Extra-Virgin Olive Oil Increases the Resistance of LDL to Oxidation More than Refined Olive Oil in Free-Living Men with Peripheral Vascular Disease[1]

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ABSTRACT Patients with peripheral vascular disease (Fontaine stage II) are characterized by ischemia of the lower extremities, atherosclerosis and alteration of blood coagulation and fibrinolysis. A randomized, two-period, crossover design was used to compare the effects of extra-virgin (VO) and refined olive (RO) oils on plasma lipids and lipoprotein composition and LDL oxidation susceptibility in free-living men with peripheral vascular disease. The oils differed in their antioxidant profile (α-tocopherol: 300 vs. 200 mg/kg; phenolic compounds 800 vs. 60) and concentration but not in their fatty acid composition. Subjects were randomly assigned to two groups. The first group ($n = 12$) received VO with which to freely cook all meals for 3 mo, followed by a 3-mo wash-out period; they then received RO for the final 3 mo. The second group ($n = 12$) consumed the oils in the opposite order. Energy, fat, polyunsaturated fatty acids (PUFA) and α-tocopherol intakes were not different when patients consumed the two oils. Profiles of the major fatty acids in plasma and LDL were not different after consumption of VO and RO. The slope of the line for LDL oxidation vs. the line for copper concentration was significantly higher after the intake of RO than after the intake of VO. Total LDL taken up by macrophages was significantly greater when the men consumed RO rather than VO. We suggest that antioxidants present in VO may protect LDL against oxidation more than does RO in men with peripheral vascular disease. J. Nutr. 129: 2177–2183, 1999.

KEY WORDS: • *lipoproteins* • *LDL* • *olive oil* • *peripheral vascular disease* • *plasma fatty acids*

Ischemia of the lower extremities or intermittent claudication is characterized by arterial stenosis due to arteriosclerosis obliterans, often complicated by coronary artery disease and leading to unstable angina or acute myocardial infarction (Kannel et al. 1970). A lower incidence of coronary heart disease (CHD)[3] in Mediterranean countries has been correlated with a diet that is rich in fruit, vegetables, legumes and grains (Keys 1995, Serra-Majem et al. 1995). In these countries, the total amount of daily energy derived from fat represents at least 35% because of the high consumption of olive oil (Moreiras-Varela 1989, Serra-Majem et al. 1995).

The concentration of antioxidants in oils is influenced by the oil extraction procedures. Extra-virgin olive oil, which is obtained from the first pressing of the ripe fruit, has a high content of unsaponifiable matter that is rich in α-*dl*-tocopherol and phenolic derivatives, i.e., tyrosol and hydroxytyrosol, both of which exhibit antioxidant properties (Litridou et al. 1997). This oil conserves all lipidic and antioxidant qualities of the olives (Papadopoulos and Boskou 1991). However, refined olive oil loses most of those antioxidants during refining procedures, although it has the same fatty acid composition as extra-virgin olive oil (Uceda and Hermoso, 1997). Virgin olive oil is the product obtained exclusively from ripened olives by physical procedures, including cleaning of fruits with water, milling, cold pressing and centrifugation. The product from unfermented olives gives a low free-acidity oil, usually <1.0%, named and classified according to the European Union standards as "extra-virgin olive oil" (EU Directives 2568/91 and 656/95). A product with acidity >1% but <2% is called "fine virgin olive oil," and that with acidity >2% but <3.3% is called "ordinary virgin olive oil." Virgin olive oils contain relatively high amounts of unsaponifiable materials, mainly phenolics compounds (tyrosol derivatives, free sterols and their precursors such as squalene) and tocopherols (α-tocopherol and other compounds responsible for flavor (Uceda and Hermoso 1997).

Low quality and high acidity virgin olive oils are usually refined by physical and chemical procedures. Basically, they are neutralized with sodium hydroxide, and the fatty acid soaps formed are eliminated by cleansing with water and centrifugation. In addition, they are passed through diatomeae or

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[3] Abbreviations used: BMI, body mass index; CHD, coronary heart disease; CVD, cardiovascular disease; DiI, 3,3′-dioctadecylindocarbocyanin; MDA, malondialdehyde; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; PVD, peripheral vascular disease; RO, refined olive oil; TBA, thiobarbituric acid; TBARS, thiobarbituric acid–reactive substances TCA, trichloroacetic acid; VO, extra-virgin olive oil.

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charcoal filters and extracted with hexane at low temperature and vacuum. The resulting oils are mostly colorless and aroma free; their fatty acid composition is close to that of virgin olive oil, but they lack the majority of unsaponifiable components, particularly phenolic compounds and tocopherols. Thus, refined olive oils have a lower stability than virgin olive oils. The category "olive oil" is a mixture of the refined oil with minor amounts of virgin olive oils, which results in the typical yellow to green color and a flavor close to that of the virgin oils.

