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In Vitro and *In Vivo* Lipoprotein Antioxidant Effect of a Citrus Extract and Ascorbic Acid on Normal and Hypercholesterolemic Human Subjects

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Abstract

Polyphenols and particularly flavonoids are well known *in vitro* antioxidants. Their consumption in foods has been shown to decrease the risk of heart disease in epidemiological studies. Because flavonoids are consumed with vitamin C in the diet, the combination may prove to be more beneficial than either alone. The combination of citrus extract and vitamin C was found to produce a synergistic antioxidant effect in an in vitro lipoprotein oxidation model. In a double-blind, placebo-controlled study with 26 normal and hypercholesterolemic subjects, the citrus extract and vitamin C, but not vitamin C or vitamin E alone, significantly lowered triglycerides. The combination of citrus extract and vitamin C increased the lag time of lipoprotein oxidation, compared with vitamin C alone or a placebo, and was a significantly better antioxidant than vitamin E. These results and other published studies are highly suggestive of *in vitro* and *in vivo* antioxidant synergism between citrus extract and vitamin C.

Introduction

Fruits and vegetables are known from epidemiological studies to be beneficial in decreasing the risk of heart disease.[1,2] It has been thought that the antioxidant vitamins C, E and β-carotene are the active ingredients in these foods. However, the consequences of dietary intakes of these antioxidants are difficult to separate by epidemiological studies from these of other important constituents such as flavonoids.[3] Flavonoids and the larger class of compounds known as polyphenols are ubiquitous in vegetables and fruits, are well known antioxidants,[4,5] and have been shown to enrich low-density lipoproteins (LDL), analogous to vitamin E.[6] These compounds also were shown to be protective against heart disease in recent epidemiological investigations.[7,8] However, the only large human trial with a large dose of citrus bioflavonoids found no decrease in lipoprotein oxidation susceptibility.[9] The latter is the ultimate test for an antioxidant to act *in vitro* in order to protect against heart disease, according to the oxidative theory of atherogenesis.[10] Flavonoids and vitamin C are consumed together in many foods, especially in citrus fruits, and are also a popular combination in supplements. We therefore decided to investigate this combination in a human supplementation study.

Materials and Methods

Chemicals and supplements

Pure ascorbic acid and DL-α-tocopherol, reagent grade, were used. Citrus extract (CE), a tan powder from Grow Company, Inc., Hackensack, New Jersey, was a 30% alcohol/water extract of the whole citrus fruit, bitter orange (*Citrus aurantium*). The polyphenol content was 565μmol/g as measured by the Folin-Ciocalteu reagent (Sigma company, St. Louis, MO) with catechin as the standard. Citrus extract with vitamin C (CEC) contained 579μmol/g polyphenols from CE and 25% ascorbate (1390μmol/g). The supplement used for the human study (CEC) was Re-Natured Vitamin C supplied by Grow Co. For human consumption Grow Company's CEC was formulated into a tablet (FOOD FORM C, Intracell Nutrition, Fort Lee, NJ), and each tablet contained 330mg of vitamin C and 900mg of CE. Vitamin E (400IU capsules) and vitamin C (500mg tablets) were from Leiner Health Products, Carson, CA. The placebo tablets were alfalfa (500mg/tablet) from General Nutrition Company, Pittsburgh, Pennsylvania.

In vitro *oxidation studies*

Pooled normal plasma was used for the oxidation under standard conditions.[5] Low-density plus very-low-density lipoproteins (LDL + VLDL) were isolated from thawed plasma with an affinity column (LDL-Direct Cholesterol,* Isolab Inc., Houston, TX). The protein in LDL + VLDL was measured by using Comassie blue reagent (Sigma) with human albumin as the standard. An aliquot of LDL + VLDL protein to equal 70μg/ml in 400μl total volume was added to a screw-capped plastic tube. CE alone, C alone and CEC alone were dissolved in 50% methanol/water and added at concentrations ranging from 0.125 to 10μM (CE and CEC as polyphenol concentration) to the tube. Also, C and CE were added together at equimolar concentrations (C & CE). Then 10mM phosphate-buffered saline (PBS) from Sigma and 10μl of 1mM cupric acetate were added to initiate oxidation. A native sample was processed with no added cupric ion. The tubes were closed and incubated at 37°C for 6 hours, after which the fluorescence of the thiobarbituric acid-reactive substances were measured in a butanol extract. All samples were processed in duplicate. The concentration necessary to inhibit oxidation by 50% compared with the control (IC_{50}) was calculated graphically after the native contribution was subtracted.

