A Citrus Extract plus Ascorbic Acid Decreases Lipids, Lipid Peroxides, Lipoprotein Oxidative Susceptibility, and Atherosclerosis in Hypercholesterolemic Hamsters

Joe A. Vinson,* Sheu-Ju Hu, Sunah Jung, and Ann M. Stanski

Department of Chemistry, University of Scranton, Scranton, Pennsylvania 18510-4626

A citrus extract containing flavonoids and ascorbic acid was used as a supplement to investigate its effect on lipids in hypercholesterolemic hamsters. Ascorbic acid or the flavonoids were without effect except that ascorbate did significantly raise HDL. After 1 month of feeding, the citrus extract plus ascorbic acid synergistically caused a significant reduction of 77%, 66%, and 40% in plasma total cholesterol, LDL + VLDL, and triglycerides, respectively, in comparison to the control group. The extract was also a synergistic inhibitor of in vitro cupric ion LDL + VLDL oxidation compared with ascorbic acid or the flavonoids alone. In a second 10-week hamster study, citrus extract plus ascorbate also significantly lowered plasma lipids, lipid peroxides, and ex vivo LDL + VLDL oxidizability vs a control group. Citrus extract plus ascorbate strongly inhibited atherosclerosis, and there was a significant correlation between several indices of oxidative susceptibility and atherosclerosis.

Keywords: *Lipids; atherosclerosis; flavonoids; lipid peroxidation; hamster*

INTRODUCTION

The importance of hypercholesterolemia, or, more specifically, an elevated low-density lipoprotein (LDL) cholesterol concentration, as a major risk factor for the development of ischemic heart disease is widely accepted. There are several classes of synthetic drugs that are effective hypocholesterolemic agents. The cost and potential side effects of these drugs have led many people to search for natural substances to lower cholesterol.

Within the last several years, the oxidative theory of atherogenesis (Steinberg et al., 1989) has provided another avenue of therapy using antioxidants. In brief, it states that lower density lipoproteins (low-density lipoprotein, LDL, and very low-density lipoprotein, VLDL) oxidized in vivo lead to foam cell production and, ultimately, atherosclerosis. If this hypothesis is correct, then antioxidants should protect lipoproteins against oxidative modification and reduce the biological consequences. Antioxidant phenols such as probucol and tocopherol (vitamin E) have been found to be effective in animal models of atherosclerosis. These compounds incorporate into LDL and decrease ex vivo LDL oxidizability in animals and humans (Jackson et al., 1993). Vitamin E has been recently shown to inhibit atherosclerosis in the vitamin E-deficient moderately hypercholesterolemic hamster without affecting cholesterol. Indeed the aortic lesion size was inversely correlated with plasma tocopherol content (Parker et al., 1995).

Vitamin C, ascorbic acid, is a well-known antioxidant. In plasma it is the major chain-breaking antioxidant against free radicals and protects LDL from oxidation in vitro although it does not bind to LDL (Retsky et al., 1993) and has no effect on atherosclerosis in the vitamin E-deficient hypercholesterolemic hamster (R. Parker, personal communication). Plasma ascorbate concentrations, however, have been inversely correlated with human coronary disease mortality (Gey, 1987).

Flavonoids are a class of some 4000 phenolic compounds that occur ubiquitously in plant foods (0.5−1.5%) and are thus important constituents of the human diet (Harborne, 1988). In general, leaves, flowers, and fruit contain flavonoid glycosides, woody tissues contain aglycones, and seeds may contain either. We have shown these compounds to be powerful antioxidants against cupric ion-induced in vitro LDL + VLDL oxidation (Vinson et al., 1995a) and also to bind to these low-density lipoproteins (Vinson et al., 1995c). A recent Dutch and seven countries epidemiology study has shown increased dietary flavonoid intake to significantly reduce deaths from heart disease (Hertog et al., 1993a, 1995). Recent evidence has shown that these compounds are absorbed in humans from foods (Hollman et al., 1995) and quercetin, a ubiquitous flavonoid, was absorbed in rats (Manach et al., 1995).

There is ample evidence that consumption of fruits and vegetables is protective against heart disease (Ames et al., 1993). Citrus fruits in contrast to many other foods provide both vitamin C and flavonoids. The present study is designed to examine both the antioxidant and lipid-lowering efficacy of a natural ascorbate- and flavonoid-containing citrus extract with both normal hamsters and a hamster model of heart disease.

MATERIALS AND METHODS

Materials. Citrus extract CEC is a light brown powder containing a 30% alcohol−water extract of whole dry ripe citrus fruit, bitter orange *Citrus aurantium*. The milled fruit is extracted 4 times with solvent with 24 h agitation. The solvent is then removed by low vacuum-drying at 60 °C. The

* To whom correspondence should be addressed [telephone (717) 941-7551; fax (717) 941-7510, e-mail vinson@tiger.uofs.edu].

S0021-8561(97)00801-7 CCC: $15.00
Published on Web 04/03/1998

Table 1. Diet Description and Body Weight for 4 Weeks Hamster Supplementation (Study 1)[a]

group	description of diet	initial weight (g)	final weight (g)
normal	rodent chow	139 ± 4	171 ± 15
control	nonpurified diet + 0.2% cholesterol and 10% coconut oil	128 ± 5	169 ± 17
C	control plus 1% ascorbic acid (57 mmol/kg diet)	134 ± 9	170 ± 15
CE	control plus 3% citrus extract (1.74 mM phenols/kg diet)	134 ± 15	166 ± 23
CE+AA	control plus 4% citrus extract and vitamin C (57 mmol/kg diet ascorbic acid and 1.70 mM phenols/kg diet)	128 ± 16	165 ± 17

[a] Values are means ± SD for 10 animals in each group.

final composition after incorporation of ascorbic acid is 25.7% ascorbic acid, 9.9% citrus flavonoids, 31.2% protein, 3.2% ash, and the remainder carbohydrates (Re-Natured Vitamin C in Citrus Fruit Media, Grow Co., Hackensack, NJ). There are four principal flavonoid glycosides in CE+AA which were identified after acid hydrolysis and HPLC (Hertog et al., 1992) as glycosides of quercetin, hesperetin, narigenin, and myricetin.

