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Antiatherogenicity of extra virgin olive oil and its enrichment with green tea polyphenols in the atherosclerotic apolipoprotein-E-deficient mice: enhanced macrophage cholesterol efflux

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Abstract

The antiatherogenic properties of extra virgin olive oil (EVOO) enriched with green tea polyphenols (GTPPs; hereafter called EVOO-GTPP), in comparison to EVOO, were studied in the atherosclerotic apolipoprotein-E-deficient (E^0) mice. E^0 mice (eight mice in each group) consumed EVOO or EVOO-GTPP (7 μl/mouse/day, for 2 months) by gavage feeding. The placebo group received only water. At the end of the study, blood samples, peritoneal macrophages and aortas were collected.

Consumption of EVOO or EVOO-GTPP resulted in a minimal increase in serum total and high-density lipoprotein (HDL) cholesterol levels (by 12%) and in serum paraoxonase 1 activity (by 6% and 10%). EVOO-GTPP (but not EVOO) decreased the susceptibility of the mouse serum to AAPH-induced lipid peroxidation (by 18%), as compared to the placebo-treated mice. The major effect of both EVOO and EVOO-GTPP consumption was on HDL-mediated macrophage cholesterol efflux.

Consumption of EVOO stimulated cholesterol efflux rate from mouse peritoneal macrophages (MPMs) by 42%, while EVOO-GTPP increased it by as much as 139%, as compared to MPMs from placebo-treated mice. Finally, the atherosclerotic lesion size of mice was significantly reduced by 11% or 20%, after consumption of EVOO or EVOO-GTPP, respectively.

We thus conclude that EVOO possesses beneficial antiatherogenic effects, and its enrichment with GTPPs further improved these effects, leading to the attenuation of atherosclerosis development.

Keywords: Olive oil; Green tea polyphenols; Macrophages; Oxidative stress; Atherosclerosis

1. Introduction

Macrophage cholesterol accumulation and foam cell formation are the hallmark of early atherogenesis [1–3]. Low-density lipoprotein (LDL) can be oxidized by macrophages [4], followed by its cellular uptake at enhanced rate, resulting in a significant increase in macrophage cholesterol mass [2,5]. Cholesterol accumulation in these cells can also result from increased cholesterol biosynthesis rate and/or decreased rate of high-density lipoprotein (HDL)-mediated cholesterol efflux from the cells.

Oxidative stress was shown to contribute to the development and progression of atherosclerosis [6]. In atherosclerotic patients, as well as in the apolipoprotein-E-deficient (E^0) mice (which develop atherosclerotic lesions with many features similar to those observed in human lesions), an increased oxidative stress was shown both in their serum lipoproteins and in their arterial (and peritoneal) macrophages [7–10]. We have previously shown that the increased oxidative stress in macrophages affects their biological activities and that "oxidized macrophages" can oxidize LDL and take up the formed oxidized LDL (Ox-LDL) at an enhanced rate [11,12].

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Olive oil contains monounsaturated fatty acids, mainly oleic acid (C-18:1), tocopherols, polyphenols and phytosterols, which possess antioxidant properties [13]. Olive oil significantly inhibited copper-ion-induced LDL oxidation in vitro [14,15], and extra virgin olive oil (EVOO) biphenols (protocatechuic acid and oleuropein) inhibited LDL oxidation by macrophages, probably as a result of cellular glutathione (GSH)-related enzyme up-regulation [16]. Dietary olive oil increased the resistance of LDL to oxidation in rabbits [17], and in rats, the activities of cardiac antioxidant enzymes were shown to be enhanced [18]. In healthy humans, short-term consumption of olive oil decreased serum oxidative stress [19], and their isolated LDL and HDL were shown to be enriched with oleic acid and resistant to oxidation [15,20]. In addition, consumption of virgin olive oil by patients with stable coronary heart disease was associated with decreased plasma oxidative stress and increased GSH peroxidase activity [21]. Other antiatherogenic properties of dietary olive oil include its ability to modulate endothelium activity [22] and to reduce blood pressure [21,23]. The beneficial effects of dietary olive oil on atherosclerosis were shown both in hyperlipidemic rabbits [24–26] and in the atherosclerotic E^0 mice [27–30].