Subjects and study design

Twenty-six subjects (11 women and 15 men; age, 53±10 years; weight 77±16 kg) participated in this study with informed consent after approval of the protocol by The University of Scranton Institutional Research Board. All subjects were non-smokers and did not take drugs, vitamins or antioxidant supplements, other than those taken during the study. Each subject had a fasting baseline blood sample measurement in heparin tubes and then was randomly assigned to receive either placebo (2 tablets / day), vitamin C (2 x 500mg / day), vitamin E (2 x 400IU / day) or CEC (3 x 330mg of C and 3 x 900mg of CE). Subjects consumed the supplements for a period of 2 months and then returned for another fasting blood measurement. A 2 month washout period ensued, followed by a third sampling. The subjects were then randomly assigned to receive another of the four supplements for 2 months, followed by a final sampling. Each plasma sample was divided into two portions and stored at -80°C until it was analysed.

Analytical methods for plasma analysis

Concentrations of cholesterol and triglycerides were determined with the use of enzyme kits from Sigma. High-density lipoprotein concentration (HDL) was determined as cholesterol by the affinity column. LDL was calculated by the Friedenwald equation. Immediately before the oxidation, LDL + VLDL was isolated from thawed plasma with the affinity column and the protein was measured as described. VLDL + LDL was added to a quartz cuvette at a final concentration of 70μg/ml in 2ml total volume. After PBS addition and pre-incubation for 5 minutes in a 37°C shaking water bath, 50μl of 1M cupric acetate was added to initiate oxidation. The resulting fluorescence was read every 15 minutes at 360nm excitation and 430nm emission and compared with a PBS blank.[11] Lag times were determined from the intersection of the initiation and propagation phase linear graphs of fluorescence versus time.

Results

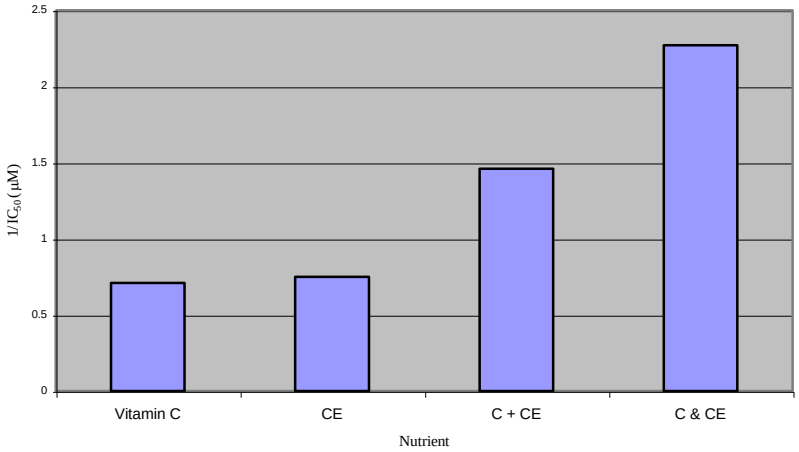
In vitro *oxidation*

For comparison purposes and to determine synergism, the values for $1/IC_{50}$ were calculated (Fig. 1). A higher value of $1/IC_{50}$ indicates a higher-quality antioxidant or antioxidants. The CE containing only polyphenol antioxidants (0.75μM) was similar to vitamin C (0.71μM) and had better-quality antioxidants compared with pure flavonoid kaempferol (0.55μM) or with hesperidin (hesperetin rutinoside from citrus fruits, 0.07μM)

* Note: this pre-packed column is no longer available, but columns can be made using 1ml of Heparin-Agarose resin from Sigma

and vitamin E (0.42μM).[5] The equimolar combination of C and CE was synergistic compared with the calculated sum of the individual effects of C and CE.

Fig. 1. Inhibition on *in vitro* cupric ion-induced LDL + VLDL oxidation by antioxidants alone and when combined in equimolar concentration (C & CE), as measured by $1/IC_{50}$. IC_{50} concentration required to inhibit oxidation by 50%.
C = vitamin C; CE = citrus extract without C; C + CE = calculated sum of C and CE.