The influence of dietary fatty acids and antioxidants on the resistance of lipoproteins to oxidation is of relevance in the development of atherosclerosis (Witztum and Steinberg 1991). Tissue membranes that are rich in monounsaturated fatty acids (MUFA) are less susceptible to oxidation by free radicals than membranes rich in PUFA (Bonanome et al. 1992). Several studies were performed in our laboratory to investigate the effect of dietary fat and dietary antioxidants in different biological models. Recently, we (Ramirez-Tortosa et al. 1997) studied the effect of different dietary fats on coenzyme Q_{10} and the hydroperoxide content of liver mitochondria in rabbits with experimental atherosclerosis. The use of extra-virgin olive oil in the dietary treatment of atherosclerosis appears to be a valid alternative for maintaining adequate levels of coenzyme Q_{10} and hydroperoxides in liver mitochondria, thus limiting their rate of lipid peroxidation. We also reported that the intake of a diet rich in extra-virgin olive oil leads to a decreased plasma-lipid concentration and a lower susceptibility of LDL to oxidation in rabbits with experimental atherosclerosis (Ramirez-Tortosa et al. 1998). In another model of oxidative stress (physical exercise), Mataix et al. (1998) found that extra-virgin olive oil affords better protection than dietary α-tocopherol against lipid peroxidation. De la Puerta et al. (1997) demonstrated anti-inflammatory activity of sterols and triterpenic dialcohols from unsaponifiable extra-virgin olive oil and Visioli et al. (1995) described the inhibition of LDL oxidation in vitro by olive oil constituents. However, no available studies have compared the effect of extra-virgin vs. refined olive oil consumption on the resistance of LDL oxidation in humans with atherosclerosis obliterans.

Spain is one of the world's major producers of olive oil (extra-virgin and refined); the Spanish population consumes high amounts of these oils. Nevertheless, it has a relatively low incidence of CHD (Serra-Majem, et al. 1995); however, lower-extremity ischemia remains as a prevalent disease within cardiovascular disease (CDV) in males.

The aim of this study was to evaluate the effect of both extra-virgin and refined olive oils on plasma lipid and lipoprotein composition and on LDL-oxidation susceptibility as markers of atherosclerosis risk in free-living men with peripheral vascular disease (Fontaine stage II). A crossover study was designed to avoid the seasonal variation of the diet and lifestyle habits of patients.

SUBJECTS AND METHODS

Subjects. A total of 24 subjects diagnosed with peripheral vascular disease (PVD) (Fontaine stage II) aged 69.9 ± 2.1 y were recruited from a group of outpatients with intermittent claudication who were being monitored by the Department of Vascular Surgery, Clinic Hospital, University of Granada, Spain. The Fontaine degree II is a degree of peripheral arterial obstruction characterized by intermittent claudication and pain while walking that disappears in a few minutes after stopping.

All subjects underwent a complete physical examination and their medical histories were compiled. Patients with endocrine or metabolic disturbances such as diabetes mellitus, hypothyroidism and obesity, those affected by cardiac episodes such as angina pectoris, previous acute myocardial infarction and some other chronic diseases were excluded from the study. PVD was diagnosed and classified by functional and clinical variables. Yao indices, defined as the ratio of systolic blood pressure in the inferior limb to the superior limb, usually measured at the ankle and arm, respectively, were calculated to recruit peripheral vascular patients in the second stage of Fontaine. In healthy subjects, both pressures are markedly similar; however, when leg arteries are partly obliterated due to atherosclerosis and thrombosis, the pressure behind the stenosis site drops. Thus, the ankle pressure is lower than the arm pressure; hence, a ratio <1 is considered pathological. Resting patients classified in stage II of Fontaine have a Yao index of ~0.5–0.7. The presence and distribution of atheroma plaques in both legs and arms were assessed for each subject by means of a scanning duplex; treadmill running was determined as the distance in meters covered after a 5-min run. All participants gave their written informed consent to participate in the study, which was approved by the Ethics Committee of the Clinic Hospital. All procedures followed institutional guidelines.

