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Olive oil preparation determines the atherosclerotic protection in apolipoprotein E knockout mice[☆]

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Abstract

Oils enriched in monounsaturated fatty acids do not seem to behave similarly in protecting against the development of atherosclerosis in animal models, which has been attributed to the presence of soluble phenolic compounds. To test the relevance of other components of oils in the prevention of atherosclerosis, two olive oils from the same cultivar devoid of soluble phenolic compounds were prepared using different procedures (pressure or centrifugation), characterized and fed to apolipoprotein E-deficient mice as 10% (w/w) of their diet. The 2 olive oils had similar levels of monounsaturated fatty acids and squalene, but they differed in their content of linoleic, phytosterols, tocopherols, triterpenes and waxes, which were particularly enriched in the test olive oil obtained by centrifugation. In mice that received a diet enriched in the olive oil derived through centrifugation, the progression of atherosclerosis was delayed compared to the mice that received standard olive oil. That effect was associated with decreases in plasma triglycerides, total and non-high-density lipoprotein cholesterol and isoprostane 8-iso-prostaglandin $F_{2\alpha}$. Our results clearly indicate that the preparation of olive oil is crucial in determining its antiatherosclerotic effect, which extends beyond the presence of phenolic compounds. The test olive oil exerted its antiatherosclerotic effects by modifying plasma lipids and oxidative stress, and it might be a good candidate to replace other fats in functional foods.
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1. Introduction

The "Seven Countries" study showed that the so-called Mediterranean diet is associated with a low cardiovascular mortality, despite a high intake of fat, mainly from olive oil [1]. In addition, diets that contain olive oil lower total and low-density-lipoprotein (LDL) cholesterol without a proportionate lowering of high-density-lipoprotein (HDL) cholesterol levels [2,3]. Other risk factors associated with the development of atherosclerosis such as postprandial triglyceride (TG) metabolism [4,5] or coagulation factors seem to be influenced by olive oil intake (reviewed in Ref. [6]).

The benefits of virgin olive oil intake cannot only be attributed to the content of oleic acid. Ruiz-Gutierrez et al. [7] observed that, in contrast to virgin olive oil, high-oleic sunflower oil did not reduce blood pressure in hypertensive patients. With similar design, Oubina et al. [8] found lower thromboxane B2 production in normal subjects and in hypercholesterolemic patients after they were fed the olive oil diet. In animal studies, the effects of olive oils on the

vascular wall were also independent of oleic acid content [9,10]. The potential factors for those effects include the TG molecular species [4–11] or non-fatty acid constituents. Therefore, the effects of olive oil on atherosclerosis need to be studied by taking into consideration that olive oil is more than a mixture of fatty acids; it contains other biologically active substances, including tocopherols, polyphenols and phytosterols, which have antioxidant and anti-inflammatory properties [12–14]. The proportions of those compounds in olive oil are influenced by the method of preparation. Pomace (also, called "orujo") olive oil is a by-product of the mechanical extraction of virgin olive oil by pressure. The residual oil in olives is commonly extracted using organic solvents (eg, hexane) and requires further refinement. The new procedure for pomace (test) olive oil extraction involves a centrifugation step, which forestalls the need for hexane, and a soft refining process, which increases the yield of olive oil from fruit [15]. The nutritional aspects of this test olive oil extracted mechanically have not been addressed.

Apolipoprotein (apo) E knockout (KO) mice develop a spontaneous atherosclerosis that mimics most of the features of human atherogenesis, such as the development of fatty streaks, necrotic cores, fibrous caps and plaque rupture [16]. Due to the rapid onset and accelerated development of atherosclerosis in apoE-KO mice, this mouse is an excellent animal model for studying the impact of environmental factors on the development and prevention of arteriosclerosis [16,17]. Using that mouse model, in the presence of low cholesterol diets, virgin olive oils obtained from different cultivars exerted specific effects on atherosclerosis [9]. Furthermore, the effect of virgin olive oil on atherosclerosis was associated with paraoxonase activity in these mice [18], which is a good intermediate biomarker of the atherosclerosis process. In the present study, to increase our understanding of the effects of olive oil on atherosclerosis, two olive oils, which were prepared using either the classical cold pressure method or a new centrifugation procedure and then refined, were characterized and administered to apoE-KO mice in low-cholesterol-content diets. To determine the effects and possible mechanisms involved, lipid, lipoproteins, paraoxonase activity and atherosclerotic lesions were examined in detail.