Supplementation study
The lipid results for the subjects are shown in Table 1. There was no significant difference between the baseline values and values obtained after washout, indicating that the washout was successful. The only supplement to influence any lipid parameter was CEC, which significantly decreased plasma triglycerides (P<0.02). There were ten subjects in this arm of the study, and seven had hypertriglyceridemia (i.e., >21.8mM). Of those seven, six experienced a decrease in triglycerides. Of the three normal subjects, two experienced a decline in triglycerides and one had an increase.

Table 1. Effect of supplementation on human plasma lipids (Mean ± Standard deviation)

Supplement (mM)	Cholesterol	HDL (mM)	LDL (mM)	Triglycerides
Baseline (n=26)	6.74 ± 1.29	1.32 ± 0.34	4.38 ± 1.14	21.3 ± 8.3
After washout	6.58 ± 1.21	1.22 ± 0.31	4.40 ± 1.42	20.8 ± 12.3
Before placebo (n=8)	6.22 ± 1.27	1.34 ± 0.34	3.76 ± 0.83	25.5 ± 8.0
After placebo	6.53 ± 1.48	1.32 ± 0.31	4.15 ± 1.11	24.1 ± 10.0
Before vitamin C (n=10)	7.23 ± 1.68	1.34 ± 0.28	5.00 ± 1.61	17.0 ± 7.8
After vitamin C	6.63 ± 1.19	1.30 ± 0.28	4.51 ± 1.61	17.7 ± 9.0
Before vitamin E (n=11)	7.10 ± 1.58	1.35 ± 0.31	3.86 ± 1.11	21.4 ± 13.4
After vitamin E	6.81 ± 1.14	1.24 ± 0.36	4.69 ± 1.04	19.0 ± 5.8
Before CEC (n=16)	6.09 ± 0.91	1.19 ± 0.28	3.89 ± 0.96	20.8 ± 10.4
After CEC	6.09 ± 0.75	1.22 ± 0.28	4.04 ± 1.06	17.4 ± 8.7[a]

[a]Significantly different from the “before” value.

Results for the lipid oxidation parameters are displayed in Table 2. There were no significant changes in the slope of the propagation curve for any of the supplements. CEC and vitamin E produced the only significant change in lag time (P<0.02). In Fig. 2 are shown the lag time results for the eight subjects who followed CEC supplementation

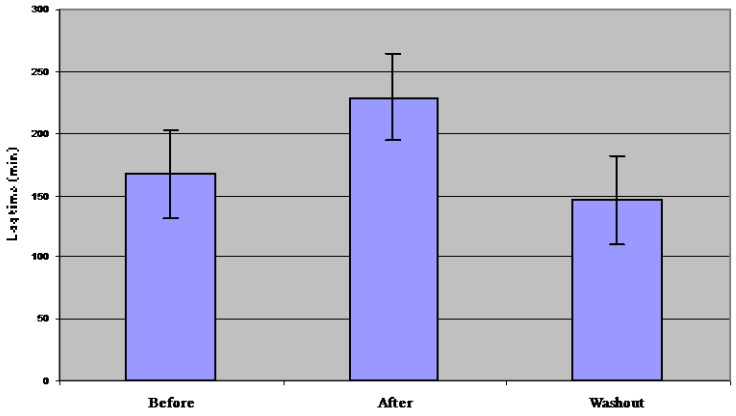
with a washout period. The supplementation produced an average 37% increase in lag time (P<0.005), and the washout significantly decreased the lag time by 36% (P<0.005).

Table 2. Effect of citrus extract supplementation on lipoprotein oxidation (Mean ± Standard deviation)

Supplement	Propagation Slope (abs/min x 10)	Lag time of propagation phase (min)	Change in lag time (min)
Before placebo	0.225 ± 0.106	192 ± 51	
After placebo	0.252 ± 0.112	196 ± 50	2 ± 31
Before vitamin C	0.274 ± 0.163	171 ± 47	
After vitamin C	0.222 ± 0.113	191 ± 47	21 ± 49
Before vitamin E	0.217 ± 0.129	160 ± 36	
After vitamin E	0.265 ± 0.093	174 ± 44[a]	18 ± 15
Before CEC	0.233 ± 0.109	168 ± 56	
After CEC	0.204 ± 0.123	203 ± 56[a]	49 ± 37[b]

[a]Significantly different from the respective "before" value.
[b]Significantly different from placebo or vitamin E.