Study design. A randomized, two-period crossover design was used to compare the effects of extra-virgin olive oil and refined olive oil on plasma lipid and lipoprotein composition as well as on LDL oxidation susceptibility and oxidized LDL uptake in men with PVD (Fontaine stage II). The study lasted for 9 mo and subjects were randomly assigned to two groups. The first group ($n = 12$) was provided extra-virgin olive oil (VO) for 3 mo with which to cook all meals freely, followed by a 3-mo wash-out period (baseline); they were then provided refined olive oil (RO) for the final 3 mo. The second group ($n = 12$) consumed the oils in the opposite order. Patients consumed their usual diets during the wash-out period. Each patient was examined at the beginning of the study and at the end of each 3-mo period. A complete clinical examination was performed by the Department of Vascular Surgery at the Clinic Hospital and a nutritional survey of food habits and lifestyle was also performed.

Diet. Before participating in the study, all men (and their wives) attended a session of dietary counseling for 1 h to receive instruction on the basic concepts of food composition and characteristics, appropriate portions, the effects of alcohol consumption and recommended cooking techniques. Patients were given strict instructions about what to eat at home and were asked to replace most of their usual saturated fat intake (butter, margarine, lard and visible fat on meat) with monounsaturated fat (refined olive oil or extra-virgin olive oil). The recommendation to all patients was to increase the consumption of fruits, legumes and vegetables to ensure adequate intake of fiber and antioxidant vitamins. Cereal products and fruit juice–based soft drinks were not prohibited. Restaurant and carry-out food was restricted to a maximum of once a week, and subjects were advised to walk at least 1 km/d and to stop smoking. The fatty acid composition of refined and virgin-olive oils used in the study, as well as the α-tocopherol and phenolic derivatives contents, are shown in **Table 1**.

Body weight and height were recorded at the beginning and every 3 mo until completion of the study, and body mass indices (BMI) were calculated. Compliance with dietary and lifestyle recommendations was assessed 1 mo after the start of the study and every 3 mo thereafter. Those not complying with dietary and lifestyle recommendations were withdrawn from the study. A dietary survey for each patient at 0, 3, 6 and 9 mo was made to estimate current dietary energy, protein, total carbohydrates, total fiber, fat, type of fat, and α-tocopherol, retinol and ascorbic-acid intakes. Adherence to recommendations was determined by two of the authors (GU and ML-J) using food records of measured and weighed food intake and all recipes of homemade dishes for 1 wk (Montellano et al. 1997).

Once the records were quantified, all foods were codified to introduce different items into a computer program. Nutrient intakes were evaluated using the program "Alimentacion y Salud" (Food and Health), developed in the Institute of Nutrition of the University of Granada (Mataix et al. 1994a). The food database used was previously published in "Spanish Food-Composition Tables" (Mataix et al. 1994b).

Determination of fatty acid composition in oils. To obtain the total fatty acid composition of the oils, fatty acids methyl esters were

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TABLE 1

Fatty acid composition and unsaponifiable matter of extra-virgin and refined olive oils used as lipid sources for dietary treatment in free-living men with peripheral vascular disease (Fontaine stage II)

	Extra-virgin olive oil	Refined olive oil
Fatty acid, *g/100g*		
16:0	6.6	9.1
16:1(n-9)	0.1	0.1
16:1(n-7)	0.4	0.6
18:0	2.8	3.4
18:1(n-9)	83.1	78.6
18:2(n-6)	5.1	6.2
18:3(n-6)	0.4	0.5
18:3(n-3)	0.6	0.4
Unsaponifiable matter, *mg/kg*		
α-Tocopherol	300	200
Squalene	4277	2598
Polyphenols (as caffeic acid)	800	60

formed according to the method of Lepage and Roy (1986). The sample (50 μL) was weighed precisely in glass tubes and dissolved in 2 mL of methanol/benzene (4:1 v/v); 50 μg of the fatty acid 13:0 and 9 μmol/L BHT were added to the samples as internal standard and antioxidant, respectively. Acetyl chloride (200 μL) was added slowly; then tubes were closed and subjected to methanolysis at 100°C for 1 h. After tubes were cooled in water, 5 mL of a 0.43 mol/L K_2CO_3 solution was slowly added to stop the reaction and neutralize the mixture. The tubes were then shaken and centrifuged; the benzene upper phase was removed and transferred to another glass tube to be dried under nitrogen and resuspended to 100 μL with hexane. A gas-liquid chromatograph (Model HP-5890 Series II, Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector was used to analyze fatty acids as methyl esters. Chromatography was performed using a 60-m capillary column, 32 mm i.d. and 20 mm thickness, impregnated with Sp 2330 FS (Supelco, Bellefonte, PA). The injector and the detector were maintained at 250 and 275°C, respectively; nitrogen was used as carrier gas, and the split ratio was 29:1. The temperature programming was as follows: initial temperature, 80°C, 15°C/min to 165°C, 3°C/min to 211°C, hold 10 min.