Citrus extract (CE) is quantitatively identical to CE+AA except that ascorbic acid is absent and the protein content is 53.2%. The total flavonoid phenol concentration in CE and CE+AA is 579 and 565 μmol/g, respectively, as measured by the Folin−Ciocalteu reagent (Sigma Chemical Co., St. Louis, MO) using catechin as a standard. AA in CE+AA was removed by ascorbic acid oxidase (Sigma Chemical Co.) prior to Folin analysis.

Animals and Diets. Male, weanling, Syrian Golden hamsters were received from Charles River Breeding Laboratories (Wilmington, MA) and given commercial (nonpurified) rodent chow (Ralston Purina, St. Louis, MO) for 4 weeks. They were then separated into groups, each with comparable average weights. The animals were housed in plastic cages, 3 or 4 animals/cage with a bedding of wood chips, in a temperature-controlled room (20 °C) and a 12-h light/dark cycle. They were allowed free access to food and water. Animals were maintained following the guidelines of the University of Scranton Institutional Animal Care and Use Committee.

Study I. The normal control group was given the commercial chow for the duration of the study, 4 weeks. Experimental groups were given a high cholesterol diet (Foxall et al., 1992). To 1 kg of powdered chow was added 600 mL of water and ascorbic acid (C), CE, or CE+AA, followed by 0.2% cholesterol and 10% coconut oil melted together. The slurry was mixed well, frozen, and cut in the form of a "bar". The diets with the initial and final weights of the animals are shown in Table 1.

After 4 weeks of feeding and following 12 h of food deprivation, the animals were anesthetized with halothane (Aldrich Chemical Co., Milwaukee, WI), and a cardiac puncture was performed. The blood was put in an EDTA microtainer and the plasma isolated and stored at −90 °C until assay. Total cholesterol and triglycerides were measured with a Sigma enzyme assay. The HDL was measured similarly after precipitation of LDL + VLDL by a Sigma phosphotungstate reagent which has been validated against ultracentrifugation for hyperlipemic hamsters (Foxall et al., 1992). Lipoproteins LDL + VLDL were calculated by difference.

Study II. The normal group in this study was also given rodent chow. Control group was given the same high cholesterol diet as in study I except that the diet was given for 10 weeks to induce atherosclerosis (Nicolosi, 1991). The CE+AA group was given the same diet as in study I. The 12 h food-deprived animals were anesthetized with pentobarbital and perfused with 10% formaldehyde in phosphate-buffered saline and the aorta isolated and prepared for histology as described (Nicolosi, 1991).

Cholesterol and triglycerides were determined as in study I. LDL + VLDL and HDL fractions were separated and isolated by affinity columns (LDL-Direct Cholesterol, Isolab, Inc., Houston, TX) using 200 μL of plasma (Bentzen et al., 1982). The HDL fraction was eluted into 1.25 mL of alpha eluting agent and LDL + VLDL into 2.5 mL of beta eluting agent. The lipoprotein fractions were collected in an ice bath and degassed with nitrogen before storage at −90 °C. Lipoproteins HDL and LDL + VLDL were assayed for cholesterol as described above and LDL + VLDL for protein using brilliant blue reagent (Sigma Chemical Co.) with human albumin as the standard. Recovery of hamster cholesterol as lipoproteins from the columns was 89.4 ± 5.9% (SD) using four samples of plasma containing from 1.64−12.8 mM cholesterol.

Atherosclerosis. Dissection and assay were by the method of Nicolosi (1991). A 4−5 mm section of the aorta at 1 mm distance from the heart was removed, cut longtitudionally, defatted, fixed, and stained with oil Red O. The sample was observed under 16 × 40 magnification, and the number, size, and distribution of foam cells were determined. The area covered by foam cells (fatty streak lesion) was measured in duplicate using a computerized digitizer pad (SigmaScan, Jandel Scientific, San Rafael, CA) after amplification with a micro projector. Oil red O was extracted from the aorta with a constant volume of 2:1 chloroform/methanol and the absorbance measured at 516 nm.

Lipid Peroxidation and Conjugated Dienes. Plasma and LDL + VLDL thiobarbituric acid reactive substances (TBARS) were measured after extraction in butanol by fluorescence at 515 nm/553 nm following a published sample preparation and derivatization method (Lepage et al., 1991). Tetraethoxypropane (Sigma Chemical Co.) was used as the internal standard. Conjugated diene absorbance (CD) was measured at 234 nm and converted into concentration (extinction coefficient 29 500).

Oxidative Susceptibility. All LDL + VLDL oxidations were done at 37 °C in a total volume of 600 μL. The LDL + VLDL was diluted with PBS to a protein concentration of 70 μg/mL and oxidation initiated with the addition of 10 μL of 1 mM cupric ion (final concentration 16.7 μM). Oxidation was stopped by the addition of 50 μL of 0.5% BHT, and proteins were precipitated with sulfuric acid and sodium tungstate prior to TBARS assay by fluorometry.