Fig. 2. Effect of citrus extract with vitamin C (CEC) supplementation on LDL + VLDL oxidizability.



Discussion

In vitro *oxidation*

The synergism found in our *in vitro* study was not caused by a chelation effect, because the flavonoids were present at 1.4μM and cupric ion at 25μM when the oxidation was inhibited by 50%. It is well known that ascorbate can assist in the regeneration of the monophenol, vitamin E, from its phenoxyl radical, resulting in a synergistic *in vitro* antioxidant effect.[12] The first example of a flavonoid synergism was between rutin and ascorbate, and rutin with vitamin E.[13] It was later shown that ascorbate spared the flavonoid myrigaline B in the presence of LDL oxidized by cupric ion.[14] Therefore, we believe that the ascorbate, which was present in our model and in the supplement in excess, spared the citrus polyphenols from oxidation or regenerated the phenoxy radicals formed by oxidation of the polyphenols or both.

Lipids

Among the 26 subjects, there were 7 who had <5.96 mM cholesterol and who would be classified as normocholesterolemic; the remainder were hypercholesterolemic. The results of these two groups of subjects were combined because there was no significant

difference between them in biochemical response after supplementation. There was no significant change in weight (i.e., ±5 kg or more) or in plasma cholesterol in any of the subjects that might have influenced the oxidative resistance of the lipoproteins. CEC was previously included as a supplement in a high-cholesterol diet used in a hamster study.[15] In that animal study, CE or vitamin C alone was without effect on triglycerides on cholesterol. However, the combination product CEC did lower triglycerides and cholesterol and was therefore synergistic *in vivo* in the hamster model of atherosclerosis. In our present human study, vitamin C and vitamin E were without effect, and only the CEC lowered triglycerides. However, the dose of CEC on a body-weight basis was much lower in the human study compared with the hamster study, in which 4% of the diet was given as CE.

Lipoprotein oxidation

Vitamin C at a dose of 1000mg/day in our study had no significant effect on LDL + VLDL oxidizability. This was the same dose of C given in a previous human study, which measured LDL susceptibility to oxidation.[16] The negative *in vivo* result derived from the fact that ascorbic acid is hydrophilic and does not bind to lipoproteins. We have also found that vitamin C added to plasma does not change the lag time of LDL + VLDL subsequently isolated and oxidised.[17]

We also corroborated earlier results with vitamin E with respect to the lag time. Princen *et al*.[18] found that supplementation with 800 IU/day of vitamin E produced a 28% increase in the lag time of LDL oxidation. Our study, using the same dose of E, found a significant 9% increase after supplementation and a 25% decrease after washout (data not shown). In this study, the CEC was found to be a significantly better *in vivo* antioxidant than this high dose of vitamin E.

We decided to investigate subjects with high cholesterol because there is some evidence indicating that high cholesterol, especially high LDL cholesterol in subjects with heart disease is associated with an increase in oxidizability.[19] Most recently, a 3-week supplementation with 1000mg/day of another commercial citrus extract was given to type 2 diabetics, and oxidatively stressed population; however, there was no effect of the flavonoid preparation alone on LDL ± VLDL oxidizability.[9] Another study of normal subjects who consumed a diet high in saturated fat found that 1L of daily consumption of freshly squeezed orange juice (containing 500mg of vitamin C and an estimated 633μmol of polyphenols) increased the lag time of LDL oxidation by 46%.[20] These results confirm those of our study at a similar dose of polyphenols and a lower dose of vitamin C. We found no *in vivo* antioxidant effect with a 1000mg dose of C alone but a significant effect with the combination of vitamin C and a concentrated flavonoid-containing citrus extract (CEC). We did not test the *in vivo* effect of the CE alone because it is not available for human consumption. We demonstrated *in vitro* antioxidant synergism between the vitamin C and citrus flavonoids using the lipoprotein oxidation model. Published results and our own work show that there is *in vivo* synergism with respect to lipoprotein oxidation for either normal- or high-cholesterol subjects.

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