Green tea contains an abundance of polyphenolic compounds called catechins, with epigallocatechin gallate being the major one [31]. Studies in mice and rabbits demonstrated that purified tea catechins, epicatechin gallate and epigallocatechin gallate lowered plasma cholesterol and reduced atherosclerosis [31,32].

In the present study, we have used, for the first time, EVOO that was enriched with green tea polyphenols (GTPPs, mostly epicatechin gallate; hereafter called EVOO-GTPP). The unique feature of this product was that GTPPs were dissolved in olive oil. The effects of EVOO-GTPP (in comparison with EVOO) on oxidative stress in serum and macrophages and on atherosclerosis development in the atherosclerotic E^0 mice were analyzed.

2. Materials and methods

2.1. Samples preparations

GTPPs were first emulsified in phosphatidylcholine, and then, EVOO was added slowly and continuously to the emulsifier–phospholipid complex (EVOO-GTPP). Control EVOO was incubated only with phosphatidylcholine.

2.2. In vitro studies

2.2.1. Polyphenol content determination

Total polyphenols were determined spectrophotometrically by the method of Singleton et al [33]. Gallic acid served as a standard. EVOO and EVOO-GTPP were diluted (×2) in water and sonicated to achieve a homogenous solution; 50 or 100 μl was taken for the assay.

2.2.2. Lipoprotein isolation

LDL was isolated from the serum of normolipidemic volunteers by density gradient ultracentrifugation [34]. LDL protein concentration was determined by the Lowry method [35].

2.2.3. LDL oxidation studies

Before oxidation, LDL was dialyzed against PBS. LDL (100 μg of protein) was then preincubated with 6 μl of the various oils. Then, the volume was completed to 1 ml with PBS, and 5 μmol/L of $CuSO_4$ was added, followed by incubation for 2 h at 37°C. At the end of the incubation, the amount of lipid peroxides [36] and thiobarbituric acid reactive substances (TBARS; [37]) was measured.

2.3. In vivo studies

2.3.1. Mice

E^0 mice (n=24, 6 weeks old) were divided into three groups. Two groups received olive oil by gavage feeding (7 μl/mouse/day, equivalent to 25 ml of olive oil/human/day) for 2 months: one of these groups consumed EVOO and the second one consumed EVOO-GTPP (mostly epicatechin gallate). EVOO contains 200 mg phenolics/L, whereas EVOO-GTPP contains 500 mg of total polyphenols/L. The placebo group received only water. At the end of the study, blood samples, mouse peritoneal macrophages (MPMs) and aortas were collected. This protocol was approved by the Committee for Supervision of Animal Experiments and complied with the Guide for the Care and Use of Laboratory Animals of the Technion-Israel Institute of Technology, Haifa.

2.3.2. Serum biochemical parameters

Serum total cholesterol, HDL cholesterol and triglyceride concentrations were measured by diagnostic kits (Raichem Co., Ltd.).

2.3.3. Serum lipid peroxidation

Serum samples were diluted (×4) in PBS and were then incubated without or with 50 mmol/L of 2,2′-azobis-2-amidinopropane hydrochloride (AAPH; Wako Co., Japan) for 2 h at 37°C [38]. The extent of lipid peroxidation was measured by the TBARS and by the lipid peroxide assays.

2.3.4. Serum paraoxonase 1 (PON1) arylesterase activity

Serum arylesterase activity was measured spectrophotometrically at 270 nm using phenylacetate (1 mmol/L) as the substrate [39]. The E_{270} for the reaction is 1310/M/cm. One unit of arylesterase activity is equal to 1 μmol of phenylacetate hydrolyzed/min/ml.

2.4. Macrophages

2.4.1. Mouse peritoneal macrophages

MPMs were harvested from E^0 mice, 4 days after intraperitoneal injection of thioglycolate (40 g/L). The cells [(10–20)×10^6/mouse] were washed and centrifuged (×3) with PBS at 1000×g for 10 min and were then resuspended

at 10^9/L in Dulbecco's Modified Eagle's Medium containing 5% fetal calf serum, 1×10^5 units penicillin/L, 100 mg streptomycin/L and 2 mmol/L glutamine. The dishes were then incubated in a humidified incubator (5% CO_2, 95% air).