Ex vivo oxidations after supplementation were used to measure the oxidative susceptibility of LDL + VLDL isolated from study II. Initial CD and TBARS (CD_i and $TBARS_i$) were assayed. The lipoproteins were then oxidized with cupric ion as described above for 6 h, when the TBARS and CD were constant and maximal as seen from pooled samples from each group. The samples were then analyzed for final CD and TBARS (CD_f and $TBARS_f$). The change in CD and TBARS (ΔCD and ΔTBARS) was calculated.

In vitro oxidations were done in duplicate using a pool of high cholesterol control hamster LDL + VLDL from study II to which freshly prepared inhibitors (C, CE, and CE+AA) were added. Aliquots were periodically taken for conjugated diene analysis for 24 h.

Statistical Methods. Results were expressed as means ± SD. The significance of differences between groups was assessed by a Student's *t* test after ANOVA testing of all the groups showed a significant difference existed. Differences were considered statistically significant at $p < 0.05$. Correlations were made using Pearson's correlation coefficient.

RESULTS

Study I. In this short-term study, there were no significant differences in initial and final weights among any of the groups in the study (Table 1). There were no significant food consumption differences between the experimental groups fed high cholesterol (results not

Table 2. Study I: Plasma Lipids and Lipoproteins of Hamsters Fed Normal and High Cholesterol Diets for 4 Weeks[a]

	fasting plasma lipids				
group	cholesterol (mM)	LDL + VLDL (mM)	HDL (mM)	cholesterol/HDL	triglycerides (mM)
normal	2.61 ± 0.44^{a}	1.20 ± 0.34^{a}	1.39 ± 0.34^{a}	1.92 ± 0.37^{a}	12.0 ± 0.50^{a}
control	10.3 ± 3.54^{b}	7.55 ± 3.98^{b}	2.84 ± 1.25^{b}	4.45 ± 3.03^{b}	41.6 ± 23.6^{b}
C	11.3 ± 3.64^{b}	6.65 ± 2.74^{b}	4.66 ± 1.21^{c}	2.42 ± 0.50^{c}	37.6 ± 20.4^{bc}
CE	11.9 ± 4.68^{b}	8.22 ± 4.83^{b}	3.72 ± 1.42^{bc}	3.86 ± 2.60^{bc}	40.8 ± 18.2^{bc}
CE+AA	5.84 ± 1.34^{c}	2.56 ± 1.12^{c}	3.31 ± 0.85^{bc}	1.85 ± 0.58^{a}	25.1 ± 11.7^{c}

[a] Values are means ± SD for 10 animals per group. Values in a column not having superscripts in common are significantly different, $p < 0.05$. C = vitamin C, CE = citrus extract, CE+AA = Citrus Extract Plus C.

Table 3. Study 2: Plasma Lipids of Hamsters after 10 Weeks of Supplementation to Animals Fed a Normal Diet or a Cholesterol and Saturated Fat Diet[a]

group	cholesterol (mM)	LDL + VLDL (mM)	HDL (mM)	triglycerides (mM)	cholesterol/HDL
normal ($n = 6$)	1.61 ± 0.06^{a}	0.33 ± 0.077^{a}	0.89 ± 0.09^{a}	8.60 ± 0.85^{a}	1.79 ± 0.13^{a}
control ($n = 13$)	15.1 ± 1.17^{b}	8.54 ± 2.39^{b}	1.48 ± 0.20^{b}	55.9 ± 42.1^{b}	10.5 ± 3.1^{b}
CE+AA ($n = 8$)	6.88 ± 1.06^{c}	3.44 ± 1.42^{c}	1.68 ± 0.35^{b}	27.1 ± 6.0^{b}	4.07 ± 1.20^{c}

[a] Values are means ± SD. Values in a column not having superscripts in common are significantly different, $p < 0.001$. CE+AA = citrus extract plus C.

Table 4. Plasma and LDL + VLDL Lipid Oxidation Measurements of Hamsters after 10 Weeks of Supplementation to Animals Fed a Normal Diet or a Cholesterol and Saturated Fat Diet[a]

	group		
lipid oxidation measurement	normal	control	CE+AA
plasma TBARS (μM)	2.70 ± 0.26^{a}	7.07 ± 0.46^{b}	5.08 ± 0.26^{c}
LDL + VLDL CD_i (nmol/mg of protein)	286 ± 5^{a}	406 ± 7^{b}	335 ± 18^{c}
LDL + VLDL CD_f (nmol/mg)	365 ± 14^{a}	698 ± 19^{b}	497 ± 30^{c}
LDL + VLDL ΔCD (nmol/mg)	79.0 ± 9.1^{a}	292 ± 15^{b}	161 ± 13^{c}
LDL + VLDL $TBARS_i$ (nmol/mg)	6.86 ± 3.57^{a}	14.9 ± 7.86^{a}	4.14 ± 2.14^{b}
LDL + VLDL $TBARS_f$ (nmol/mg)	292 ± 28.1^{a}	532 ± 27.0^{b}	333 ± 14.3^{a}
LDL + VLDL $\Delta TBARS$ (nmol/mg)	282 ± 63.8^{a}	515 ± 87.0^{b}	329 ± 35.3^{a}

[a] Values are means ± SD. Values in a row not having superscripts in common are significantly different, $p < 0.05$. CE+AA = citrus extract plus C, CD_i = initial conjugated dienes, CD_f = final conjugated dienes after oxidation, $\Delta CD = CD_f - CD_i$, $TBARS_i$ = initial thiobarbituric acid reactive substances, $TBARS_f$ = final thiobarbituric acid reactive substances after oxidation, $\Delta TBARS = TBARS_f - TBARS_i$.

shown). All the experimental groups consumed significantly less food than the normal group ($p < 0.05$). The plasma lipid results following the supplementations are shown in Table 2. Control group had over a 3-fold elevation of cholesterol compared to the normal group. LDL + VLDL cholesterol was increased 6-fold and triglycerides over 3-fold. Vitamin C did not significantly affect cholesterol, LDL + VLDL, or triglycerides compared to the control group. However, HDL was significantly greater vs the control group, and the atherogenic index (cholesterol/HDL) was significantly lowered. The lipids in the CE group were not significantly different from the control group. CE+AA produced a significant improvement in all the lipid parameters including the atherogenic index.