2. Material and methods

2.1. Animals

Homozygous apoE-KO mice were bred in the Unidad Mixta de Investigación, Zaragoza, Spain. To estimate initial plasma cholesterol and TG levels, 26 two-month-old males were fasted overnight and anesthetized with isofluorane, and blood samples were collected using retroorbital bleeding. The mice were assigned randomly to one of three groups that had the same average levels of plasma cholesterol and TGs and were housed in sterile filter-top cages in rooms maintained on a 12-h light/12-h dark cycle. Animals had ad libitum access to food and water. Body weights were recorded throughout the experiment. The Ethical Committee for Animal Research of the University of Zaragoza approved the protocol.

2.2. Diets

The three groups included a control group ($n=9$) fed a chow diet, a standard olive oil group ($n=9$) fed a chow diet enriched with (10%, w/w) olive oil freshly prepared using cold pressure and a test olive oil group ($n=8$) fed a chow diet enriched with (10%, w/w) olive oil prepared using a new procedure. The standard mouse chow was Teklad Mouse/Rat Diet no. 2014 (Harlan Ibérica, Barcelona, Spain). To avoid the potential confounding effects of variation among batches of chow, 25 kg from a single batch was reserved and used to prepare diets throughout the experiment. All of the diets were prepared weekly and stored in N_2 atmosphere at $-20°C$. Fresh food was provided daily. The experiment ran for 11 weeks, and all of the diets were well received.

Both of the olive oils were refined using nitrogen at low temperature, active carbon and low concentrations of phosphoric (to remove phospholipids). Those processes remove undesirable flavors, odors and colors and improve transparency. The new procedures are protected by patents ES2048667 and ES200400775 and are partly described by Alba et al. [19] To determine their composition, the olive oils were analyzed in duplicate following the standard regulations of the European Union [20]. Distinctive general features of both oils were phytosterols, waxes, triterpenes (erythrodiol, uvaol and maslinic) and tocopherols, which were higher in the test olive oil than in the standard olive oil (Table 1). Both of the oils lost soluble phenolic components in the refinement process due to its hydrophilic character (data not shown) and contained similar amounts of squalene, linolenic and monounsaturated fatty acids. The test olive oil also had a slightly lower proportion of saturated fatty acids and a higher proportion of linoleic acid than did the standard oil. The new procedure permits the inclusion of a number of components from the skin and pit of the olive, such as phytosterols and waxes, which are present in low concentrations in virgin olive oil.

2.3. Blood cell analysis of surface molecule expression

After 10 weeks, the mice were fasted overnight and anesthetized using isofluorane, and blood samples were collected using retroorbital bleeding. Approximately 1×10^6 white blood cells were resuspended in phosphate-buffered saline (PBS) supplemented with 0.1% (w/v) bovine serum albumin and 10 mmol/L sodium azide and analyzed for the expression of Mac-1 (anti-CD11b from Becton-Dikinson, Madrid, Spain) using fluorescence-activated cell sorter analysis. The results are expressed as the proportion (%) of the marker-positive cells recovered in the region corresponding to monocytes.

Table 1
Composition of the used olive oils

Component	Standard olive oil	Test olive oil
Eritrodiol+uvaol	0.0017	0.05
Maslinic acid	0	0.011
Squalene	0.26	0.25
Total phytosterols	0.112	0.224
Total tocopherols	0.022	0.098
Waxes	0.012	0.34
Fatty acids		
Myristic (14:0)	0.02	0.02
Palmitic (16:0)	10.98	10.29
Stearic (18:0)	3.53	2.95
Arachidic (20:0)	0.42	0.45
Behenic (22:0)	0.12	0.17
Lignoceric (24:0)	0.05	0.07
Palmitoleic (16:1)	0.82	0.76
Oleic (18:1)	77.8	74.27
Gadoleic (20:1)	0.25	0.33
Linoleic (18:2n-6)	4.52	8.07
Linolenic (18:3n-3)	0.62	0.70
Saturated	15.14	13.95
Monounsaturated	78.87	75.36

Dietary components are expressed as g% (w/w).
P/S ratio, polyunsaturated/saturated fatty acid ratio.