2.5. Macrophage oxidative status

2.5.1. Macrophage peroxide content (DCFH assay)

Cellular peroxide levels were determined by the flow cytometric assay with dichlorofluorescein diacetate (DCFH-DA; [40]). Cellular fluorescence was determined with a flow cytometry (FACS) apparatus (FACS-SCAN, Becton Dickinson, San Jose, CA, USA). Measurements were done at 510–540 nm after excitation of cells at 488 nm with an argon ion laser.

2.5.2. Macrophage-reduced GSH content

The MPMs from triplicate dishes (2×10^6 per dish) were washed, scraped and sonicated in an ultrasonic processor (3×20 s at 80 W). The protein content was measured by the Lowry method, and the GSH level was measured by the DTNB-GSSG reductase recycling assay [41].

2.5.3. Macrophage-mediated LDL oxidation

Human LDL (after dialysis) was incubated (100 μg of protein/ml) with no cells or with the MPMs (2×10^6) in the presence of 5 μmol/L $CuSO_4$ for 3 h at 37°C, and the extent of cell-mediated LDL oxidation was determined [4].

2.6. Macrophage cholesterol metabolism

2.6.1. LDL and Ox-LDL uptake by macrophages

LDL (1 mg/ml) was incubated for 18 h at 37°C with 5 μmol/L of $CuSO_4$. The extent of LDL oxidation was determined by the TBARS assay. LDL and Ox-LDL were conjugated to fluoroisothiocyanate (FITC) for cellular uptake studies [42]. MPMs were incubated at 37°C for 3 h with FITC-conjugated LDL or Ox-LDL at a final concentration of 25 μg of protein/ml. Measurements of mean cellular fluorescence intensity by FACS were done at 510–540 nm after excitation of the cells at 488 nm with an argon ion laser.

2.6.2. Cholesterol efflux determination

MPMs were incubated with [^{3}H]-labeled cholesterol (2 μCi/ml) for 1 h at 37°C, followed by cell wash in ice-cold PBS (×3) and a further incubation in the absence or presence of 100 μg of HDL protein/ml for 3 h at 37°C [43]. The percentage of HDL-mediated macrophage cholesterol efflux was determined as described [43].

2.6.3. Macrophage cholesterol biosynthesis

MPMs were incubated with [^{3}H] acetate, and cellular lipids were extracted with hexane:isopropanol (3:2, v:v) and separated by thin-layer chromatography [44]. The spots that corresponded to unesterified cholesterol were visualized by iodine vapor, scraped into scintillation vials and counted for radioactivity.

2.7. Atherosclerotic lesion size

2.7.1. Histopathology of aortic atherosclerotic lesions

After olive oil consumption by E^0 mice for 2 months, the heart and entire aorta were rapidly dissected out and immersion fixed in 3% glutaraldehyde in 0.1 mol/L of sodium cacodylate buffer with 0.01% calcium chloride, pH 7.4, at room temperature. The lesion sections were prepared for histomorphometrical analyses [45]. Only the area of the aortic arch was examined because previous studies showed that this area is especially prone to atherosclerosis in E^0 mice, and this area is well defined with a clear starting point (aortic valves). Histopathology determinations of the lesion size were performed using an Olympus Cue-2 image analysis system with appropriate morphometric software (Olympus Corporation, Lake Success, NY). Measurements were made in standardized "windows" (fields) with an area of 176,758 μm^2.

2.8. Statistical analyses

Student's *t* test was performed for all statistical analyses. ANOVA was used when more than two groups were compared, and results are expressed as mean±S.D.

3. Results

3.1. In vitro study

The phenolic content in EVOO was 36% higher than that found in a commonly used olive oil (Fig. 1). In EVOO-GTPP, the polyphenol concentration was increased by 99%, as compared to their concentration in EVOO (Fig. 1). In corn, soy or fish oils, phenolic content was only about half that found in commonly used olive oil (Fig. 1). We next analyzed the ability of EVOO or EVOO-GTPP to inhibit copper-ion-induced LDL oxidation, in comparison to the other food oils (Fig. 2). The addition of 6 μl of the oils to LDL (100 μg protein) in 1 ml solution revealed that corn oil, soy oil and fish oil did not significantly affect the extent of copper-ion-induced LDL oxidation (as measured by the lipid peroxide level; Fig. 2), in comparison to control LDL (with no additions). In contrast, the three types of olive oils significantly inhibited LDL oxidation. While the commonly used olive oil decreased LDL oxidation by 54%, EVOO decreased the oxidation by 83% and EVOO-GTPP decreased it by 86% (Fig. 2).

