

# Phenolic Content of Virgin Olive Oil Improves Ischemic Reactive Hyperemia in Hypercholesterolemic Patients

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<b>OBJECTIVES</b>	The goal of this study was to evaluate the effects of the phenolic content of virgin olive oil on endothelial reactivity.
<b>BACKGROUND</b>	Endothelial-dependent vasodilatation is impaired during the postprandial state, and oxidative stress could play a key role in its development.
<b>METHODS</b>	Twenty-one hypercholesterolemic volunteers received two breakfasts, using a randomized sequential crossover design. Both arms received the same olive oil, but one had its phenolic acid content reduced from 400 to 80 ppm. Ischemic reactive hyperemia (IRH) was measured with a laser-Doppler procedure at baseline and 2 h and 4 h after oil intake. Postprandial plasma concentrations of lipid fractions, lipoperoxides (LPO), 8-epi prostaglandin-F <sub>2α</sub> , and nitrates/nitrites (NO <sub>(x)</sub> ) were obtained at baseline and after 2 h of the fat meal.
<b>RESULTS</b>	The intake of the polyphenol-rich breakfast was associated with an improvement in endothelial function, as well as a greater increase in concentrations of NO <sub>(x)</sub> (p < 0.001) and a lower increase in LPO (p < 0.005) and 8-epi prostaglandin-F <sub>2α</sub> (p < 0.001) than the ones induced by the low polyphenol fat meal. A positive correlation was found to exist between NO <sub>(x)</sub> and enhanced endothelial function at the second hour (r = 0.669; p < 0.01). Furthermore, a negative correlation was found between IRH and LPO (r = -0.203; p < 0.05) and 8-epi prostaglandin-F <sub>2α</sub> levels (r = -0.440; p < 0.05).
<b>CONCLUSIONS</b>	A meal containing high-phenolic virgin olive oil improves ischemic reactive hyperemia during the postprandial state. This phenomenon might be mediated via reduction in oxidative stress and the increase of nitric oxide metabolites. (J Am Coll Cardiol 2005;46:1864-8) © 2005 by the American College of Cardiology Foundation

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Atherosclerosis and cardiovascular disease are the major cause of death in developed countries (1). Endothelial dysfunction, characterized by reduced bioavailability of nitric oxide, is considered the first pathological symptom of atherosclerosis (2). Recent evidence suggests that oxidative stress plays a role in the process of endothelial dysfunction (3).

Several observational and epidemiological studies have associated the Mediterranean diet with a lower incidence of coronary artery disease (4). It has been postulated that the high content of certain antioxidant compounds on the diet may slow the atherogenic process by inhibiting oxidative damage and restoring endothelial function (5,6). Virgin olive oil is a major constituent of the Mediterranean diet (7). Olive oil, in addition to oleic acid, contains a range of micronutrients, such as phenolic compounds, which have been shown to possess antioxidant (8), anti-inflammatory (9), and antithrombotic (10) activities in cell cultures and in vitro studies. Our group has previously demonstrated that long-term consumption of a Mediterranean-type diet im-

proves endothelial dysfunction in patients with hypercholesterolemia (11). Two other studies, based on the consumption (sharp intake) of olive oil rich in phenolic compounds, have proven in vitro its ability to reduce the low-density lipoprotein susceptibility to oxidative modifications and to increase the antioxidant capacity of plasma (12,13). A recent study has shown its capacity to improve oxidative status in the postprandial state after a short period of consumption of olive oil rich in phenolic compounds (14). Nevertheless, at present there are no data showing a direct benefit of these compounds on endothelial function in vivo. Because of the short life of these micronutrients in plasma, to observe its potential benefit the studies should be carried out during the postprandial state, immediately after its intake.

The purpose of this study was to determine whether a meal containing virgin olive oil, rich in phenolic compounds, could improve endothelial function in hypercholesterolemic patients.