Vitamin E determination in oils. The concentration of vitamin E in the samples was determined by HPLC with a Beckman in-line Diode Array Detector, model 168 (Fullerton, CA), a Water (Milford, MA) 717 plus autosampler and a Beckman Ultrasphere 5-μm silica (250 mm × 4.6 mm) column. The eluates were detected at 292 nm. The flow rate was 2 mL/min and the mobile phase was methanol/water (99:1, v/v). For the treatment of the samples, the technique of Ueda and Igarashi (1990) was followed. Samples were dissolved directly in hexane in proportions of 1:10 v/v for olive and virgin olive oils and shaken for 2 min; 100 μL of this solution was injected into the column, with 5,7-dimethyltocol as internal standard. The tocopherol peaks were identified by predetermining the retention times of individual tocopherol standards; results are expressed as tocopherol equivalents.

Colorimetric evaluation of total phenolic acids in oils. The total phenols were extracted from the oils according to the method described by Vázquez-Roncero et al. (1973). Oil (10 g) was dissolved in 50 mL hexane; the phenolic solution was extracted three times with 30 mL of a methanol/water mixture (60:40, v/v) and shaken for 2 min. The combined extracts were evaporated completely in a rotary evaporator at 40°C, dissolved in 1 mL methanol and stored at −80°C.

The concentration of total phenols in the methanolic extracts was estimated with the Folin-Ciocalteu reagent using caffeic acid as standard. The procedure consisted of dilution of 0.1 mL of the extracts with water to 5 mL and the addition of 0.5 mL Folin-Ciocalteu reagent. After 3 min, 1 mL of Na_2CO_3 (35 g/L, wt/v) solution was added, mixed with a vortex and incubated at room temperature for 1 h. Absorbance was measured after 1 h at 725 nm against a blank.

Squalene determination in vegetable oils. A simple and rapid analytical method for the quantification of squalene in vegetable oils was used as described (Lanzón et al. 1995). The method consists of a cold alkaline methylation (KOH 2 mol/L in methanol) of the oil sample (0.2 g of oil) diluted with hexane (5 mL) followed by quantitation by gas chromatography. The internal standard used was squalene. A calibration curve was obtained by using different standard solutions.

Blood sampling and biochemical determinations. Blood for plasma lipid and lipoprotein determinations was drawn from fasting patients at 0, 3, 6 and 9 mo. Blood (25 mL) was collected by venipuncture into EDTA-containing vacutainer tubes. Samples were kept on ice before centrifugation at 1700 × *g* for 15 min at 4°C to obtain plasma.

Plasma total cholesterol and triglyceride concentrations were measured by enzymatic colorimetric methods using commercial kits (Preciset Cholesterol and Peridochrom Trigliceridos PAP, respectively, Boehringer Mannheim, Germany). Serum HDL cholesterol was determined in the supernatant after precipitation with phosphotungstic acid and magnesium chloride as described (Burstein et al. 1970). LDL cholesterol was calculated according to Friedewald et al. (1972).

Lipoprotein isolation. VLDL, LDL and HDL were isolated by a single discontinuous density-gradient ultracentrifugation in a vertical rotor using a discontinuous NaCl/KBr density gradient (Chung et al. 1981). Isolated LDL were exhaustively dialyzed against 150 mmol/L NaCl, pH 7.4, at 4°C overnight. LDL protein content was measured according to Bradford (1979) using bovine serum albumin as a standard. Plasma and LDL fatty acid patterns were determined by gas-liquid chromatography as previously described above for the oils.

Determination of LDL oxidation susceptibility. LDL protein (200 mg/L) was oxidized in the presence of copper (Cu^{2+}) (1.25, 2.5, 5, 10 and 20 μmol/L) in PBS for 24 h at 37°C. After incubation, oxidation was stopped by cooling samples to 4°C and adding 100 mmol/L EDTA and 4.5 μmol/L BHT (Jialal et al. 1991). The lipid peroxide content of oxidized LDL was determined as thiobarbituric acid–reactive substances (TBARS). Oxidized LDL were combined with 1 mL of a mixture of trichloroacetic acid (TCA), thiobarbituric acid (TBA) and hydrochloric acid (0.92 mol/L TCA, 25.7 mmol/L TBA, 25 mol/L HCl) and mixed with a vortex as described by Buege and Aust (1978). The solution was heated for 20 min in a water bath at 100°C for color development. After cooling, samples were centrifuged at 1700 × *g* for 20 min. The supernatant was taken, and absorbance was determined at 532 nm against a blank containing all reagents except LDL. Absorbance units were converted to malondialdehyde (MDA) equivalents/mg LDL protein using a standard curve obtained with 1,1,3,3-tetramethoxypropane. The MDA concentrations formed after 24 h of LDL oxidation with different copper concentrations were plotted against copper concentrations; the slopes of the corresponding curves were calculated using Slidewrite software (Sunnyvale, CA).