Study II Lipids. At the end of the 10 week supplementation, the normal group gained significantly less weight than the cholesterol-fed control and CE+AA groups, 50 ± 13, 69 ± 15, and 68 ± 16 g, respectively ($p < 0.05$). The weight gains of the control and CE+AA groups were not significantly different. As shown in Table 3 the control group had a significant 8-fold elevation in cholesterol and a 6-fold increase in triglycerides compared to the normal group. The CE+AA lowered cholesterol 55% and triglycerides 52%, and the atherosclerotic index was improved 61% ($p < 0.000l$).

Study II Lipid Peroxidation and Oxidative Susceptibility. Lipid oxidation products were measured in plasma as TBARS and in LDL + VLDL before oxidation as $TBARS_i$ and CD_i. Oxidative susceptibility was measured in LDL + VLDL as CD_f, ΔCD, $TBARS_f$, and $\Delta TBARS$. The results are shown in Table 4. The control group had the highest lipid peroxidation in both plasma and LDL + VLDL before and after oxidation and was significantly higher than the normal group except for $TBARS_i$. CE+AA was significantly lower than the control group for all lipid peroxidation and oxidative susceptibility measurements ($p < 0.01$). The oxidative susceptibility of CEC, as measured by the change in TBARS of LDL + VLDL ($\Delta TBARS$), was not significantly different from the normal group.

Table 5. Atherosclerosis Measurements of Hamsters after 10 Weeks of Supplementation to Animals Fed a Normal Diet or a Cholesterol and Saturated Fat Diet[a]

	group		
measurement	normal	control	CE+AA
area of foam cells (mm^2)	0	2.53 ± 1.04^{b}	0.93 ± 0.29^{c}
foam cell density (cells/mm^2)	0	55.64 ± 14.96^{b}	26.93 ± 6.41^{c}
% of aorta with foam cells	0	14.22 ± 3.664^{b}	5.54 ± 1.65^{c}
intimal oil red 0 (ng/mm^2)	59 ± 7^{a}	99 ± 25^{b}	73 ± 7^{c}

[a] Values are means ± SD. Values in columns not having superscripts in common are significantly different, $p < 0.001$. CE+AA = citrus extract plus C.

Atherosclerosis. Measurements of the extent of atherosclerosis are given in Table 5. In all cases, the CE+AA group had significantly less severe atherosclerosis than the control group ($p < 0.001$). The fatty streak lesion area was reduced 63% with CE+AA supplementation, and the density of foam cells was decreased 52% with CEC. Correlations of atherosclerosis and oxidative analyses are shown in Table 6.

In Vitro Oxidation. Oxidations of LDL + VLDL from a pool of control high cholesterol animals were

Table 6. Correlations of Lipid Oxidation and Lipid Oxidizability with Atherosclerosis[a]

	atherosclerosis			
oxidation	foam cell density	foam cell area	Oil Red 0	% atherosclerosis
plasma TBARS	0.655 (0.002)	0.525 (0.015)	0.615 (0.001)	0.557 (0.013)
LDL+VLDL CD_i	0.520 (0.019)	0.523 (0.022)	0.560 (0.003)	0.675 (0.002)
LDL+VLDL CD_f	0.602 (0.004)	0.513 (0.0001)	0.576 (0.002)	0.753 (0.0001)
LDL+VLDL ΔCD	0.608 (0.004)	0.475 (0.024)	0.563 (0.003)	0.747 (0.0001)
LDL+VLDL $TBARS_i$	0.259 (NS)	0.520 (0.019)	0.429 (0.041)	0.499 (0.035)
LDL+VLDL $TBARS_f$	0.531 (0.016)	0.474 (0.030)	0.460 (0.018)	0.787 (0.0001)
LDL+VLDL $\Delta TBARS$	0.550 (0.012)	0.616 (0.001)	0.584 (0.0001)	0.709 (0.0001)

[a] The first number in a column is the Pearson correlation coefficient (r). The second number is the statistical significance (p value). CD_i = initial conjugated dienes, CD_f = final conjugated dienes after oxidation, $\Delta CD = CD_f - CD_i$, $TBARS_i$ = initial thiobarbituric acid reactive substances, $TBARS_f$ = final thiobarbituric acid reactive substances after oxidation, $\Delta TBARS = TBARS_f - TBARS_i$.