## METHODS

**Subjects.** Twenty-one hypercholesterolemic subjects (5 men and 16 women) from the Lipids and Atherosclerosis Unit at Hospital Universitario Reina Sofia (Cordoba, Spain) participated in the study. The average age was 59 years (5 patients; range, 53 to 68 years), with body mass index of

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#### Abbreviations and Acronyms

AUC	= area under the curve
HDL-C	= high-density lipoprotein cholesterol
IRH	= ischemic reactive hyperemia
LDL-C	= low-density lipoprotein cholesterol
LPO	= lipoperoxides
NO <sub>(x)</sub>	= nitrates/nitrites
TC	= total cholesterol
TG	= triglycerides
TRL	= triglyceride-rich lipoproteins
8-epi-F <sub>2α</sub>	= 8-epi prostaglandin-F <sub>2α</sub>

25.4 kg/m<sup>2</sup> (range, 23.5 to 27.1 kg/m<sup>2</sup>); total cholesterol (TC) plasma levels were between 200 and 350 mg/dl, and triglyceride (TG) plasma levels below 200 mg/dl. The women were postmenopausal, and none were using hormone replacement therapy. In none of the subjects was there evidence of chronic diseases (hepatic, renal, thyroid, cardiac), smoking, alcohol consumption, or family history of early onset cardiovascular disease. The study was approved by the Human Investigation Review Committee at the Reina Sofia University Hospital. All the participants gave their informed consent before joining the study.

**Experimental design.** The participants were instructed not to take vitamins, soy supplements, or any drug treatments in the six weeks preceding the study. Only three patients were taking atorvastatin, 10 mg, which was discontinued six weeks before the randomization. To eliminate the potential effect that might exist in their usual dietary habits, all subjects were instructed to follow a low-fat, carbohydrate-rich diet during the duration of the study. Compliance with the stabilization diet was assessed after two and four weeks using a three-day record and a food frequency dietary questionnaire. Participants were instructed to avoid consuming polyphenol-rich foods such as fruit or juices, wine, grape juice, chocolate, coffee, tea, olive oil, or soya in the 24 h before the study meal, and to refrain from intense physical exercise during that period. After a 12 h fasting, the participants reported to the hospital and, as per the randomized sequential crossover design, received two fat meals consisting of 60 g of white bread, 40 ml of virgin olive oil (Carapelli Firenze S.p.A., Florence, Italy) with high (400 ppm) or low (80 ppm) content in phenolic compounds, and 60,000 IU of vitamin A per m<sup>2</sup> of body surface. Olive oil B was obtained as a result of extraction by physical procedures of most of the phenolic compounds in olive oil 400 ppm, so that both oils kept a similar composition of the remaining macro- and micronutrients. Throughout the 4-h duration of the study, the subjects performed no physical activity, nor did they consume anything but water. Venous blood was obtained at 0, 30, 60, 120, and 240 min after consumption of the fat meal.

**Study of the endothelial function.** Laser-Doppler linear Periflux 5000 (Perimed S.A., Stockholm, Sweden) was used to measure ischemic reactive hyperemia (IRH). With the patient

lying in the supine position in a room with stable temperature (20°C to 22°C), the blood pressure cuff (HG Erkameter 300, Erka, Bad Tolz, Germany) was placed 5 cm above the elbow, while the laser probe was attached to the palmar surface of the second finger of the same dominant hand. After a 5-min resting period, basal capillary flow was measured for 1 min (t<sub>0</sub>). Thereafter, 4-min distal ischemia was induced by inflating the cuff to suprasystolic pressure (200 to 220 mm Hg). Subsequently, the cuff was deflated and, after 30 s, the flow was recorded for 1 min (t<sub>d</sub>). The data obtained were recorded and stored using the software PeriSoft for Windows. The values of the area under the curve (AUC) of the t<sub>0</sub> and t<sub>d</sub> times were analyzed. These data were used to calculate the increase in postischemic flow by means of this formula: IRH = (AUC<sub>t<sub>d</sub></sub> - AUC<sub>t<sub>0</sub></sub>) × 100 / AUC<sub>t<sub>0</sub></sub>. This calculation was carried out using the basal determinations 120 and 240 min after the consumption of both breakfasts. **Analytic methods.** Determination of tocopherols was carried out by separating the different tocopherol isomers by high performance liquid chromatography (Beckman, Palo Alto, California), and posterior analysis was done by means of a Jasco FP-920 spectrofluorimeter (Jasco, Tokyo, Japan). A spectrophotometer (UNICAM 5625, Cambridge, United Kingdom) was used to determine total carotenoid and chlorophyll contents.