Labeling and macrophage uptake of oxidized LDL. LDL was labeled with 3,3′-dioctadecylindocarbocyanin (DiI) by the method of Zouhair and Edna (1993). A stock solution of fluorescent probe DiI (Molecular Probes, Eugene, OR) was prepared by dissolving 30 mg DiI in 1 mL of dimethyl sulfoxide. This solution was added to LDL samples to a final ratio of 0.32 mmol DiI/mg LDL protein. After the incubation of this mixture for 18 h at 37°C, labeled LDL were isolated by ultracentrifugation (189,000 × *g* for 24 h at 4°C) and dialyzed against normal and filter-sterilized PBS (0.22 μm). A standard solution of DiI-LDL was prepared in PBS to give a concentration range of 100-1600 mg/L. Fluorescence was determined in a Perkin Elmer Model LD-50 (Norwalk, CT) with excitation and emission wavelengths set at 520 and 578 nm, respectively.

To assay oxidized-LDL macrophage uptake, we used the U-937 macrophage human cell line obtained from American Tissue Culture Collection (ATCC, Rockville, MD). U-937 cells were maintained in RPMI-1640 media containing 100 mL/L heat-inactivated (56°C for 30 min) fetal bovine serum; cells were plated at 2 × 10^6 macrophages

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TABLE 2

Calculated daily intake of energy and nutrients in free-living men with peripheral vascular disease (Fontaine stage II) after crossover dietary treatments using extra-virgin olive oil and refined olive oil as the main source of dietary lipid[1]

	Peripheral vascular disease	
	Extra-virgin olive oil	Refined olive oil
Energy, *kJ/d*	10216 ± 423	10134 ± 445
Protein, *g/d*	78.9 ± 3.4	79.0 ± 3.4
Carbohydrates, *g/d*	296.2 ± 12	290.2 ± 13
Fiber, *g/d*	22.4 ± 1.9	19.3 ± 1.1
Fat, *g/d*	87.2 ± 5.1	85.1 ± 4.9
Saturated, *% total energy*	7.5 ± 0.2	7.8 ± 0.2
Monounsaturated, *% total energy*	15.1 ± 0.6	14.6 ± 0.8
Polyunsaturated, *% total energy*	0.76 ± 0.05	0.75 ± 0.06
PUFA/Saturated[2]	0.5 ± 0.03	0.5 ± 0.04
PUFA/MUFA	0.2 ± 0.01	0.2 ± 0.01
Cholesterol, *mg/d*	261 ± 22	260 ± 21
α-Tocopherol, *mg/d*	6.7 ± 0.5[b]	4.1 ± 0.7[a]
Retinol, *μg/d*	924 ± 249	832 ± 250
Ascorbic acid, *mg/d*	142 ± 14	127 ± 13

[1] Values are means ± SEM, $n = 24$. Values in a row with different letter superscripts differ, $P < 0.05$.
[2] PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids.

per well. DiI-LDL protein (100 g/L) was incubated with U-937 cells for 24 h at 37°C in 5% CO_2/95% air. After incubation, the cells and medium were centrifuged at 1700 × *g* for 15 min and fluorescence was determined in the supernatant. Cells were added to 1 mL of isopropanol and shaken for 2 min. Isopropanol-extracted DiI was centrifuged at 1700 × *g* for 15 min, and fluorescence was determined in the supernatant. The percentage of fluorescence relative to initial value was considered to be the macrophage LDL uptake.

LDL tocopherol, retinol and β-carotene determinations. LDL-protein (200 μg) was mixed with 250 μL cold methanol containing 100 μL α-*dl*-tocopherol acetate as internal standard and extracted with hexane (2.5 mL). The hexane phase was dried under N_2 and reconstituted in ethanol (50 μL) as described by Frei and Gaziano (1993). Ethanol extracts were analyzed by reversed-phase HPLC on a Lichrospher 60 RP-select B column (Merck, Darmstadt, Germany) using methanol/water (99:1 v/v) as the mobile phase and a flow rate of 1 mL/min. Under these conditions, retinol, β-carotene and α-tocopherol were determined simultaneously.

Statistical analysis. Values in the text are means ± SEM To evaluate the effect of the diets, an ANOVA for crossover studies was performed using the SPSS 8.0 version for windows (Salem, OR). Homogeneity of variances was tested by Levene's test. When variances were heterogeneous, data were transformed to natural logarithms or reciprocals. When a significant effect was found, post-hoc comparisons of means were made using the *t*-adjusted Bonferroni test (Dixon et al. 1990). Differences were considered different at $P < 0.05$.