2.4. Plasma determinations

At the end of the experiment, the mice were fasted overnight and sacrificed by suffocation in CO_2, and blood was drawn from their hearts. Total plasma cholesterol and TG concentrations were measured using commercial kits from Sigma (Madrid, Spain). HDL cholesterol levels were determined in a similar manner after the phospho-tungstic acid $MnCl_2$ (Roche, Barcelona, Spain) precipitation of apoB-containing particles [21]. Total isoprostane 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) was measured by immunoassay (Cayman Chemical, Ann Arbor, MI, USA). Paraoxonase was assayed using arylesterase [18] or lactonase activities [22] and were expressed as (IU $L^{-1} \times 1000$). To analyze plasma lipoprotein profiles, 100 µl of pooled plasma samples from within each dietary group were subjected to fast protein liquid chromatography gel filtration using a Superose 6B column (GE Healthcare, Barcelona, Spain) (see Ref. [9]).

2.5. RNA isolation and analysis

At the moment of sacrifice, livers were removed and immediately frozen in liquid nitrogen. RNA was prepared from each liver using Trigent reagent MRC (Cincinnati, OH, USA) following the manufacturer's instructions. Total RNA was subjected to Northern blot analysis [23]. The mouse clones for apoa5 (4196296 IMAGE Clone) and paraoxonase1 (4158951 IMAGE Clone) were obtained from The MGC Geneservice (Cambridge, UK). For descriptions of the apoa5 and pon-1 probes, see Ref. [24]. To standardize the amount of RNA loaded onto a gel, a mouse â-actin was used as a housekeeping gene probe [24]. Probes were labeled using [$_\alpha$-^{32}P]-dCTP and Rediprime. Filters were exposed to Biomax film (Kodak, Amersham), and the

intensity of bands in films was quantified using a laser LKB 2202 densitometer (GE Healthcare).

2.6. Evaluation of atherosclerotic lesions

The heart and arterial tree were perfused with PBS and, later, with phosphate-buffered formalin (4%, pH 7.4, Panreac, Barcelona, Spain) under physiological pressure. Hearts and aortas were dissected out, cleaned and stored in neutral formaldehyde. Heart bases were placed in OCT (Bayer Diagnostic, Germany) on a cryostat chuck (Microm HM505E, Barcelona, Spain). Serial cryosections of the proximal aorta and the aortic sinus were stained with Sudan IV B (Sigma) and counterstained with hematoxylin and eosin (Sigma) [9]. Images were captured and digitized using a Nikon microscope equipped with a digital camera. Morphometric analyses were evaluated blindly using Scion Image software.

2.7. Statistical analysis

A Mann–Whitney U test or one-way analysis of variance (ANOVA) followed by post hoc analysis were used for comparisons, and differences were considered statistically significant at $P < .05$. The relationships among variables were assessed by Spearman rank-order correlation coefficient (rs) using Instat 3.02 for Windows software (GraphPad, San Diego, CA, USA).

3. Results

3.1. Effects of diet on somatic and plasma parameters

After the 11-week experiment, the average body weight gain and liver weight of apoE-deficient mice did not differ significantly among the control group (fed a chow diet) and treatment groups (fed a chow diet supplemented with either standard olive oil or test olive oil (Table 2). Plasma cholesterol levels increased significantly in mice fed a diet supplemented with standard olive oil, but not in mice fed a diet supplemented with the test olive oil. Although HDL cholesterol and TG levels of mice fed the chow diet enriched with 10% (w/w) olive oil did not increase significantly, mice fed a diet supplemented with the test olive oil had significantly lower plasma TG levels than did mice in the control group and those fed a diet supplemented with standard olive oil. Differences in non-HDL cholesterol levels mirrored those of total cholesterol.