3.2. In vivo study

The antiatherogenic effects of consumption of purified GTPPs such as catechins, epicatechin gallate and epigallocatechin gallate by E^0 mice [32] or by cholesterol-fed rabbits [31] were previously studied. These studies clearly demonstrated that green tea consumption significantly attenuated atherosclerosis development. Since the antiatherogenic properties of EVOO were previously studied [27–30], the aim of the present study was to evaluate, for the first time, the

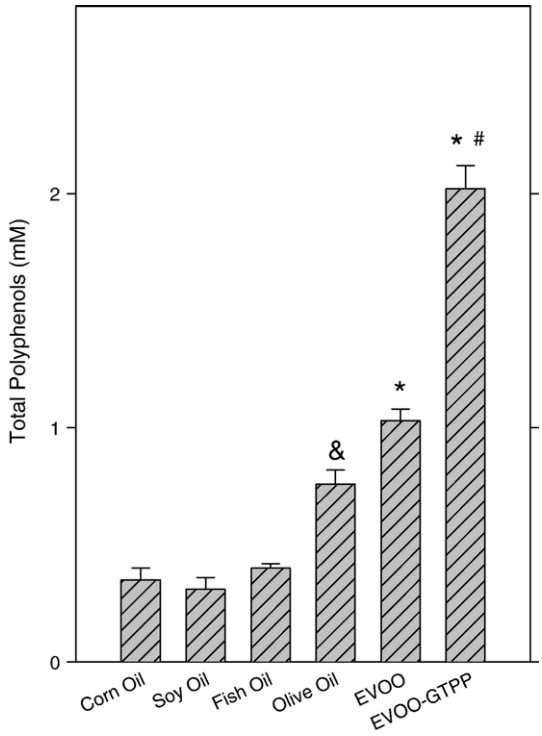

Fig. 1. Total polyphenol content of EVOO or EVOO-GTPP in comparison to other oils. Corn, soy and fish oils were compared to olive oil, including commonly used olive oil, EVOO or EVOO-GTPP. The concentration of total polyphenols in these oils was determined as described in Section 2. Results are expressed as mean±S.D. of three different measurements. $^{\&}P<.01$ versus corn oil, soy oil or fish oil; $^{*}P<.01$ versus olive oil; $^{\#}P<.01$ versus EVOO.

antiatherogenic properties of EVOO-GTPP versus EVOO on atherogenic parameters in vivo. As EVOO and EVOO-GTPP were found to be potent antioxidants against in vitro LDL oxidation, we next analyzed their effects also in vivo in the atherosclerotic E^0 mice that were previously shown to be under increased oxidative stress [7,8,11,12].

3.2.1. The effect of EVOO or EVOO-GTPP consumption by E^0 mice on atherosclerotic lesion size

Consumption of EVOO or EVOO-GTPP (7 μl/mouse/day) by E^0 mice for 2 months resulted in a significant reduction in the atherosclerotic lesion area, as compared to the lesion area in the placebo group. Consumption of EVOO significantly reduced the aortic lesion size of mice by 11% (Fig. 3), whereas consumption of EVOO-GTPP decreased lesion size by 20% (Fig. 3).

3.3. Mechanisms for olive oil antiatherogenicity

In order to find out the possible mechanisms by which consumption of EVOO or EVOO-GTPP attenuated atherosclerotic lesion development in E^0 mice, we next analyzed the effects of their consumption on serum lipid pattern, on serum oxidative stress and on macrophage oxidative stress and cellular cholesterol metabolism.

3.3.1. The effect of EVOO and EVOO-GTPP consumption by E^0 mice on serum lipid levels.

Consumption of EVOO or EVOO-GTPP (7 μl/mouse/day) by the mice for 2 months, as compared to the placebo-treated mice, resulted in a minimal increase in serum total cholesterol levels by 12% or 13% (549±19 or 554±24 mg/dl vs. 490±15 mg/dl, respectively), which were paralleled by a similar increase in serum HDL cholesterol levels by 11% or 14% (239±12 or 245±10 mg/dl vs. 215±11 mg/dl, respectively). EVOO consumption also resulted in some increase (by 14%) in serum triglyceride levels (from 470±25 mg/dl in placebo-treated mice to 536±26 mg/dl in mice that consumed EVOO), whereas EVOO-GTPP consumption decreased serum triglyceride levels by 15% (down to 400±10 mg/dl).

