Curcuma longa Extract Supplementation Reduces Oxidative Stress and Attenuates Aortic Fatty Streak Development in Rabbits

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Objective—This study evaluates the effect of a Curcuma longa extract on the development of experimental atherosclerosis (fatty streak) in rabbits and its interaction with other plasmatic antioxidants.

Methods and Results—Two experimental groups of male New Zealand White rabbits, a control group and a curcuma-extract (CU) group, were fed an atherogenic diet. Additionally, the CU group received an oral curcuma hydroalcoholic extract. Six animals from each experimental group were killed after 10, 20, and 30 days. Compared with the CU group, the control group showed significantly higher plasma lipid peroxide at all experimental times (10, 20, and 30 days) and significantly lower α-tocopherol and coenzyme Q levels at 20 and 30 days. Histological results for the fatty streak lesions revealed damage in the thoracic and abdominal aorta that was significantly lower in the CU group than in the control group at 30 days.

Conclusions—Supplementation with Curcuma longa reduces oxidative stress and attenuates the development of fatty streaks in rabbits fed a high cholesterol diet. (Arterioscler Thromb Vasc Biol. 2002;22:1225-1231.)

Key Words: Curcuma longa ■ antioxidants ■ aorta ■ fatty streak ■ atherosclerosis ■ rabbits

A plant of Indian origin, Curcuma longa L (Zingiberaceae) has a rhizome of bright orange color under a fine light brown cell layer. It is in common use as a spice in Asian cultures, where it is considered to be a magical plant because of its organoleptic properties and undoubted therapeutic and protective effects, especially for the skin and liver.1

Since ancient times, many properties have been ascribed to extracts of Curcuma longa. The plant has been applied for the prevention and cure of skin and hepatic conditions and of ulcers and digestive disorders. It has also been used in the treatment of intestinal parasites and as a remedy for poison, snakebites, and various other complaints.2

Many studies have shown the capacity of curcumin to prevent lipid peroxidation, a key process in the onset and progression of many diseases. The capacity of curcumin to stabilize membranes has also been demonstrated.3 Our research group has found that a dose of 2.4 to 9.6 μmol/L curcumin inhibits the oxidation of human LDL in vitro.4 Venkatesan5 observed a protective effect of curcumin against the cardiotoxicity produced by adriamycin in rats, showing a reduction in the parameters that indicate lipid peroxidation.

Atherosclerosis is a multifactorial disease in which a major alteration of the vascular lipid metabolism is produced. It has been observed that curcumin reduces plasmatic lipid peroxides, molecules that play an important role in the pathogenesis of the disease.6 Curcumin also has different properties that contribute to combat this disease: it reduces the susceptibility of LDL to oxidation,7 inhibits the proliferation of vascular smooth muscle cells,8 has an antithrombotic effect, has a transient hypotensive effect, and inhibits platelet aggregation in vivo and ex vivo.9

Sreejayan et al10 claimed that the presence of phenolic groups in the structure of curcumin are fundamental in explaining its ability to eliminate oxygen-derived free radicals from the medium largely responsible for the peroxidation of cell lipids.11 They are able mainly to eliminate the hydroxyl radical,12 superoxide radical,13 singlet oxygen,14 nitrogen dioxide,15 and NO.16 It has also been demonstrated that curcumin inhibits the generation of the superoxide radical.17

Despite all these positive effects attributed to curcumin, to date, there have been no time-course studies with this extract investigating the development of fatty streak lesions in the atherosclerotic process. Thus, the aim of the present study was to evaluate the effect of our curcumin extract on the development of experimental atherosclerosis (fatty streaks) in rabbits and its interaction with other plasmatic antioxidants.
Figure 1. Main curcuminoids of Curcuma longa extract.

Such a study could determine the utility of the extract in patients with peripheral vascular disease.