The distribution of cholesterol among the different plasma lipoproteins was analyzed by Superose 6B column chromatography of plasma from animals in each experimental group and is shown in Fig. 1. After 11 weeks, LDL cholesterol levels in mice fed standard olive oil increased, while very low-density lipoprotein (VLDL) levels decreased in mice fed test olive oil (Fig. 1), which is consistent with the patterns in plasma TG and cholesterol levels (Table 2). Those results indicate that the preparation of olive oil had marked effects on the lipid and lipoprotein profiles of apoE-KO mice.

Table 2
Effects of experimental diets on apoE-KO mice

	Chow ($n=9$)	Standard olive oil ($n=9$)	Test olive oil ($n=8$)
Body weight change g	3.4 ± 0.4	4.4 ± 0.2	4 ± 0.3
Liver weight (g)	0.78 ± 0.02	0.79 ± 0.05	0.76 ± 0.02
Plasma cholesterol (mmol L^{-1})	15.0 ± 0.8	$17.4\pm0.8\ddagger$	$15.1\pm1.1*$
HDL cholesterol (mmol L^{-1})	0.56 ± 0.1	0.45 ± 0.02	0.56 ± 0.1
VLDL+ LDL cholesterol (mmol L^{-1})	14.3 ± 0.7	$16.9\pm0.7\ddagger$	$14.6\pm1.0*$
Plasma TGs (mmol L^{-1})	1.7 ± 0.1	2.4 ± 0.4	$1.1\pm0.1*\cdot\dagger$
Aryl esterase (IU $L^{-1}\times1000$)	87 ± 4	97 ± 3	$72\pm4*\cdot\ddagger$
Lactonase (IU $L^{-1}\times1000$)	19 ± 1.5	16 ± 3.3	18 ± 0.5
8-iso-PGF$_{2\alpha}$ (pg ml^{-1})	309 ± 22	$192\pm59\dagger$	$97\pm7**\cdot\dagger$

Results are expressed as means±S.E.M. Mice were fed chow or experimental diets for 11 weeks and fasted overnight before blood collection.

Statistical analysis to evaluate dietary response was done using nonparametric one-way ANOVA and Dunn Multiple Comparisons test as post hoc analysis.

   * $P=.05$ vs. olive.
   ** $P=.01$ vs. olive.
   † $P=.01$.
   ‡ $P=.05$ vs. chow diet.

To determine the effect of diet on the antioxidative mechanism, we measured the activity of serum paraoxonase, an antioxidant enzyme in HDL, and 8-iso-PGF$_{2\alpha}$ levels. The arylesterase and lactonase activities of paraoxonase are shown in Table 2. The lactonase activity of paraoxonase did not change significantly in any of the groups, but arylesterase activity decreased significantly in mice that receive the diet supplemented with the test olive oil (Table 2). Mice fed an olive oil-enriched diet had significantly decreased plasma levels of 8-iso-PGF$_{2\alpha}$, and the effect was most pronounced in mice that received the test olive oil.