Venous blood was extracted after a 12-h fast, and after 30, 60, 120, and 240 min after consumption of the breakfast. The samples were collected in tubes containing 1 g · l<sup>-1</sup> EDTA or 3.8% citrate, and were stored in containers with ice and kept in the dark. A similar procedure was used for the different determinations of the samples in all the other periods, avoiding exposure to air, light, and room temperature. Plasma was obtained by low speed centrifugation at 1,500 × g for 15 min at 4°C within one hour of extraction. Lipid parameters were assessed with the modular autoanalyzer DDPPII Hitachi (Roche, Basel, Switzerland), using specific reagents (Boehringer-Mannheim, Mannheim, Germany). Total cholesterol and TG levels were determined by colorimetric enzymatic methods (15,16). High-density lipoprotein cholesterol (HDL-C) levels were measured using colorimetric assay after precipitating the lipoproteins containing apolipoprotein B with polyethylene-glycol (17). Low-density lipoprotein cholesterol (LDL-C) levels were estimated using the Friedewald formula based on the CT, TG, and HDL-C values (18). Apolipoprotein AI and apolipoprotein B levels were measured by immunoturbidimetry (19). The chylomicron and large very low-density lipoprotein fraction of large triglyceride-rich lipoproteins (TRL) (Sf > 400) was isolated from 4 ml plasma overlaid with 0.15M NaCl, 1 mM EDTA (pH 7.4; density, 1.006 kg·l<sup>-1</sup>) by a single ultracentrifugal spin (28,000 × g, 30 min, 4°C) in a 50-type rotor (Beckman Instruments, Fullerton, California). Chylomicrons contained in the top layer were removed by aspiration after cutting the tubes. The infranatant was centrifuged at a density of 1.019 kg·l<sup>-1</sup> for 24 h at 115,000 × g in the same rotor. The nonchyl-

**Table 1.** AUC of the Different Parameters After the Ingestion of a Phenol-Rich Olive Oil Meal (400 ppm) as Compared to a Low Phenol Olive Oil Meal (80 ppm)

Lipoprotein Fractions	AUC		
	Olive Oils		p Value
	80 ppm	400 ppm	
TG (mg·dl <sup>-1</sup> ·h <sup>-1</sup> )	8,515 ± 5,760	8,646 ± 6,484	0.707
Large TRL-C (mg·dl <sup>-1</sup> ·h <sup>-1</sup> )	1,398 ± 948	1,444 ± 903	0.823
Large TRL-TG (mg·dl <sup>-1</sup> ·h <sup>-1</sup> )	6,387 ± 3,186	6,692 ± 4,613	0.616
Small TRL-C (mg·dl <sup>-1</sup> ·h <sup>-1</sup> )	1,680 ± 503	1,687 ± 493	0.951
Small TRL-TG (mg·dl <sup>-1</sup> ·h <sup>-1</sup> )	2,325 ± 1,773	2,204 ± 2,091	0.667

Student *t* test for paired samples. Values expressed as mean ± SD.