Methods

Animals and Diets
Forty-two male New Zealand White rabbits (University of Granada Laboratory Animals Service, Granada, Spain), weighing 2500 g and kept 1 per cage, were put on a 12-hour light/12-hour dark cycle with free access to food (150 g/d) and water. All the animals were fed rabbit chow for 10 days. At this point, 6 animals were killed as a baseline group. The remaining animals were divided into 2 experimental groups: the control group and the curcuma extract (CU) group. Both groups were fed an atherogenic diet (Abbott Laboratories SA) containing 95.7% standard chow, 3% lard, and 1.3% cholesterol to provoke an atherosclerotic process. Additionally, the CU group received an oral hydroalcoholic solution of curcuma at a dosage of 1.66 mg/kg body wt, and the control group received an oral curcuma-free hydroalcoholic solution daily during the experiment. The atherogenic meal was kept in darkness at 4 °C to avoid peroxidation until use. During the study, 6 animals from each experimental group were killed after 10, 20, and 30 days, respectively. The animals were clinically observed and weighed weekly. The University of Granada Ethics Committee approved the present study, and the animals were handled according to the guidelines of the Spanish Society for Laboratory Animal Sciences for the care and use of laboratory animals.

Extraction and Curcuminoids Composition of Curcuma longa
The hydroalcoholic extract of curcuma was provided by A.S.A.C. Pharmaceutical International A.I.E. For the extraction, the rhizome of Curcuma longa was macerated with hot water (80 °C) for 4 hours, and the aqueous extract was evaporated under vacuum at 60 °C. The rhizome residue was reextracted with ethanol at 60 °C for 2 hours, filtered, and evaporated under vacuum. The final extract is a 1:1 mixture of both the aqueous extract and the alcoholic extract, which are redissolved with water and alcohol, respectively. Curcuminoids composition of the extract was carried out by high-performance liquid chromatography (HPLC) with a Beckman In-line Diode Array Detector (model 168) and a Supelcosil LC-18 column (150 mm × 4.6 mm and 5 μm, Supelco). The mobile phase was 0.05 mol/L sodium acetate and 55:45 (vol/vol) acetonitrile (pH 4.2 at 1 mol/L) and vortexed, as described by Buege and Aust. Results were combined with a 1-mL mixture of trichloroacetic acid (15% [wt/vol]), thiobarbituric acid (0.37%), and hydrochloric acid (25 mol/L) and vortexed, as described by Buege and Aust. Results were expressed as nanomoles of TBARS per milliliter of plasma, with 1,1,3,3-tetramethoxypropane used as a standard.

Determination of LDL Oxidation Susceptibility
LDL was isolated by a single discontinuous density-gradient ultracentrifugation in a vertical rotor as described previously. Total LDL cholesterol level was measured by using a commercially available kit (Boehringer-Mannheim). LDL protein was measured by the Bradford method, with BSA used as a standard. To study the susceptibility to oxidation of LDL, 2 determinations (TBARS and conjugated dienes) were performed. Two hundred milligrams of LDL protein per liter was oxidized in the presence of 25 μmol/L Cu2+ in PBS for 6 hours at 37 °C. After incubation, oxidation was terminated by cooling the samples to 4 °C and adding 100 mmol/L EDTA and 4.5 μmol/L butylated hydroxytoluene. The lipid-peroxide content of oxidized LDL was determined as TBARS, according to Beugel and Aust (1978). Absorbance units were converted to malondialdehyde equivalents per milligram LDL protein with the use of a standard curve obtained with 1,1,3,3-tetramethoxypropane. Conjugated dienes in LDL was carried out according to Puhl et al (1989) in a Perkin-Elmer UV-VIS Lambda 40 spectrometer equipped with an auto-cell holder and controlled by a Peltier element at a temperature of 37 °C. Twenty milligrams of LDL protein per liter was oxidized in the presence of Cu2+ (5 μmol/L) in PBS. LDL-conjugated dienes were measured every 2 minutes at 234 nm for 60 minutes at 37 °C. Results are expressed as nanomoles of conjugated dienes per milligram LDL protein.

Coenzyme Q10, Retinol, and α-Tocopherol Determinations
Analyses of coenzyme Q10, retinol, and α-tocopherol in plasma were assayed according to the method of MacCrehan by reversed-phase HPLC with a Spherisorb S5 ODS1 (Merck, Darmstadt, Germany) column and ethanol/purified water (97:3 [vol/vol]) used as the mobile phase. The HPLC system was a Beckman In-line Diode Array Detector (model 168) connected to a Water 717 Plus Autosampler (Milford). Coenzyme Q10, retinol, and α-tocopherol were identified by predetermining the retention times of individual pure standards.