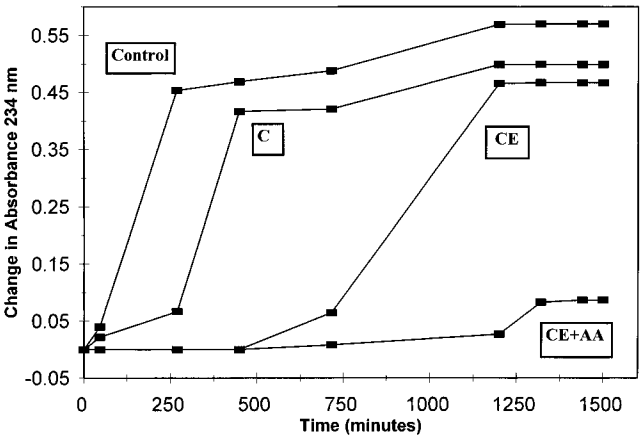

Figure 1. Effect of added antioxidants on cupric ion induced LDL + VLDL oxidation from a pooled hypercholesterolemic hamster plasma sample. C = ascorbic acid (2.5 μM), CE = citrus extract phenols (0.64 μM), CE+AA = citrus extract phenols + ascorbic acid (0.64 μM and 2.5 μM, respectively).

done in the presence of the components of CE+AA (C and CE) and followed by conjugated diene formation in order to observe the kinetics of the reaction (Figure 1). The concentration of ascorbic acid was the same in the C and CE+AA incubations. Flavonoid phenol concentrations were also equalized in CE and CE+AA. The lag time (time when the oxidation rate changed abruptly) and the maximum rate and extent of oxidation (maximum change of absorbance) were measured. The lag times of control, C, CE, and CE+AA were 23, 222, 650, and 1063 min, respectively. The maximum rates were 2.01×10^{-3}, 9.93×10^{-4}, 1.93×10^{-3}, and 4.76×10^{-4} absorbance unit/min, respectively. The extent of oxidation was 0.570, 0.467, 0.499, and 0.087 absorbance unit, respectively. The combinations of these two components in CE+AA were synergistic inhibitors of LDL + VLDL oxidation as seen in lag time, rate, and extent of oxidation.

DISCUSSION

The hyperlipemic hamster model has been used to examine the effects of drugs (Nicolosi, 1991) and diet (Kahlon et al., 1992) on lipids and the development of atherosclerosis. The hamster is an excellent model as it has an omnivorous diet, its hepatic cholesterol synthesis is like that in humans, and it has a significant proportion of LDL in the plasma and develops atheromas in a human-like progression (Gaviraghi et al., 1997). We tested CE+AA because it had several components known to beneficially affect lipids in animals, namely, ascorbic acid and flavonoids. The effect of vitamin C on cholesterol has recently been reviewed (Hemila, 1992) and has been shown to be salutary for animals fed an atherogenic diet. The hypolipemic and antiaggregating effects of flavonoids have also been reviewed (Stavric and Matula, 1992). CE+AA has a high concentration of flavonoids (9.9%) and has been shown to increase the absorption of ascorbate in guinea pigs following long-term supplementation (Vinson and Bose, 1983). CE+AA and ascorbate, when given to normal subjects and smokers, significantly decreased plasma TBARS (Vinson and Hsu, 1992; Vinson et al., 1992).

Study I Lipids. After 1 month, the HDL content changed from 54% of total cholesterol in the normal group to 28% of cholesterol in the cholesterol-fed control group, a trend seen by Surette et al. (1992). As there were no significant differences in weight gain or food consumption among the high cholesterol groups, the differences in plasma lipids cannot be due to differences in food consumption.

In the present study, ascorbate did not lower cholesterol but did significantly increase HDL. This latter result agrees with a guinea pig study in which HDL levels were raised with vitamins supplemented with the atherogenic diet (Sharma et al., 1988). In our hamster study, ascorbate significantly improved the atherogenic index but did not normalize it.

CE containing flavonoids, but no ascorbate, did not significantly change any lipid in study I. Yugarani and co-workers (1992) also did not find any effect of the isolated flavonoid quercetin, a compound present in CE as the glycoside, on cholesterol in the cholesterol-fed rat. In the present study, CE+AA, containing both flavonoids and ascorbate, had the strongest beneficial effect on the hamster lipids. CE+AA was the only supplement to lower triglycerides compared to the control. It was the only supplement to significantly decrease cholesterol and LDL + VLDL and improved the atherogenic index so that it was not significantly different from the group fed normal hamster chow without cholesterol. There appears to be an in vivo synergism of the flavonoids and ascorbate in the CE+AA with respect to cholesterol, LDL + VLDL, triglycerides, and the atherogenic index. A longer study was then initiated with CE+AA to determine its effect on lipids, lipid oxidation, and atherosclerosis.

Study II Lipids and Atherosclerosis. As in study I, CE+AA had a highly beneficial effect on cholesterol, LDL + VLDL, and the atherogenic index. The cholesterol in the CE+AA group in study II after 10 weeks was not significantly different from the CE+AA group in study I after 4 weeks, 6.88 and 5.84 mmol/L, respectively. This result shows that the CE+AA stabilized cholesterol during long-term feeding. The percent lowering of cholesterol and triglycerides by supplementation with CE+AA (54 and 52%, respectively) was very comparable to that from the highest dose of the

drug doxazosin given to cholesterol-fed hamsters for 10 weeks (Foxall et al., 1992).

The calculated consumption of flavonoids from the CE+AA was 2.4 mg/day per animal which transforms into 1120 mg/day for a 70 kg human. This is a reasonable human dose since the daily intake of flavonoids from a Western diet has been estimated to be 500−1000 mg/day (Kuhnau, 1976).

The mild atherogenic diet induced fatty streak involvement in the aortic arch close to the heart as found previously (Kowala et al., 1991). The oil red O stained lipid was found within macrophage-derived foam cells below the endothelium. CE+AA significantly decreased all measures of atherosclerosis in this hamster model including foam cell area, density, percent of fatty streak involvement, and oil red O lipid staining.

Study II Lipid Peroxides and Oxidative Susceptibility. To examine closely the relationship of the oxidative theory to hyperlipidemia, several oxidation and oxidative susceptibility measurements were made. All measurements, except LDL + VLDL $TBARS_i$, were significantly elevated in the control cholesterol-fed animals compared with those from the normal group given chow alone as seen in Table 4. Other investigators have shown increased plasma TBARS in cholesterol-fed rabbits (Kisanuki et al., 1992) and hypercholesterolemic humans (Yalcin et al., 1989) and increased oxidative susceptibility of LDL from hypercholesterolemic humans (Lavy et al., 1991).