AUC = area under the postprandial curve; C = cholesterol; TG = triglycerides; TRL = triglyceride-rich lipoproteins.

micron fraction of TRL (also referred to as small TRL, Sf 20 to 400) was removed from the top of the tube. All operations were done in subdued light. Large and small TRL fractions were kept at -70°C until TC and TG levels of the same were ready to be analyzed. Using the frozen samples of plasma or serum, we determined the levels of lipoperoxides (LPO) by colorimetric method (LPO-CC Assay, Kamiya Biomedical Company, Seattle, Washington); 8-epi prostaglandin-F<sub>2α</sub> (8-epi-F<sub>2α</sub>) by immunoenzymatic assay (Bioxytech 8-isoprostane Assay, Oxis Research, Portland, Oregon); and nitrates/nitrites (NO<sub>(x)</sub>) by colorimetric method (Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemical, Ann Arbor, Michigan). All the determinations were carried out in duplicate.

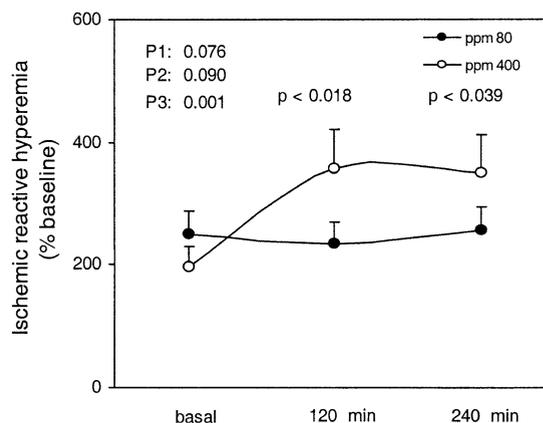
**Statistical analysis.** Several variables were calculated to define the postprandial response of lipid parameters in plasma and triglyceride-rich particles. The AUC is defined as the area between the plasma concentration versus time curve and a line traced parallel to the horizontal axis through the 0 h concentration. This value was calculated using the trapezoidal method with MedCalc statistics package (Medcalc Software, Mariakerke, Belgium). The data were analyzed using Student *t* test for paired data analysis and analysis of variance for repeated measures. In this analysis we studied the statistical effects of the type of fat meal ingested, independent of time (represented by P1), the effect of time (represented by P2), and the interaction of both factors, indicative of the degree of the postprandial response in each group of subjects with each breakfast (represented by P3). A value of *p* < 0.05 was considered statistically significant. Pearson's linear correlation coefficient was calculated, and a multiple linear regression analysis was carried out. Statistical analysis was performed using SPSS 9.0 for Windows (SPSS Inc., Chicago, Illinois). All data presented in the text and tables are expressed as mean ± SD.

## RESULTS

**Basal and postprandial lipid parameters.** No significant differences were found for any of the basal lipid parameters of the participants before each meal (data not shown). No statistically significant differences were found in the postprandial AUC of plasma TG, nor TG in small TRL and large TRL, after the intake of the fat meal based on olive oil

with a high content of phenolic compounds versus that containing a lower content of the same substances (Table 1). **IRH.** After the intake of the fat meal based on olive oil with a high content of phenolic compounds, we observed a greater increase in IRH at 120 and 240 min as regards the basal values, compared to the olive oil with a low content of the same substances (Fig. 1). After the intake of the olive oils with low and high content in polyphenols, there were no differences in the basal and the peak IRH (AU) values when data were compared at 0 min (basal: 21 ± 9 vs. 19 ± 11, *p* = 0.85; and peak: 51 ± 32 vs. 37 ± 41, *p* = 0.23), 120 min (basal: 24 ± 12 vs. 34 ± 17, *p* = 0.235; and peak: 56.4 ± 35 vs. 121.7 ± 73, *p* = 0.327), and 240 min (62 ± 47 vs. 140.5 ± 82, *p* = 0.224).