Histological Analysis of Aortic Atherosclerotic Lesions
Entire aortas were rapidly dissected out, and ~1 cm of the aortic arch and of the thoracic and abdominal aortas was selected and standard-ized for all the animals. For histological examinations, buffered 4% formaldehyde-fixed paraformaldehyde-embedded tissue sections were stained with hematoxylin and eosin (H&E), Masson-Goldner trichrome, and van Gieson’s elastin stains. Samples were examined in a blinded manner to evaluate the presence of the fatty streak. Lesions were scored on a 4-point–intensity semiquantitative scale for the damage present (1, absence of damage; 2, mild damage; 3, moderate damage; and 4, intense damage).

Determination of Lipid Aortic Composition
Similar aortic segments of ~2 cm for all rabbits were weighed and minced in a tissue homogenizer. Total lipids were extracted by using 1.5 mL of 0.01 mol/L HCl, 0.1 mL of 1% MgCl2, and 5 mL hexane/isopropanol (3:2 mixture). The organic phase was separated by centrifugation at 1750g for 10 minutes. Standard procedures were used to measure TC and esterified cholesterol (EC) with the use of commercial analytical kit (Boehringer-Mannheim).
commercially available kits (Boehringer-Mannheim). The fatty acid pattern of aortic lipids was determined by gas-liquid chromatography as previously described. The monounsaturated fatty acid (MUFA)/polyunsaturated fatty acid (PUFA) index was calculated with the following fatty acids: MUFA (16:1n-9, 18:1n-9, 20:1n-9, and 24:1n-9) and PUFA (18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6, 22:5n-6, 18:3n-3, 20:5n-3, 22:5n-3, and 22:6n-3).

All the chemical products and solvents, of high quality, were acquired from Sigma and Merck.

Statistical Analyses
Before any statistical analysis, all variables were checked for normality and homogeneous variance by using the Kolmogorov-Smirnov and the Levene tests, respectively. When a variable was found not to follow normality, it was logarithmically transformed and reanalyzed. All parameters for the control and CU groups and baseline levels were analyzed by a 1-way ANOVA; to evaluate mean differences for groups at each time point (10, 20, and 30 days), multiple comparison tests adjusted by Bonferroni corrections were performed. Changes in aortic lesions were analyzed by the Kruskal-Wallis test, and a Mann-Whitney U test was used a posteriori to evaluate mean differences between groups. A value of \( P<0.05 \) was considered significant. Data were analyzed by using a statistical software package (SPSS for Windows, 9.0.1, 1999, SPSS Inc).

Results
No differences were found among the experimental groups with respect to the weight gain at the different experimental times (data not shown). Plasma and LDL cholesterol values were significantly higher at 10, 20, and 30 days for the control and CU groups than for the baseline group. However, no differences were found among the periods of time for each group (plasma cholesterol, 13.7±3.7 mg/dL for baseline group and mean value 2977±51.8 mg/dL at 10, 20, and 30 days for control and CU groups; LDL cholesterol, 42.9±1.5 mg/mg protein for baseline group and mean value 93.2±9.5 mg/mg protein at 10, 20, and 30 days for control and CU groups). TBARS have been used to achieve the degree of lipid peroxidation in the plasma of the animals (Figure 2A). Baseline levels of TBARS were lower than those found in the control group at the 3 experimental times. No differences were found between the baseline group and the CU group at 10, 20, and 30 days. Additionally, for each experimental time, the control group showed significantly higher levels of TBARS than did the CU group. The susceptibility of LDL to oxidation was determined by conjugated dienes (Figure 2B) and TBARS (Figure 2C). No differences were found for conjugated dienes between the groups. However, compared with the CU group, the control group showed a significantly higher level of LDL TBARS at 30 days.

Figure 3A shows plasma coenzyme Q10 concentration in all experimental groups. A decrease in the levels of this antioxidant was found for all times (10, 20, and 30 days) in the experimental groups compared with the baseline group. This decrease was higher in the control group than in the CU group. No differences were found in either group regarding the experimental time. Plasma retinol levels (Figure 3B) showed results similar to those described for coenzyme Q10 regarding the decrease in the levels of this antioxidant compared with the baseline levels. Rabbits fed curcumin extract for 10 days reflected significantly higher levels of retinol than did the control group for this experimental time.

Figure 3C shows plasma α-tocopherol levels in the studied animals. No significant differences were found among experimental times in the control group compared with the baseline group. The plasma α-tocopherol level of the CU group at 10 days was no different from that of the baseline group. However, the CU group at 20 and 30 days reached values of α-tocopherol that were significantly higher than those at 10 days and at baseline.