As a measure of lipoprotein oxidative susceptibility, we studied the cupric ion-induced oxidation of LDL + VLDL. Previous lipoprotein oxidation has been done with LDL or LDL + VLDL isolated by ultracentrifugation (Rayssiquier et al., 1993). The heparin−agarose micro-affinity column used in this study has been found to quantitatively separate HDL from LDL + VLDL in both normal and hyperlipemic plasma and has been found to correlate with separation by ultracentrifugation (Bentzen et al., 1982). We have found the affinity column to be an inexpensive, quick, and reproducible means of both hamster and human liporotein isolation prior to oxidation (Vinson et al., 1995a,b,c).

CE+AA significantly decreased both the oxidizability of plasma as measured by TBARS and LDL + VLDL as measured by CD_f or $TBARS_f$. The oxidative susceptibility of LDL + VLDL, as determined by the change ΔCD or $\Delta TBARS$, was also significantly reduced by CE+AA. This suggests that flavonoids from CE+AA, after absorption into the bloodstream, bind to the lipoproteins and decrease the oxidizability of lipoproteins both in vivo and ex vivo. A similar result was seen in a human study after supplementation with red wine, in which polyphenols were found bound to LDL (Fuhrman et al., 1995). We have found that flavonoids bind to LDL + VLDL following plasma spiking and inhibit ex vivo LDL + VLDL oxidation (Vinson et al., 1995c).

Heretofore, the lag times or maximum propagation rates of LDL oxidation have been the measures of oxidative susceptibility with in vitro or ex vivo studies (Esterbauer and Jurgens, 1993). These parameters have been correlated with the extent of human atherosclerosis (Regnstrom et al., 1992). In our study, all oxidation and oxidative susceptibility measurements correlated with atherosclerosis with coefficients ranging from 0.429 to 0.709 ($p < 0.05$ to $p < 0.0001$). Surprisingly our data also indicated that CD_i, normally considered an in vivo oxidation measurement, is very highly correlated with oxidative susceptibility parameters such as CD_f, ΔCD, $TBARS_f$, $\Delta TBARS$; correlation coefficients 0.951, 0.905, 0.849, and 0.851, respectively ($p < 0.0001$). CD_i measures minimally modified lipoproteins (containing lipid peroxides) present in circulating plasma and formed during the isolation procedure. Recently Mosinger (1995) has also found that the oxidizability of human LDL with cupric ion was strongly correlated to preformed conjugated dienes, $r = 0.91$.

Mechanism. In study I we found that CE+AA but not CE or C lowered cholesterol and triglycerides in hypercholesterolemic hamsters. Thus, our studies indicate that there exists a powerful hypolipemic synergism of C and flavonoids in hypercholesterolemic hamsters. The mechanism for this beneficial synergism is not known.

Flavonoids have a unique ability to chelate metal ions (Afanas'ev et al., 1989) as well as possess antioxidant properties. However, we have found no spectral shift for flavonoids or cupric ion when mixing them in the concentrations used in the in vitro oxidations. From stoichiometric considerations, the lack of chelation is reasonable given the 26:1 cupric ion-to-flavonoid ratio during the in vitro oxidation. Vitamin C is also a well-known in vitro LDL antioxidant in plasma and LDL (Retsky et al., 1993). However, it has not been shown to improve LDL oxidative susceptibility following supplementation to normal human subjects (Belcher et al., 1993).

The in vitro oxidation model showed that ascorbate and the flavonoids in CE+AA were powerful synergistic inhibitors. Synergism between flavonoids and ascorbate has been known for a long time (Cotereau et al., 1948). Negre-Salvayre and co-workers (1991) have found synergistic antioxidant inhibition of the glycosylated flavonoid rutin with vitamin C in other lipid oxidation models. Recently, lag time synergism of phenolic acids with ascorbate was observed during the oxidation of LDL (Viera et al., 1997). We believe that the in vitro and ex vivo synergism occurs by the following mechanism: after oxidation of the flavonoid, the vitamin C regenerates the flavonoid phenoxy radical present in the lipoprotein and forms the nontoxic ascorbyl radical. This mechanism is analogous to that proposed for probucol and ascorbate which synergistically inhibit the oxidation of LDL (Kalyanaraman et al., 1992).

CONCLUSION

The results of our study suggest that CE+AA is an effective agent for decreasing lipids and for the inhibition of atherosclerosis in the cholesterol-fed hamster model. Relevant to the oxidized LDL theory of atherogenesis is the fact that CE+AA contains two antioxidants, ascorbate and flavonoids, which act synergistically, not only to improve lipoprotein oxidative susceptibility but also to improve the lipid profile. CE+AA may prove to be a therapy that both lowers cholesterol and prevents the oxidation of LDL and thus may have a dual action in preventing and treating human atherosclerosis. Studies are under way in our laboratory to test this hypothesis.

ACKNOWLEDGMENT

We thank Grow Co. for supplying the supplements for use in this study. Credit must be given to A. Pastorino for suggesting the hamster model, to Robert

Nicolosi for advice with the hamster study, and to John Nunnari for expertise on the sample preparation and staining of the aortas. We are indebted to Kibbe Manning, Diana Sater, Ray Correlle, and Stephanie Sninsky for assistance with the animals. Lifu Tang did the HPLC analysis of the flavonoids, and Songhuai Chang performed the Folin assay. Grateful acknowledgment is given to Thomas Bocan for reviewing the manuscript and to Debbie Jennings for splendid secretarial assistance.