TABLE 3

Plasma triglycerides, total cholesterol, LDL cholesterol and HDL cholesterol in free-living men with peripheral vascular disease (Fontaine stage II) after crossover dietary treatments using extra-virgin and refined olive oils as the main source of dietary lipid[1]

	Peripheral vascular disease	
	Extra-virgin olive oil	Refined olive oil
	mmol/L	
Triglycerides	1.81 ± 0.17[b]	1.30 ± 0.09[a]
Total cholesterol	6.18 ± 0.22	6.40 ± 0.22
LDL cholesterol	4.82 ± 0.19	5.03 ± 0.21
HDL cholesterol	1.38 ± 0.06	1.35 ± 0.06

[1] Values are means ± SEM, $n = 24$. Values in a row with different letter superscripts differ, $P < 0.05$.

TABLE 4

Lipid lipoprotein composition in free-living men with peripheral vascular disease after crossover dietary treatments using extra-virgin olive oil and refined olive oil as the main source of dietary lipid[1]

		Peripheral vascular disease	
		Extra-virgin olive oil	Refined olive oil
		% total lipoprotein lipids	
Triglycerides	VLDL	59.9 ± 2.0	63.7 ± 1.4
	LDL	8.4 ± 0.9[b]	6.3 ± 0.4[a]
	HDL	10.9 ± 0.8	9.5 ± 0.9
Phospholipids	VLDL	16.1 ± 1.2	16.7 ± 0.3
	LDL	21.5 ± 0.5	19.1 ± 0.8
	HDL	35.8 ± 2.1	38.4 ± 2.5
Total cholesterol	VLDL	23.0 ± 1.6	21.3 ± 1.2
	LDL	69.9 ± 1.0[a]	74.5 ± 0.9[b]
	HDL	53.1 ± 2.2	52 ± 2.7
Cholesterol esters	VLDL	12.8 ± 1.2	12.9 ± 1.05
	LDL	50.7 ± 0.8[a]	57.7 ± 1.5[b]
	HDL	39.4 ± 2.5	41.3 ± 2.3
Free cholesterol	VLDL	10.1 ± 0.6[b]	8.4 ± 0.5[a]
	LDL	19.2 ± 0.7[b]	16.7 ± 0.9[a]
	HDL	13.8 ± 1.1[b]	10.7 ± 0.5[a]

[1] Values are means ± SEM, $n = 24$. Values in a row with different letter superscripts differ, $P < 0.05$.

RESULTS

Weight and BMI did not change in the patients over the study. Body weights and BMI were 72.8 ± 2.8 kg and 24.8 ± 1.1 kg/m^2, respectively. Smoking habits were not altered during the crossover study. Physical activity, treadmill running and clinical and functional variables (e.g., Yao indices) did not differ between the VO and RO periods.

Intakes of energy, proteins, carbohydrates, lipids and fiber did not differ between the two diet periods. In addition, the proportion of saturated, mono- and polyunsaturated fatty acids and the intake of cholesterol, retinol and ascorbic acid also did not differ. However, the intake of α-tocopherol was greater when subjects consumed VO than when they consumed RO **(Table 2)**. The fatty acid compositions of the extra-virgin and refined olive oils used in this study for the dietary treatment of PVD patients were quite similar (Table 1). However, the unsaponifiable matter in extra-virgin olive oil reflected a higher concentration of α-tocopherol and polyphenols than did the refined olive oil.

Plasma triglyceride concentrations were significantly lower when patients ingested RO than when they ingested VO, but

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TABLE 5

Profile of the major fatty acids of plasma and LDL, and α-tocopherol, retinol and β-carotene contents of LDL in free-living men with peripheral vascular disease (Fontaine stage II) after crossover dietary treatments using extra-virgin and refined olive oil as the main source of dietary lipid