3.3.2. The effect of EVOO or EVOO-GTPP on serum oxidative stress

The susceptibility of the serum samples from the mice that consumed EVOO to oxidation induced by the free radical generator AAPH was not significantly affected, as compared to the serum lipid peroxidation of placebo-treated mice (Fig. 4A). In contrast, consumption of EVOO-GTPP

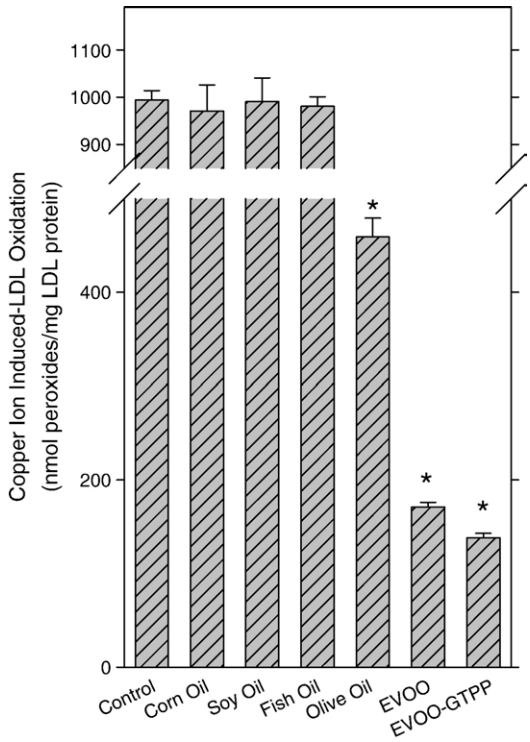

Fig. 2. Antioxidative effects of EVOO or EVOO-GTPP in comparison to other oils. LDL (100 μg protein) was preincubated with no addition (control) or with the addition of 6 μl of corn oil, soy oil, fish oil, olive oil, EVOO or EVOO-GTPP. Then, the sample volume was completed up to 1 ml with PBS, and 5 μmol/L $CuSO_4$ was added. The samples were further incubated for 2 h at 37°C. The extent of LDL oxidation was measured by the TBARS assay. Results are expressed as mean±S.D. of three different measurements. $^{*}P<.01$ versus control.

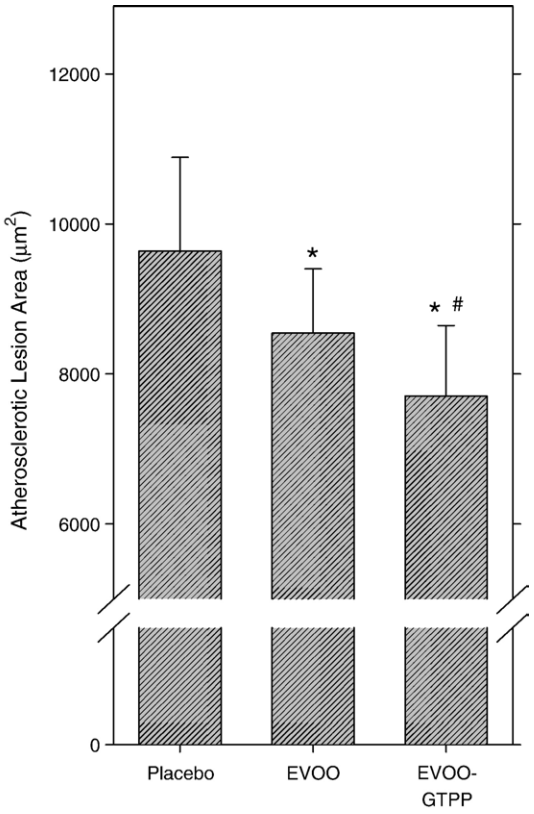

Fig. 3. The effect of EVOO or EVOO-GTPP consumption by E^0 mice on atherosclerotic lesion development. E^0 mice consumed EVOO or EVOO-GTPP by gavage feeding (7 μl/mouse/day for 2 months). The placebo-treated mice received only water. At the end of the study, the mice were sacrificed and their aortas were collected. The atherosclerotic lesion size was measured as described in Section 2. Results are expressed as mean±S.D. of eight mice in each group. *P<.01 versus placebo; #P<.01, EVOO-GTPP versus EVOO.

resulted in a significant decrement, by 18%, in the susceptibility of the mouse serum to AAPH-induced lipid peroxidation (Fig. 4A). Serum PON1 arylesterase activity was minimally but significantly increased, by 10% or 6%, after EVOO or EVOO-GTPP consumption, in comparison to serum PON1 activity in the placebo-treated mice (Fig. 4B).