LITERATURE CITED

Afanas'ev, I. B.; Dorozhko, A. I.; Brodskii, A. V.; Kostyuk, A.; Potapovitch, A. I. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem. Pharmacol.* **1989**, *36*, 1763−1769.

Ames, B. N.; Shigenaga, M. K.; Hazen, T. M. Oxidants, antioxidants and degenerative diseases of aging. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7915−7922.

Belcher, J. D.; Balla, G.; Jacobs, D. R., Jr.; Gross, M.; Jacob, H. S.; Vercelotti, G. M. Vitamin E, LDL and endothelium. Brief vitamin supplementation prevents oxidized LDL-mediated injury in vitro. *Arteriosler. Thromb.* **1993**, *13*, 1779 (abstr.).

Bentzen, C. L.; Acuff, K. J.; Marechal, B.; Rosenthal, M. A.; Volk, M. E. Direct determination of lipoprotein cholesterol distribution with microscale affinity chromatography columns. *Clin. Chem.* **1982**, *28*, 1451−1455.

Cotereau, H.; Gabe, M.; Géro, E.; Parrot, J. L. Influence of vitamin P (vitamin C_2) upon the amount of ascorbic acid in the organs of the guinea pig. *Nature* **1948**, *161*, 577−578.

Esterbauer, H.; Jurgens, G. Mechanistic and genetic aspects of susceptibility of LDL to oxidation. *Curr. Opin. Lipidol.* **1993**, *4*, 114−124.

Foxall, T. L.; Shwaery, G. T.; Stucchi, A. F.; Nicolosi, R.; Wong, S. S. Dose-related effects of doxaosin on plasma lipids and aortic fatty streak formation in the hypercholesterolemic hamster model. *Am. J. Pathol.* **1992**, *140*, 1357−1363.

Fuhrman, B.; Lavy, A.; Aviram, M. Consumption of red wine with meals reduces the susceptiblity of human plasma and low-density lipoprotein to lipid peroxidation. *Am. J. Clin. Nutr.* **1995**, *61*, 549−554.

Gaviraghi, G.; Pastorino, A. M.; Ratti, E.; Trist, D. G. Antioxidant dihydropyridines, a new and comprehensive therapy for free-radical induced cardiovascular diseases. In *Free Radicals in Biology and Environment*; Kluwer: Dordrecht, The Netherlands, 1997; pp 193−221.

Gey, K. F.; Brubacker, G. B.; Stähelin, H. B. Plasma levels of antioxidant vitamins in relation to ischemic heart disease and cancer. *Am. J. Clin. Nutr.* **1987**, *45*, 1368−1377.

Harborne, J. B. *The Flavonoids; Advances in Research since 1980*; Chapman and Hall: New York, 1988.

Hemila, H. Vitamin C and plasma cholesterol. *Crit. Res. Food Sci. Nutr.* **1992**, *32*, 33−57.

Hertog, M. G. L.; Hollman, P. C. H.; Venema, D. P. Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in 28 vegetables and fruits. *J. Agric. Food Chem.* **1992**, *40*, 1591−1598.

Hertog, M. G. L.; Feskens, E. J.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart diseases: The Zutphen elderly study. *Lancet* **1993a**, *342*, 1007−1011.

Hertog, M. G. L.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutr. Cancer* **1993b**, *20*, 21−29.

Hertog, M. G. L.; Kromhout, D.; Aravanis, C.; Blackburn, H.; Buzina, R.; Fidanza, F.; Gaimpaoli, S.; Jansen, A.; Menotti, A.; Nedeljkovic, S. L.; Pekkarinen, M.; Simic, B. S.; Toshima, H.; Feskens, E. J. M.; Hollman, P. C. H.; Katan, M. B. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Arch. Intern. Med.* **1995**, *155*, 381−386.

Hollman, P. C. H.; de Vries, J. H. M.; van Leeuwen, S. D.; Mengelers, M. J. B.; Katan, M. B. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am. J. Clin. Nutr.* **1995**, *62*, 1276−1282.

Jackson, R. L.; Ku, G.; Thomas, C. E. Antioxidants: a biological defense mechanism for the prevention of atherosclerosis. *Med. Res. Rev.* **1993**, *13*, 161−182.

Kahlon, T. S.; Chow, F. I.; Sayre, R. N.; Betschart, A. A. Cholesterol-lowering in hamsters fed rice bran at various levels, defatted rice bran and rice bran oil. *J. Nutr.* **1992**, *122*, 513−519.

Kalyanaraman, B.; Darley-Usmar, V. M.; Wood, J. J.; Parthasarathy, S. Synergistic interaction between the probucol phenoxyl radical and ascorbic acid in inhibiting the oxidation of low density lipoprotein. *J. Biol. Chem.* **1992**, *267*, 6789−6795.

Kisanuki, A.; Asada, Y.; Hatakeyama, K.; Hayashi, T.; Sumiyoshi, A. Contribution of the endothelium intimal thickening in normocholesterolemic and hypercholesterolemic rabbits. *Arteriosler. Thromb.* **1992**, *12*, 1198−1205.

Kühnau, J. The flavonoids: a class of semi-essential food components: their role in human nutrition. *World Rev. Nutr. Diet.* **1976**, *24*, 117−120.

Lavy, A.; Brook, G. J.; Dankner, G.; Amota, A. B.; Aviram, M. Enhanced in vitro oxidation of plasma lipoproteins derived from hypercholesterolemic patients. *Metab. Clin. Exp.* **1991**, *40*, 794−799.