The results of the histological examination for fatty streak lesions in the aortic arch are shown in Figure 4A. No lesions were found in the baseline group or in the control or CU groups at 10 days. For 20 and 30 days, a dramatic and similar increase in fatty streak was found in the control and CU groups, with no differences between these groups. Concerning the presence of fatty streaks in thoracic aortas, Figure 4B shows results similar to those in Figure 4A for the baseline group and for the control and CU groups at 10 days. The control and CU groups at 20 and 30 days showed an increase in fatty streaks; this increase was significantly lower in the CU group than in the control group at 30 days. The histolog-
A study of fatty streaks in the abdominal aorta is presented in Figure 4C. There was an absence of fatty streaks in the baseline group and in the groups studied at 10 days and 20 days. After 30 days, an increase in this lesion was found in both experimental groups, although a significantly higher lesion was shown in the control group compared with the CU group.

TC, EC, and free cholesterol (FC) were determined in all aortic fractions (Table). The aortic fraction with the highest concentration of cholesterol was the aortic arch, followed by thoracic and abdominal aortas. The aortic fraction with more significant differences between the groups and among the times was the aortic arch. Compared with the control group, the CU group showed significantly higher concentrations of TC and EC in the aortic arch at 30 days. However, only TC concentration was significantly higher in the CU group versus the control group at 30 days. No differences were found in TC, EC, and FC between the control and CU groups in the aortic fraction at 10 and 20 days. Briefly, TC, EC, and FC concentrations were significantly different among the periods of time (at baseline and at 10, 20, and 30 days) for both experimental groups in all aortic fractions; the highest cholesterol level always corresponded with the final period of time. As a marker of lipid oxidation in the aorta, the MUFA/PUFA index was determined, as shown in the Table. All rabbits had the highest values in the abdominal aorta, followed by the thoracic aorta and, finally, the aortic arch. Significant differences were found at the various times, and the highest susceptibility to oxidation was at 30 days. On the other hand, rabbits fed curcumin showed a lipid oxidation susceptibility index that was significantly higher in all aortic fractions than that for the control group at 20 and 30 days.

Discussion

The best-known antioxidant mechanism of curcuma and its components is their capacity to eliminate reactive oxygen species, such as hydroxyl radical, superoxide radical, singlet oxygen, and NO. The results of the present study
show other points of view regarding the antioxidant mechanism of curcuma extract by protection of the endogenous antioxidants from oxidative damage.

Lipid peroxidation is a fundamental process in atherogenesis. LDL is modified by free radical–mediated reactions, both in their lipid and protein moieties, leading to the alterations that are the starting point for the first events in the outcome and development of the atherogenic process. Because plasma contains various antioxidants, the extent to which LDL oxidation occurs in the circulation was thought to be limited until (at least) a sufficiently active amount of such antioxidants was available. In the LDL in the arterial wall, the lipid peroxidation proceeds as a chain reaction, which may be terminated only by suitable antioxidants within the LDLs themselves. Moreover, the presence of several co-antioxidants is also required for effectively scavenging free radicals, because some antioxidant molecules, per se, would act paradoxically as pro-oxidant once activated in the lipid moiety. This is the case for α-tocopherol: its interaction with a peroxyl radical converts it to an α-tocopheroxyl radical, which, in turn, is alternatively regenerated to α-tocopherol by specific co-antioxidants (such as coenzyme Q10); if the co-antioxidants fail, or are limited, a so-called tocopherol-mediated peroxidation has been identified in which the α-tocopherol itself does not act as a chain-breaking antioxidant but rather facilitates the transfer of radical reactions from the aqueous phase inside the lipophilic environment, thus mediating radical chain reactions within the lipid moieties.

The situation found in the plasma of the control rabbits suggests that even if α-tocopherol does not change, a great deal of oxidative damage still occurs. In fact, plasma retinol and the essential co-antioxidant coenzyme Q10 were lost, and their concentrations were drastically reduced within the first 10 days of treatment. A concomitant increase in peroxidation products was also found in the control group, confirming that coenzyme Q10 (as well as retinol) is essential for antioxidant activity by α-tocopherol to attenuate plasma peroxidation.