3.3.3. The effect of EVOO or EVOO-GTPP consumption by E^0 mice on macrophage oxidative stress

The cellular level of total peroxides in the MPM was measured by the DCFH assay. No significant effect on total lipid peroxide level was observed in MPM harvested from the mice that consumed EVOO or EVOO-GTPP, as compared to MPM from placebo-treated mice (205±13 or 197±11 arbitrary units vs. 198±12 arbitrary units, respectively).

The following cellular GSH levels in MPM were observed: from placebo-treated mice, it was 5.8±0.4 nmol/mg cell protein, and from mice that consumed EVOO or EVOO-GTOO, values were 5.6±0.3 or 5.5±0.2 nmol/mg cell protein, respectively. The cells' ability to oxidize LDL in the presence of copper ions was not significantly affected (460±30 nmol TBARS/mg cell protein in MPM from placebo-treated mice vs. 440±25 or 505±23 nmol TBARS/mg cell protein in MPM from mice that consumed EVOO or EVOO-GTPP, respectively). These results indicate that EVOO consumption did not reduce macrophage oxidative

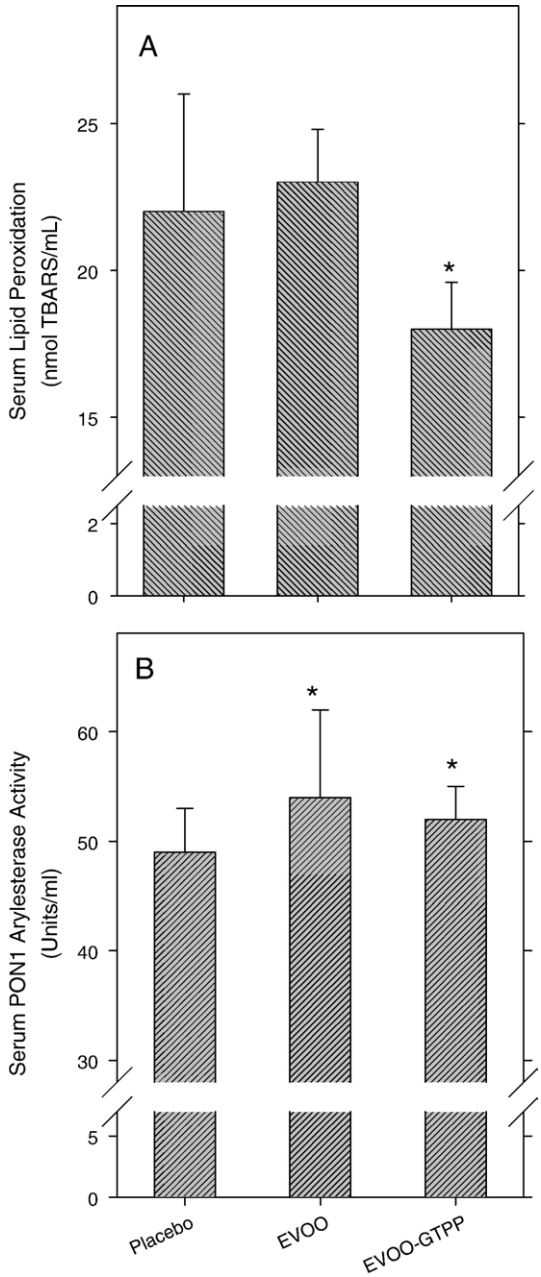

Fig. 4. The effect of EVOO or EVOO-GTPP consumption by E^0 mice on AAPH-induced serum lipid peroxidation and on serum PON1 activity. E^0 mice consumed EVOO or EVOO-GTPP by gavage feeding (7 μl/mouse/day for 2 months). The placebo-treated mice received only water. At the end of the study, mouse blood samples were collected. (A) The blood samples were diluted (×4) with PBS and incubated with AAPH (50 mmol/L) for 2 h at 37°C. The extent of serum lipid peroxidation was determined by the TBARS assay. (B) Serum PON1 arylesterase activity was determined using 1 mmol/L phenyl acetate as substrate. Results are expressed as mean±S.D. of eight mice in each group. *P<.01 versus placebo.

state and that the addition of GTPPs to EVOO had no antioxidant effect in the macrophage system, as opposed to the serum system.