Lepage, G.; Munoz, G.; Champagne, J.; Roy, C. C. Preparative steps necessary for the accurate measurement of malondialdehyde by high-performance liquid chromatography. *Anal. Biochem.* **1991**, *277*, 277−283.

Manach, C.; Morand, C.; Texier, O.; Favier, M.-L.; Agullo, C. D.; Régerat, F.; Rémesty, C. Quercetin metabolites in plasma of rats fed diets containing rutin or quercetin. *J. Nutr.* **1995**, *125*, 1911−1922.

Mosinger, B. J. Copper-induced and photosensitive oxidation of serum low-density lipoprotein. The relation of cholesterol level and inter-species differences. *Biochim. Biophys. Acta* **1995**, *1270*, 73−80.

Negre-Salvayre, A.; Affany, A.; Hariton, C.; Salvayre, R. Additional antilipoperoxidant activities of alpha-tocopherol and ascorbic acid on membrane- like systems are potentiated by rutin. *Pharmacology* **1991**, *42*, 262−272.

Nicolosi, R. J. Doxazosin and cholestryamime similarly decrease fatty strak formation in the aortic arch of hyperlipemic hamsters. *Atherosclerosis* **1991**, *91*, 35−49.

Parker, R. A.; Sabrah, T.; Cap, M.; Gill, B. T. Relation of vascular oxidative stress, α-tocopherol, and hypercholerolemia to early atherosclerosis in hamsters. *Arteroscler. Thromb. Vasc. Biol.* **1995**, *15*, 349−358.

Rayssiguier, Y.; Gueux, E.; Bussire, L.; Mazur, A. Copper deficiency increases the susceptibility of lipoproteins and tissues to lipid peroxidation in rats. *J. Nutr.* **1993**, *123*, 1343−1348.

Regnstrom, J. N.; Nilsson, J.; Tornavall, P.; Landou, C.; Hamsten, A. Susceptibility of low-density lipoprotein oxidation and coronary atherosclerosis in man. *Lancet* **1992**, *339*, 1183−1186.

Retsky, K. L.; Freeman, M. W.; Frei, B. Ascorbic acidoxidation product(s) protect human low density lipoprotein against atherogenic modification. *J. Biol. Chem.* **1993**, *268*, 1304−1309.

Sharma, P.; Pramod, J.; Sharma, P. K.; Chaturvedi, S. K.; Kothari, L. K. Effect of vitamin C administration on serum and aortic lipid profile of guinea pigs. *Indian J. Med. Res.* **1988**, *87*, 283−289.

Stavric, B.; Matula, T. I. Flavonoids in foods: Their significance for nutrition and health. In *Lipid-soluble antioxidants: Biochemical and clinical applications*; Ong, A. S. H., Packer, L., Eds.; Birhauser and Verlag: Basel, Switzerland, 1992.

Steinberg, D. S.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witzum, J. C. Beyond cholesterol: modifications of low-

density cholesterol that increase its atherogenicity. *N. Engl. J. Med.* **1989**, *320*, 915−924.

Surette, M. E.; Whelan, J.; Lu, G.-P.; Broughton, K. S.; Kinsella, J. E. Dependence of dietary cholesterol for n-3 polyunsaturated fatty acid-induced changes in plasma cholesterol in the syrian hamster. *J. Lipid Res.* **1992**, *20*, 263−271.

Viera, O.; Laranjinha, J.; Madeira, V.; Almeida, L. Cholesteryl ester hydroperoxide formation in myoglobin-catalyzed low-density lipoprotein oxidation. *Biochem. Pharmacol.* **1997**, *55*, 334−340.

Vinson, J. A.; Bose, P. Comparative bioavailability of synthetic and natural vitamin C in guinea pigs. *Nutr. Rep. Int.* **1983**, *27*, 875−880.

Vinson, J. A.; Hsu, C. Effects of vitamins A, E, and a citrus extract on in vitro and in vivo lipid peroxidation. *Med. Sci. Res.* **1992**, *20*, 145−146.

Vinson, J. A.; Chang, J. Y.; Nykaza, L. Effect of ascorbic acid supplementation on plasma ascorbate and lipid peroxidation parameters in nonsmokers and smokers. *Med. Sci. Res.* **1992**, *20*, 483−484.

Vinson, J. A.; Dabbagh, Y. A.; Serry, M. M.; Jang, J. Plant flavonoids, expecially tea flavonols, are powerful antioxidants using an *in vitro* oxidation model for heart disease. *J. Agric. Food Chem.* **1995a**, *43*, 2800−2802.

Vinson, J. A.; Hontz, B. A. Phenol antioxidant index: comparative antioxidant effectiveness of red and white wines. *J. Agric. Food Chem.* **1995b**, *43*, 401−403.

Vinson, J. A.; Jang, J.; Dabbagh, Y. A.; Serry, M. M.; Cai, S. Plant polyphenols exhibit lipoprotein-bound antioxidant activity using an *in vitro* model for heart disease. *J. Agric. Food Chem.* **1995c**, *43*, 2798−2799.

Yalcin, A. S.; Sabuncu, N.; Kilinic, A.; Gulcan, G.; Emerk, K. Increased plasma and erythrocyte lipid peroxidation in hyperlipemic individuals. *Atherosclerosis* **1989**, *80*, 169−170.

Yugarani, T.; Tan, B. K. H.; Das, N. P. Effects of polyphenolic natural products on the lipid profiles of rats fed high fat diets. *Lipids* **1992**, *27*, 181−186.

Received for review September 15, 1997. Revised manuscript received January 30, 1998. Accepted February 10, 1998.

JF970801U