3.2. Influence of diet on the atherosclerotic lesion development in apoE-deficient mice

To determine whether the type of olive oil added to the diet influenced the degree of activation of circulating blood cells or the development of atherosclerosis, circulating monocytes expressing Mac-1 and cross-sectional atherosclerotic areas of aortic roots were analyzed. Mac-1 (CD11b) is a monocyte integrin molecule, referred to be the ligand for intercellular adhesion molecule 1 and 2 and involved in the recruitment of monocytes in the development of atherosclerosis [25]. The proportion of monocytes expressing Mac1 was lower in mice fed the test olive oil-enriched diet than in mice fed the chow or standard olive oil diet (data not shown). After the 11-week experiment, the infiltration of macrophages and transformation into foam cells were the main features of the aortic root atherosclerotic lesions (data not shown). Mice that received a diet enriched in standard olive oil had more lesions than did controls (Fig. 2); however, mice fed a test olive oil-enriched diet had significantly less area covered by atherosclerotic lesions than did mice in the control and standard olive oil groups. The proportion of circulating blood cells expressing Mac-1
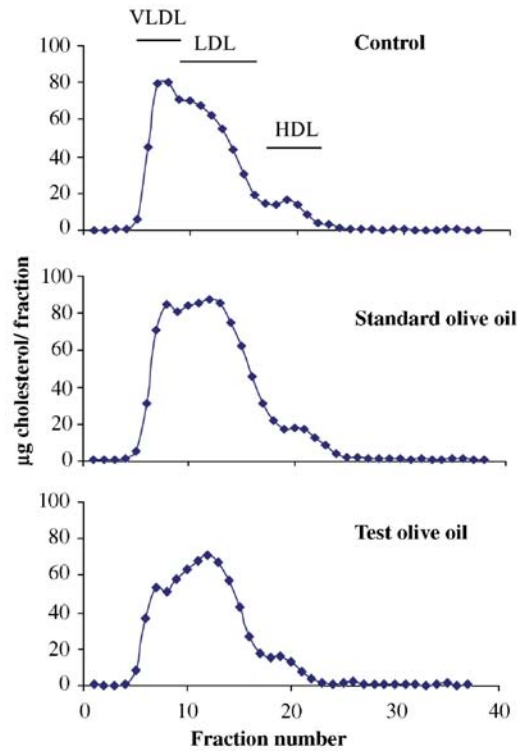

Fig. 1. FPLC plasma lipoprotein profile from apoE-KO mice following the different experimental diets for 11 weeks. Representative profiles were based on pooled plasma samples from animals within each experimental group [no olive oil ($n=9$), standard olive oil ($n=9$), test olive oil ($n=8$)]. Results are expressed as micrograms of cholesterol per fraction. Fraction numbers 4–8, 9–17 and 18–24 correspond to triglyceride-rich lipoproteins, LDL and HDL, respectively.

and the area of aortic atherosclerotic lesions were strongly positively correlated ($r=0.53$), as was the amount of area covered by lesions and lactonase activity ($r=-0.67$). Although of lesser magnitude, the correlation coefficients between total cholesterol and lesion area ($r=0.40$) and between 8-iso-PGF$_{2\alpha}$ and lesion area ($r=0.43$) were significant. Those results indicate that the preparation of olive oil influences the progression of atherosclerosis in apoE-deficient mice, and the effect is associated with
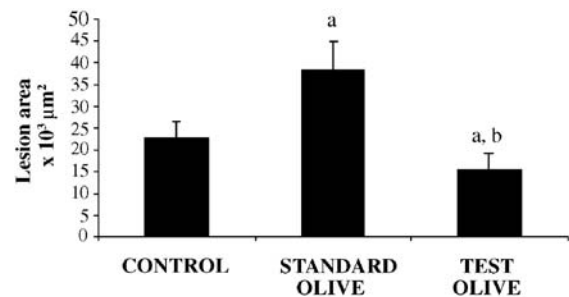

Fig. 2. Aortic atherosclerotic lesions in apoE-KO mice fed one of three diets (with or without olive oil) for 11 weeks. Cross-sectional analysis of aortic lesions of control ($n=9$), standard olive ($n=9$) and test olive groups ($n=8$). The results are expressed as mean±S.E.M. Statistical analyses were done according to Mann–Whitney U test. [a,b]$P < .05$ vs. control and olive, respectively.
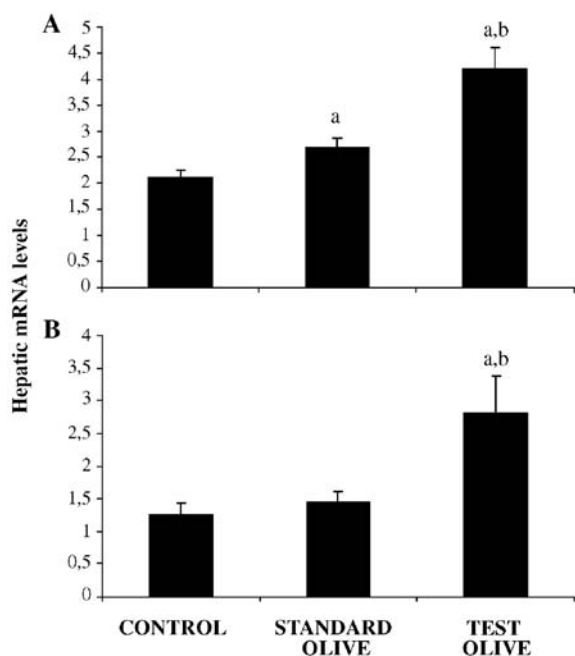
Fig. 3. Hepatic gene expression (mRNA levels) in apoE-KO mice fed one of three diets (with or without olive oil) for 11 weeks. (A) *apoa5* and (B) *pon1* genes. Results are expressed as mean±S.E.M. in relative arbitrary absorbance units normalized to the expression of the β-actin gene (used as a housekeeping gene). Total RNA (5 μg) from animals in each group was subjected to Northern blot analysis. Statistical analyses are based on the Mann–Whitney *U* test. [a,b]*P* < .05 vs. control and olive, respectively.