**Reproducibility of the method.** In a preliminary study performed in nine healthy subjects with measurements three weeks apart, we found an interstudy variability of 8.85%. A total of 10 measurements within the same day in one single healthy volunteer renders an intrastudy variability of 8.7%. **NO<sub>(x)</sub>, LPO, and 8-epi-F<sub>2α</sub> concentrations.** Similarly, 120 min after the intake of the meal with the high-phenol olive oil, we observed a greater increase in NO<sub>(x)</sub>, a lower increase in LPO, and a significant higher decrease in 8-epi-F<sub>2α</sub> as regards the basal values of each participant, compared to the olive oil with a low content of phenolic substances (Table 2).



**Figure 1.** Postprandial values of ischemic reactive hyperemia after the ingestion of a phenol-rich olive oil meal (400 ppm) as compared to a low phenol olive oil meal (80 ppm). Analysis of variance for repeated samples. P1 = effect of the type of meal; P2 = effect of time; P3 = interaction meal-time.

**Table 2.** Postprandial Changes of Nitrates/Nitrites, Lipoperoxides, and 8-epi Prostaglandin Plasma Levels After the Ingestion of a Phenol-Rich Olive Oil Meal (400 ppm) as Compared to a Low Phenol Olive Oil Meal (80 ppm)

Variables	Oils	Time		p Value
		0 min	120 min	
NO <sub>(x)</sub> (μmol·l <sup>-1</sup> )	80 ppm	25.4 ± 4.9	26.4 ± 4.1	<0.001
	400 ppm	26.3 ± 4.5	32.9 ± 4.6	
LPO (μg·ml <sup>-1</sup> )	80 ppm	0.4 ± 0.5	3.2 ± 2.4	<0.005
	400 ppm	0.7 ± 0.7	2.3 ± 2.1	
8-epi-F <sub>2α</sub> (ng·ml <sup>-1</sup> )	80 ppm	3.6 ± 0.7	4.5 ± 0.6	<0.001
	400 ppm	4.6 ± 0.7	3.2 ± 0.6	

Analysis of variance for repeated samples.

LPO = lipoperoxides; NO<sub>(x)</sub> = nitrates/nitrites; 8-epi-F<sub>2α</sub> = 8-epi prostaglandin-F<sub>2α</sub>.

**Multiple linear regression analysis.** We evaluated the possible relation between the different lipoprotein fractions and changes in the basal situation (by percentage) of the plasma level of LPO, 8-epi-F<sub>2α</sub>, NO<sub>(x)</sub>, and IRH measured by laser-Doppler (Table 3); IRH correlated positively with the variations in HDL-C and NO<sub>(x)</sub>, and negatively with TG, TC, LDL-C, LPO, and 8-epi-F<sub>2α</sub>. The variations in plasma TG levels correlated positively with LPO and 8-epi-F<sub>2α</sub>, and negatively with NO<sub>(x)</sub>. A negative correlation was observed between LPO and NO<sub>(x)</sub>. Multiple linear regression analysis indicated that HDL-C and TG plasma levels and the variations in NO<sub>(x)</sub> may account for 23.9%, 14.7%, and 20.2% of the variability of IRH during the postprandial period. Similarly, the changes in LPO and 8-epi-F<sub>2α</sub> levels were significant predictors of NO<sub>(x)</sub> increase in plasma during the postprandial period, and justify up to 20.8% and 19.1%, respectively, of these variations.

## DISCUSSION

Our study shows that the consumption of a meal based on virgin olive oil with a high phenolic compound content improves endothelium-dependent microvascular vasodilatation during the first 4 h of the postprandial period in patients with hypercholesterolemia. This improvement is associated with a decrease in oxidative stress and an increase of the final products of nitric oxide.