	Peripheral vascular disease	
Fatty acids	Extra-virgin olive oil	Refined olive oil
	g/100 g fatty acids	
Plasma		
16:0	21.4 ± 0.34	20.8 ± 0.5
18:0	6.6 ± 0.5	6.7 ± 0.5
Total saturated	28 ± 0.3	28 ± 0.5
18:1(n-9)	25.6 ± 0.8	25.1 ± 0.8
Total monounsaturated	31.4 ± 0.8	31.9 ± 0.7
18:2(n-6)	25.6 ± 0.7	26.3 ± 0.8
20:4(n-6)	6.8 ± 0.3	6.7 ± 0.3
18:3(n-3)	0.33 ± 0.02	0.37 ± 0.03
20:5(n-3)	0.88 ± 0.01	0.88 ± 0.01
22:6(n-3)	2.7 ± 0.15	2.7 ± 0.14
Total polyunsaturated	40.1 ± 0.9	39.7 ± 0.8
LDL		
16:0	21.5 ± 1.1	20.2 ± 1.2
18:0	7.6 ± 0.6	6.9 ± 0.15
Total saturated	30.3 ± 0.8	29.5 ± 0.4
18:1(n-9)	22.0 ± 0.9	22.1 ± 0.5
Total monounsaturated	29.0 ± 1.2	27.3 ± 0.6
18:2(n-6)	26.4 ± 0.8	28.6 ± 0.8
20:4(n-6)	6.7 ± 0.5	6.2 ± 0.3
18:3(n-3)	0.25 ± 0.01	0.22 ± 0.01
20:5(n-3)	0.96 ± 0.1	0.85 ± 0.1
22:6(n-3)	2.54 ± 0.17	2.26 ± 0.12
Total polyunsaturated	40.4 ± 1.6	43.1 ± 0.6
	mg/mg LDL protein	
α-Tocopherol	17.1 ± 2.5	15.1 ± 3.4
Retinol	0.30 ± 0.04	0.27 ± 0.03
β-Carotene	3.2 ± 0.8	3.1 ± 0.8

[1] Values are means ± SEM, $n = 24$.

there were no differences between periods in HDL-cholesterol, total cholesterol or LDL cholesterol (**Table 3**).

LDL triglycerides and VLDL, LDL and HDL free cholesterol were significantly higher when the patients consumed VO rather than RO, whereas LDL total cholesterol and LDL cholesterol esters were lower after VO intake (**Table 4**).

Profiles of the major fatty acids of plasma and LDL did not differ after consumption of VO and RO. No significant differences were found for LDL α-tocopherol, retinol and β-carotene levels between the VO and RO periods (**Table 5**). By contrast, the slope of the line obtained when LDL oxidation vs. copper concentration was plotted was significantly higher after the intake of RO compared with VO (**Fig. 1**). Moreover, the macrophage uptake of plasma-oxidized LDL and LDL macrophage uptake were significantly higher after the intake of RO compared with VO (**Fig. 2**)

DISCUSSION

We compared the effects of VO and RO on plasma lipids and lipoprotein composition and oxidative susceptibility of LDL in free-living men suffering occlusive PVD without dramatically altering their lifestyles. The daily physical activity and smoking frequency did not change during the course of the crossover study (data not shown).

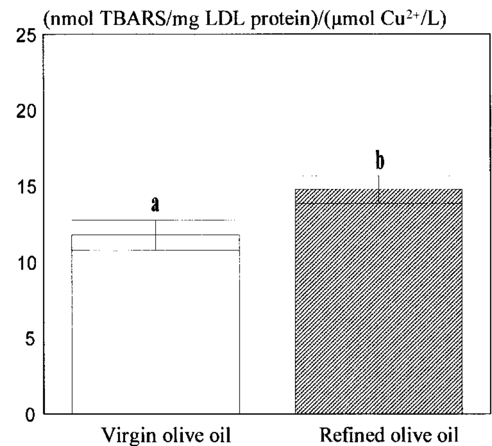

FIGURE 1 Cu^{2+}-mediated LDL oxidation in men with peripheral vascular disease (Fontaine stage II) after crossover dietary treatments using extra-virgin and refined olive oils as the main source of dietary lipid. Values are means ± SEM of the slope of the lines generated when LDL is oxidized in the presence of copper (Cu^{2+}), $n = 24$. Bars with a different letter are significantly different, $P < 0.05$.

The intake of VO and RO did not have a substantial effect on the clinical and functional variables or the plasma lipid and LDL compositions in patients; however, after consuming RO, the patients had lower plasma total and LDL triglyceride concentrations and after the intake of VO, they had higher percentages of free cholesterol in HDL. The reason for these results may be that the intake of energy derived from fat, as well as the dietary fatty acid pattern, were very similar for both periods of olive oil consumption. Similarly, our results suggest that the unsaponifiable fraction of VO, composed mainly of lineal isoprenoids (i.e., squalene, sterols, α-tocopherol and phenolic compounds Uceda and Hermoso 1997) may influence the metabolism of both LDL and HDL in humans by unknown mechanisms. Wiseman et al. (1996) found similar changes for plasma lipoproteins in rabbits fed extra-virgin olive oil, refined olive oil and Trisun high oleic sunflower seed oil; our laboratory (Ramirez-Tortosa et al. 1998) also showed that rabbits with experimental atherosclerosis fed refined olive