3.3.4. The effect of EVOO or EVOO-GTPP consumption by E^0 mice on macrophage cholesterol metabolism

The extent of native LDL uptake by the MPM was not significantly affected after consumption of EVOO or EVOO-GTPP, as compared to its uptake by MPM from placebo-treated mice (80±3 or 78±3 arbitrary units vs. 75±5 arbitrary units, respectively). In contrast, the uptake of Ox-LDL by MPM harvested from the mice that consumed EVOO or EVOO-GTPP was slightly but significantly inhibited, by 4% or 6%, respectively, in comparison to Ox-LDL uptake by MPM from placebo-treated mice (Fig. 5A). EVOO consumption also resulted in a significant but small decrement, by 11%, in cholesterol biosynthesis rate in the MPM, as compared to MPM from placebo-treated mice (770±37 vs. 685±27 cpm/mg cell protein, respectively). EVOO-GTPP, however, did not inhibit cellular cholesterol biosynthesis rate (785±32 cpm/mg cell protein). Finally, the most impressive effect of EVOO or EVOO-GTPP consumption by E^0 mice was demonstrated in the rate of HDL-mediated cholesterol efflux from the MPM. Consumption of EVOO stimulated HDL-mediated cholesterol efflux rate from the MPM by 42%, as compared to that from the MPM of placebo-treated mice (Fig. 5B). Consumption of EVOO-GTPP stimulated the extent of cholesterol efflux from the MPM by HDL, by as high as 139%, in comparison to that from the MPM of placebo-treated mice (Fig. 5B). Similar results were observed upon using ApoA-I instead of HDL as the acceptor for cholesterol (data not shown).

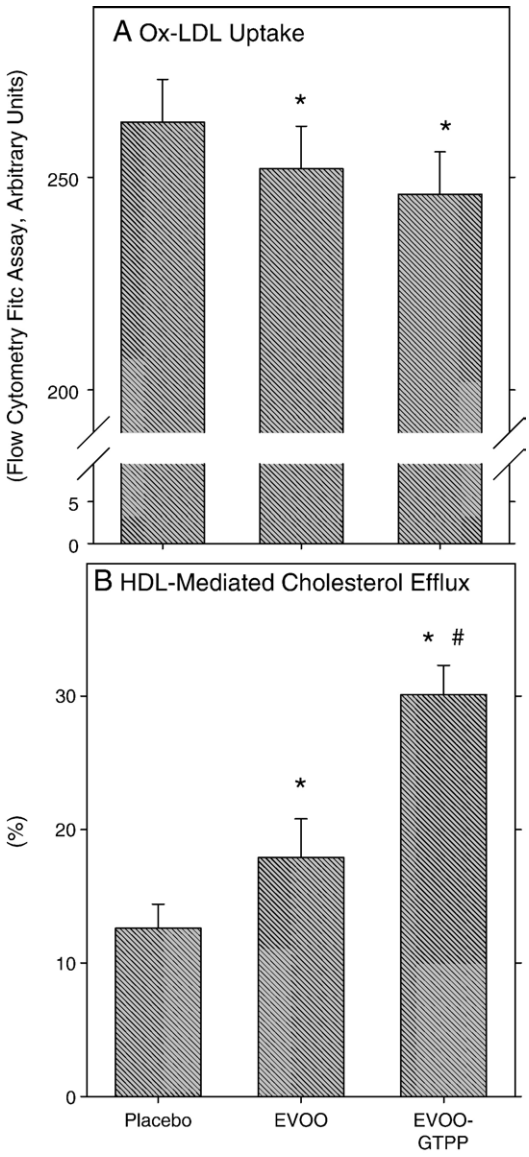

Fig. 5. The effect of EVOO or EVOO-GTPP on macrophage cholesterol metabolism. E^0 mice consumed EVOO or EVOO-GTPP by gavage feeding (7 μl/mouse/day for 2 months). The placebo-treated mice received only water. At the end of the study, MPMs were harvested. (A) The cells were washed, and the extent of FITC-labeled Ox-LDL (25 μg protein/ml) uptake was determined by flow cytometry. (B) After cell wash, the extent of HDL-mediated macrophage cholesterol efflux was determined after 3 hours of incubation. Results are expressed as mean±S.D. of three different experiments. *$P<.01$ versus placebo; $^{\#}P<.01$, EVOO-GTPP versus EVOO.