changes in the levels of cholesterol and 8-*iso*-PGF$_{2\alpha}$, lactonase activity of paraoxonase and the expression of Mac-1 by circulating monocytes.

3.3. Effects of olive oil preparation modulates hepatic gene expression in apoE-deficient mice

To determine whether hepatic *pon1* and *apoa5* messages were involved in changes in serum paraoxonase activity and in plasma TG, we determined their levels of hepatic mRNA expression using Northern blot analyses (Fig. 3). The mRNA levels of *apoa5* were elevated in mice that received an olive oil-enriched diet, but the levels of *pon1* were not influenced by this diet (Fig. 3). The increases were most pronounced in mice fed a diet enriched with test olive oil. Those results suggest that the phytochemical enrichment in the test olive oil influenced the expression of the *pon1* and *apoa5* genes in apoE-KO mice.

4. Discussion

This study evaluated the biologic properties of a new preparation of olive oil that is enriched in linoleic, phytosterols, tocopherols, triterpenes (erythrodiol, uvaol and maslinic acid) and waxes. A diet enriched with the test olive oil was well tolerated and retarded the development of atherosclerosis in apoE-KO mice more effectively than did a chow diet rich in carbohydrates and even more so than did a standard olive oil that had a similar content of oleic acid,

squalene and linolenic acid, but was devoid of the components indicated above. Soluble phenolic compounds were not responsible for the differences between the two olive oils in their effects on the development of atherosclerosis because they were removed during the refining process. The striking antiatherosclerotic effect of the test olive oil can be attributed to a suite of factors, such as decreases in the total and non-HDL cholesterol and plasma TGs, reduced oxidative stress (as estimated by plasma levels of isoprostane 8-*iso*-PGF$_{2\alpha}$) and a reduction in the proportion of circulating monocytes expressing Mac-1.

This is the first study to assess the influence of the method of olive oil preparation on the development of atherosclerosis in mice. To that end, two olive oil procedures were used to obtain olive oil from the same cultivar — the standard cold pressure method and a new procedure that involves the centrifugation of moltured olives [26]. Both of the olive oils we tested were refined to eliminate soluble phenolic compounds and characterized to determine whether compounds in olive oils were influenced by the method of production. In this study, the method of preparing the olive oil influenced the content of linoleic and the unsaponifiable fraction characterized as waxes, phytosterols, tocopherols, erythrodiol, uvaol and maslinic acid, without influencing the proportion of squalene and oleic and linolenic acids.