Curcuma treatment (CU group) significantly buffered such impaired antioxidant unbalance, leading to levels of TBARS similar to baseline levels and to less reduced coenzyme Q10 levels. Moreover, similar (for 10 days) or higher plasma levels of α-tocopherol (20 and 30 days), compared with baseline levels, were found. Different reasons may account for the higher levels of α-tocopherol found in the CU group at 20 and 30 days: (1) It has been estimated that >90% of the body pool of α-tocopherol is located in the adipose tissue. This tissue does provide a source of vitamin E for the rest of the body. Maybe a mobilization of α-tocopherol from the adipose tissue of rabbits fed curcuma occurs, thus protecting the body against the oxidative damage produced during the development of atherosclerosis. (2) Plasma α-tocopherol concentration is also maintained by the secretion of nascent VLDL by the liver that is due to the hepatic α-tocopherol transfer protein that incorporates α-tocopherol into VLDL. Rabbits fed curcuma extract could transport more nascent VLDL by the liver that is due to the hepatic α-tocopherol transfer protein that incorporates α-tocopherol into VLDL. Rabbits fed curcuma extract could transport more nascent VLDL by the liver that is due to the hepatic α-tocopherol transfer protein that incorporates α-tocopherol into VLDL.
VLDL in plasma, increasing the levels of α-tocopherol. (3) Both these hypotheses plus a better plasma antioxidant defense of the CU group, which recycles the α-tocopherol radical, could also help us to understand the high plasma levels of α-tocopherol at 20 and 30 days.

Concerning the histological analysis, the intensity and extension of the lesions in the aortas of the control and CU groups are in accordance with the study of Wójcicki et al., in the sense that the highest grade of lesion was found in the aortic arch, followed by the thoracic aorta and, finally, by the abdominal aorta (Figures 4 and 5). This finding could indicate a major susceptibility of the aortic arch to LDL permeability, leading to the conclusion that the antioxidant mechanisms are not capable of protecting the high levels of modified LDL deposited in the intima because of the rapid and intense development of the fatty streak in both groups (control and CU) at 20 and especially at 30 days (Figures 4 and 5), when LDL susceptibility to oxidation increased. This is also supported by a study reporting that a higher cholesterol accumulation and a lower MUFA/PUFA index (a marker of lipid oxidation susceptibility) are found in the aortic arch compared with the thoracic and abdominal aortas.

All the morphological modifications took place after the antioxidant systems were impaired. In fact, the main changes were found after 20 days, and in any case, curcuma treatment (which corresponded to better antioxidant status and lower levels of TBARS in plasma and LDL) gave better morphological parameters in the thoracic and abdominal aortas.

Why did the CU group report higher levels of TC in all aortic fractions at 30 days despite having fewer histological lesions and similar plasma and LDL TC values? A possible explanation is that this cholesterol comes from LDL that is more resistant to oxidation. Thus, HDL would remove the excess of cholesterol from peripheral tissues before the macrophage uptake of these LDL particles, thus generating foam cells and attenuating the development of atherosclerosis. This is also supported by the fact that rabbits fed curcumin extract had a significantly higher aortic MUFA/PUFA index than did the control group, inasmuch as their plasma antioxidant mechanisms were impaired. In fact, the main changes were found after 20 days, and in any case, curcuma treatment (which corresponded to better antioxidant status and lower levels of TBARS in plasma and LDL) gave better morphological parameters in the thoracic and abdominal aortas.

Figure 5. Histological photomicrographs of aortic segments from atherosclerotic rabbits. Photomicrograph A, No lesion (score 1) on luminal surface of aortic arch (H&E stain). Bar = 100 μm. This type of lesion was observed in the baseline group and in the control [C] and CU groups at 10 days for all aortic segments. Photomicrograph B, High subintimal deposits of foam cells and extracellular lipids showing the extension and thickness of the fatty streak (H&E stain, score 4). Bar = 100 μm. This type of lesion was observed in both treatment groups (C and CU), mainly at 30 days. Photomicrograph C, Development of fatty streak in the intima of abdominal aortoiliac bifurcation (H&E stain, score 2). Bar = 100 μm. This type of lesion was more observed in group C at 20 days, when the score was mainly 2 in the thoracic aorta. Photomicrograph D, Development of fatty streak in the intima of abdominal aortoiliac bifurcation (H&E stain, score 2). Bar = 100 μm. This lesion (score 2) in the abdominal aorta at 30 days was more frequent in the CU group than in group C, for which the lesion was scored 3.


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