**Table 3.** Multiple Regression Analysis for Variations in Ischemic Reactive Hyperemia, Oxidative Stress Markers, and NO<sub>(x)</sub> Plasma Concentrations

Dependent Variables	Independent Variables	Beta Coefficients	R <sup>2</sup>	p Value
IRH	NO <sub>(x)</sub>	0.380	0.588	0.013
	TG	-0.384		
	HDL-C	0.489		
LPO	8-epi-F <sub>2α</sub>	0.438	0.191	0.028
	TG	0.679		
8-epi-F <sub>2α</sub>	LPO	0.394	0.155	0.038
	TG	0.579		
NO <sub>(x)</sub>	LPO	-0.457	0.399	0.025
	8-epi-F <sub>2α</sub>	-0.438		

HDL-C = high-density lipoprotein cholesterol; IRH = ischemic reactive hyperemia; LPO = lipoperoxides; NO<sub>(x)</sub> = nitrates/nitrites; TG = triglycerides; 8-epi-F<sub>2α</sub> = 8-epi prostaglandin-F<sub>2α</sub>.

Dietary fat is one of the main environmental factors associated with the development of endothelial dysfunction. Vogel et al. (20) reported a reduction of up to 31% in mean arterial diameter during postischemic vasodilatation response, when studying the effect of meals based on virgin olive oil, canola oil, and salmon. Similar findings had been observed with regard to the intake of saturated fat, suggesting that such an effect was dependent on the presence of fat, irrespective of the type of fat or whether it had undergone high temperatures during cooking (21). The administration of vitamins and other antioxidant compounds like salad, wine vinegar, and vitamins C and E in tablet form produced an improvement of up to 71% in postischemic arterial vasodilatation. These observations suggested the possibility that this effect might be due to the increase in oxidative stress concomitant with the postprandial increase of triglyceride-rich particles. These findings are significant because human beings consume complete foods, and the presence of antioxidants may well counteract the effect produced by the fat content of the food itself. Williams et al. (22) observed that endothelial dysfunction does not occur after the intake of olive oil, a foodstuff rich in phenolic compounds of high antioxidant value. Weinbrenner et al. (14) have recently shown that the polyphenols in olive oil improve the oxidative state and that this finding is directly associated with the bioavailability of these compounds in the postprandial period. In this respect, our findings demonstrate that the same dietary constituent—virgin olive oil—may have different effects depending on its micronutrient content.

It should be noted that in our research we used IRH, determined by laser-Doppler technique, previously considered an indicator of endothelial dysfunction (23). In this study we observed that endothelial-dependent vasodilatory response was positively related with HDL-C plasma levels and with NO<sub>(x)</sub> variations, and negatively with total triglyceride plasma levels, TC, LDL-C, and LPO and 8-epi-F<sub>2α</sub> variations in plasma. The source of LPO and 8-epi-F<sub>2α</sub> generation is likely to be derived from the oxidation of free fatty acids resulting during the hydrolysis of postprandial TRL. Any intervention reducing the TG peak or the source of reactive species would induce a drop in the production of

these markers for lipid oxidation. Our study did not find any effect on the postprandial TG levels. Because the only difference between the two oils was the concentration in phenolic compounds, we would suggest that the improvement in the endothelial response may be due to the oxidative protection related to the effect of these substances during the postprandial period.

Reduced nitric oxide availability is another mechanism related to the alteration of the vasomotor response that accompanies endothelial dysfunction. This fact may be a result of diminished production of nitric oxide (due to lower expression of nitric oxide synthase, the reduction of its precursor L-arginine, and its main cofactor tetrahydrobiopterine or to the action of the natural inhibitor of nitric oxide synthase, asymmetric dimethyl-L-arginine). Alternatively, it could result from higher consumption of nitric oxide after the interaction with radical species, especially hypochloric acid and superoxide anion (24). In hypercholesterolemic patients, it has been shown that oxidative stress and the presence of higher asymmetric dimethyl-L-arginine levels (25) are the main mechanisms that could account for the diminished availability of nitric oxide. The fact that a negative correlation has been observed between the variations of LPO and NO<sub>(x)</sub> could provide the link for the oxidative hypothesis and the alteration in nitric oxide bioavailability.

In conclusion, the consumption of a diet containing olive oil rich in phenolic compounds seems to prevent the endothelial dysfunction associated with acute fat intake. A reduction in oxidative stress and an increase of nitric oxide metabolites could explain these reported findings.

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