In this study, the test olive oil-enriched diet retarded the development of atherosclerosis more effectively than did the chow diet or the standard olive oil-enriched diet, which indicates that the olive oil devoid of phenolic compounds requires the presence of those compounds to behave as an antiatherosclerotic agent and as an antithrombotic [27]. This might explain the higher atherosclerosis observed in several animal models after they consumed monounsaturated fatty acid-containing diets [28]. To date, no attention has been paid to the effects of the method of preparing olive oil on atherosclerosis [9–29,10–34]. In our study, the dramatic differences in the composition of olive oil, depending on the method of preparation, and the effects that such changes exert on the development of atherosclerosis might explain the apparent discrepancies in the reported effects of olive oil action, are against the general belief that all diets containing monounsaturated fatty acids behave similarly and provide further support for the view that not all monounsaturated fatty acids-containing oils behave in a similar way [35]. Consequently, *monounsaturated fatty acid-enriched oils* is no longer a term that reflects adequately the complexities of these oils, and future studies should be aware of the mentioned facts to avoid conflicting results that are creating confusion in the nutritional and arteriosclerosis fields as well as for the consumers. Once again, the use of the apoE-KO mice as helpful models to delineate the influence of food on the development of atherosclerosis is an important tool to rapid advance in these complex issues with potential human implications.

The antiatherosclerotic capabilities of the test olive oil might have resulted from the synergistic action of all its minor components. Thus, diets high in linoleic acid were associated with lower atherosclerosis in mice [28]. Phytosterols are hypocholesterolemic and act as antiatherosclerotic agents in hamsters when they are provided in the diet at concentrations of 0.2% (w/w) or higher [36]. Vitamin E (tocopherol) is antiatherosclerotic [37], and the pentacyclic triterpenoid, maslinic acid in the test olive oil is an antioxidant [38]. Moreover, erythrodiol (3b-olean-12-en-28-diol), a pentacyclic triterpenoid alcohol, is a potent vasorelaxant [39]. Finally, dietary waxes are important modulators of lipid metabolism [40]. Whatever the mechanisms of actions of the various compounds, our results demonstrated the role of these components in the global action of olive oil, but our experimental design does not allow discriminating between them.

In our study, the mice fed the test olive oil-enriched diets had decreased F_2-isoprostanes. The analysis of those compounds is a specific and reliable marker of oxidant stress in vivo [41], and Fontana et al. [42] found that F_2-isoprostanes activated Mac-1 integrin (CD11b) expression through a very restricted signaling pathway. Our results are consistent with that finding because apoE-KO mice fed the test olive oil-enriched diet had the lowest levels of plasma F_2-isoprostanes and a smaller number of circulating monocytes that expressed Mac-1. Given the role of Mac-1 in capillary recruitment of monocytes, a lower level of this integrin would result in a smaller proportion of monocytes in subendothelial space and, hence, contribute to the decrease in the amount of atherosclerotic lesion observed in mice fed the test olive oil-enriched diet. Yaqoob et al. [43] reported a trend for a decrease in Mac-1 in circulating monocytes of human subjects that consumed olive oil, but they did not analyze the compounds in the olive oil. Their findings and our results suggest that minor compounds in olive oil might be crucial in the modulation of the levels of Mac-1 in circulating monocytes.

For the first time, our results showed that the apoA-V poses a dietary regulation through the influence of the method by which olive oil is prepared. It has been proposed that apoA-V modifies plasma TG levels by modulating hepatic VLDL assembly and secretion because apoA-V overexpression lowers plasma TG levels [44]. The increased expression of *apoa5* might explain the decrease in plasma TG levels observed in our study through the effect of a new method of preparing olive oil. The preparation of olive oil with a different recovery of minor compounds exerts important effects through changes in gene expression. Posttranscriptional or posttranslational mechanisms of paraoxonase expression might be influenced by the test olive oil because the increase in the expression of hepatic *pon1* was not paralleled by a change in plasma paraoxonase activity, which has been observed in diets high in linoleic acid because of the inhibitory effect of this fatty acid [24] that is more abundant in the test olive oil than in the standard olive oil.

In conclusion, the method used to prepare olive oil is crucial in determining the oil's antiatherosclerotic properties, which can arise from compounds other than phenols. The action of the test olive oil used in our study is associated with a hypolipidemic action, lower oxidative stress and reduced activation of circulating monocytes. Moreover, the test olive oil might be a safe strategy for retarding the development of atherosclerosis lesions and a good candidate to replace other fats in functional foods, although human clinical trials are required to validate that proposition.

Acknowledgments

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