-C</td>
<td>1.17 ± 0.34</td>
<td>1.24 ± 0.35</td>
<td>1.12 ± 0.29</td>
</tr>
<tr>
<td>Vitamin C reduced ((\mu mol/L))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-C</td>
<td>21.15 ± 2.5</td>
<td>13.16 ± 1.90</td>
<td>13.05 ± 2.45</td>
</tr>
<tr>
<td>High-C</td>
<td>24.46 ± 3.19</td>
<td>13.49 ± 1.98</td>
<td>51.71 ± 3.05(^4)</td>
</tr>
</tbody>
</table>

\(^1\) \(\bar{x} \pm \text{SEM}\). Supplementation was introduced after 1 mo of the low–vitamin C diet. Low-C, received \(\approx 50\) mg ascorbic acid/d for the entire study period; high-C, received \(\approx 50\) mg ascorbic acid/d for 1 mo and then \(\approx 500\) mg ascorbic acid/d for the next 2 mo.

\(^2\) Significantly different from 0 mo (two-factor, repeated-measures ANOVA): \(^3\) \(P < 0.05\), \(^4\) \(P < 0.01\).

\(^3\) Significantly different from 1 mo (two-factor, repeated-measures ANOVA): \(^3\) \(P < 0.05\), \(^4\) \(P < 0.01\).
the oxidized and reduced states of vitamin E, which might not be represented by a change in the total vitamin E concentration (26).

Vitamin C in doses of 0.5–1.0 g/d may decrease total cholesterol under certain conditions, eg, in subjects with hyperlipidemia or in subjects with an initial marginal ascorbic acid intake (27, 28). However, in some studies ascorbic acid supplementation was reported to be neutral in relation to total cholesterol in subjects with plasma concentrations of ascorbic acid > 41 μmol/L (29–31). The relation between vitamin C and HDL concentrations is variable, with reports of a positive correlation in the elderly, shown clearly in men but not so clearly in women; reports of effects depending on plasma ascorbic acid status (32, 33); and finally reports of an inverse relation (34); summarized by Trout (9). Some studies showed no correlation between vitamin C status and heart disease in women (35), whereas others did (36).

The finding that vitamin C status may be correlated with plasma cholesterol was noted in a previous study in which cholesterol (and perhaps saturated fatty acid) intake was high (37). Ginter (38) claimed that ascorbic acid was necessary for the conversion of cholesterol to bile salts by activating 7α-hydroxylation, and thus that when ascorbic acid was deficient, the cholesterol concentration was high (38). He and his colleagues also showed that with a similar high-fat diet, supplementation with 300 mg ascorbic acid/d lowered cholesterol concentrations predominantly in hypercholesterolemic subjects (39). A more recent study confirmed this observation (40); vitamin C in amounts of 1.0 g/d was given to patients with type 2 diabetes and hyperlipidemia over a period of 4 mo. Total and LDL cholesterol were reduced and the percentage increase in plasma ascorbic acid correlated with the percentage decline in LDL (r = 0.44) (40). Our results showed a small increase in total and LDL cholesterol in the citrus-treated group. The values were all within the normal range despite being on a background of a diet high in saturated fatty acids. These differences, therefore, may be of statistical rather than biological significance.

Attempts to reconcile these variations in the results from different studies are complicated by several factors: populations; background diet, in particular the amounts of other antioxidants and their balance with the polyunsaturated fats in the diet; and the manner in which the vitamin C is added, whether from natural (food) sources or by tablet supplementation. Also, we found no differences in fibrinogen and factor VII between the high-C and low-C groups. Thus, we were unable to confirm the inverse relation found in elderly subjects by Khaw and Woodhouse (8).

Concentrations of ascorbic acid in the present study were in the range found in the literature (41). The dietary enrichment resulted in concentrations considered to maximize protection from ischemic heart disease (40–50 μmol/L) (42). Note that dose responses for diet-plasma vitamin C concentrations are not known, but are the subject of several ongoing studies (43). The plasma concentrations reached in response to the two diet regimens, representing a 10-fold dietary difference, support a relatively slow saturation curve. With the 50-mg/d low-C diet for 3 mo, plasma concentrations stayed just above the lower limit of normal at 10 μmol/L (41).

### TABLE 4

Concentrations of fibrinogen, factor VII, and vitamin E after 2 mo of supplementation with citrus fruit

<table>
<thead>
<tr>
<th></th>
<th>Low-C (n = 17)</th>
<th>High-C (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen (mg %)</td>
<td>155.0 ± 6.23</td>
<td>163.5 ± 7.41</td>
</tr>
<tr>
<td>Factor VII (× 10^3 U/L)</td>
<td>1.098 ± 0.031</td>
<td>1.031 ± 0.042</td>
</tr>
<tr>
<td>Plasma vitamin E (mg/L)</td>
<td>11.6 ± 0.9</td>
<td>12.2 ± 1.3</td>
</tr>
<tr>
<td>Vitamin E in LDL (mg/g LDL protein)</td>
<td>3.9 ± 0.4</td>
<td>4.3 ± 0.5</td>
</tr>
</tbody>
</table>

1 ± SE. Low-C, received <50 mg ascorbic acid/d for the entire study period; high-C, received 50 mg ascorbic acid/d for 1 mo and then <500 mg ascorbic acid/d for the next 2 mo. There were no significant differences between groups.

2 Vitamin E status was measured in only six subjects in the low-C group and seven in the high-C group.
The current recommended dietary allowance for vitamin C is 60 mg/d for adults (44), but 20–30% of US adults fail to reach this intake (45); furthermore, the recommendations for consuming five servings of fruit and vegetables, which would provide > 200 mg vitamin C/d, are also poorly adhered to. The Paleolithic diet has been estimated to have contained ≈350 mg vitamin C/d (46); amounts > 500 mg/d are difficult to obtain from the diet alone without supplementation, and may also be harmful to patients with hyperoxaluria and a tendency to form oxalate kidney stones (47). It is not known whether such adverse reactions also occur with natural supplementation (48). This study showed that high concentrations of ascorbic acid can be obtained with citrus fruit supplementation and that these high concentrations have beneficial effects on lipoprotein oxidation on the background of a diet high in saturated fatty acids.

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