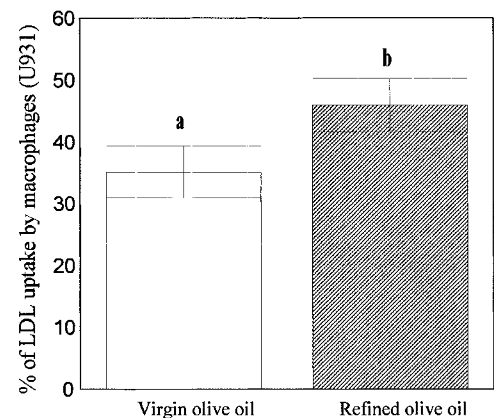

FIGURE 2 Macrophage uptake of plasma oxidized LDL in free-living men with peripheral vascular disease (Fontaine stage II) after crossover dietary treatments using extra-virgin olive oil and refined olive oil as the main source of dietary lipid. Values are means ± SEM, $n = 24$. Bars with a different letter are significantly different, $P < 0.05$.

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oil had lower plasma and LDL triglyceride concentrations than did rabbits fed extra-virgin olive oil.

The free radical process of lipid peroxidation is involved in the pathogenesis of atherosclerosis through the formation of oxidized LDL. Consequently, the prevention of LDL oxidation by exogenous antioxidants could have a great potential for prevention of this major disease (Esterbauer et al. 1992). The resistance of lipoproteins to lipid peroxidation is modulated by both dietary fatty acids and antioxidants. Studies have shown that the intake of MUFA in the diet can modulate the susceptibility of LDL to oxidative modification. The susceptibility of LDL to oxidation depends on the PUFA/MUFA ratio, their oleic acid and antioxidant contents, as well as on the size of the LDL particle (Aviram and Fuhrman. 1998, Parthasarathy et al. 1990).

In quantitative terms, α-tocopherol is the major antioxidant among those present in LDL; thus, it is considered the first line of defense against oxidation (Reaven et al. 1994). Furthermore, β-carotene and retinol have been shown to have important effects on increasing the lipoprotein resistance to oxidation (Jialal et al. 1991, Livrea et al. 1995, Tertov et al. 1998). It has been shown that LDL resistance varies widely among subjects; this variation is caused in part by differences in the α-tocopherol content of LDL, but also depends on other variables that are yet to be identified (Esterbauer 1992). In this study, the exclusive source of variation in the diet was the type of oil consumed as visible fat and indeed the intake of olive oil antioxidants, which was higher when the patients consumed VO rather than RO (Table 1). There were no differences in the LDL levels of α-tocopherol, retinol and β-carotene during the crossover study. However, the LDL susceptibility to oxidation, measured as the slope of the line reflecting TBARS formation, was significantly lower after the VO period than after RO consumption (Fig. 1). In addition, total LDL uptake by human macrophages was significantly greater in LDL isolated when the patients consumed RO rather than VO. These results agree with those of others, which show that extra-virgin olive oil increases the resistance of LDL to oxidation in rabbits (Wiseman et al. 1996) and in healthy subjects (Carmena et al. 1996)

The lower LDL oxidation rate, as well as the reduced uptake of oxidized LDL by macrophages in patients after VO intake, may be due to the high concentration of both phenolic compounds as well as other unsaponifiable compounds present in extra-virgin olive oil. Flavonoids and isoflavonoids as well as other linear isoprenoids exhibiting in vitro antioxidant activity (Baldioli et al. 1996) could help limit LDL peroxidation. Although in vivo studies on the kinetics and metabolism of olive oil phenolics are scarce, there is evidence that lipoproteins from animals fed phenol-rich olive oils are less susceptible to oxidation than those isolated from controls fed refined oils (Wiseman et al. 1996). Dietary supplementation in humans, using nutrients rich in polyphenols such as black or green tea (Serafini et al. 1994, licorice extract (Fuhrman et al. 1997), grapes (Meyer et al. 1997) or red wine (Fuhrman et al. 1995) has been associated with increased resistance of LDL to oxidation, increased plasma antioxidant capacity and inhibition of the development of aortic atherosclerotic lesions (Hertog et al. 1993). In this study, we estimated that 32–35 mg/d of total phenols were supplied by extra-virgin olive oil because the extra-virgin olive oil intake ranged from 40 to 42 g/d. Population groups in Mediterranean countries typically consume similar amounts of dietary antioxidants, which could explain in part the lower incidence of CHD in those areas.

In conclusion, antioxidants present in extra-virgin olive oil seem to protect more LDL against oxidation than does refined olive oil in men with PVD.

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