4. Discussion

In the present study, we have clearly demonstrated, for the first time, that enrichment of EVOO with GTPPs improved the antiatherogenic properties of the olive oil, resulting in enhanced macrophage cholesterol efflux and a significant attenuation of atherosclerosis development, as shown in the atherosclerotic E^0 mice.

EVOO-GTPP contains twice as much polyphenols in comparison to EVOO, but at the relatively high concentration used in this study, EVOO and EVOO-GTPP similarly inhibited copper-ion-induced LDL oxidation in vitro, in comparison to other commonly used oils such as corn, soy and fish oils. In contrast, in vivo, EVOO consumption by the atherosclerotic E^0 mice did not affect the extent of serum lipid peroxidation. Some other studies performed in healthy or atherosclerotic patients [15,19–21] or in hyperlipidemic rabbits [24,25] using diet that was enriched with olive oil demonstrated that the phenolic compounds in olive oil were absorbed from the intestine [46] and that serum oxidative state was decreased. Similarly, a study using E^0 mice showed a reduction in the level of serum isoprostanes [25]. The discrepancy among various oxidative stress studies could be related to differences in the oil preparation, the concentration used, the manner of olive oil feeding and/or the extent of the basal oxidative stress present in the animals used. The current study regarding copper-ion-induced LDL oxidation in vitro was performed in order to compare the antioxidative capacities of EVOO and EVOO-GTPP with other commonly used oils. Since we observed a significant inhibition in the extent of in vitro LDL oxidation, by EVOO and more so by EVOO-

GTPP, we next performed the in vivo study using the atherosclerotic E^0 mouse model, as these mice are under high oxidative stress. In vivo, only EVOO-GTPP consumption decreased serum oxidative stress in these mice. Since Ox-LDL is cytotoxic to arterial wall cells, and as its enhanced uptake by macrophages converts them to foam cells [2,5], it is considered a reliable index for atherogenesis, and the beneficial effects of EVOO or EVOO-GTPP on LDL oxidation could have contributed to the attenuation of atherosclerosis development.

The extent of LDL oxidation in vitro is usually related to serum lipid peroxidation ex vivo, but in serum, other factors that can affect the oxidation process exist. Indeed, whereas in vitro copper-ion-induced LDL oxidation was similarly decreased by EVOO and EVOO-GTPP, ex vivo AAPH-induced serum (not isolated LDL) lipid peroxidation was reduced only after EVOO-GTPP consumption. The inability of EVOO to decrease serum lipid peroxidation, as opposed to its inhibitory effect on copper-ion-induced LDL oxidation, could indeed be related to serum components that are not present in isolated LDL. Alternatively, the in vivo results are reflected on the background of high oxidative stress, which is present in E^0 mice [8], as well as on the background of high cholesterol levels (more substrate is available for oxidation). Furthermore, it could be that the concentration of EVOO given to the mice was not sufficient to affect serum oxidative stress. Only EVOO that contained GTPPs and thus possesses increased antioxidant capability affected serum oxidative stress. Such a reduction could have resulted also from an elevation in serum paraoxonase activity. PON1 is an HDL-associated esterase/lactonase [47,48], which was shown to protect lipoproteins in serum from lipid peroxidation [49,50]. Since serum PON1 activity was similarly increased after EVOO and EVOO-GTPP consumption, the inhibitory effect of EVOO-GTPP consumption on serum oxidative stress may be mostly related to a direct effect of the GTPPs present in this olive oil preparation [51–53].

Both EVOO and EVOO-GTPP consumption by the mice increased serum total cholesterol, mostly as a result of increasing serum HDL cholesterol levels. Similar results were observed in other studies after dietary olive oil intake by healthy subjects [54,55] or by E^0 mice [30].

Although, in vitro, olive oil was shown to reduce macrophage oxidative stress [16,56,57] and GTPPs inhibited LDL oxidation by endothelial cells [58], no effect of EVOO consumption by the mice on their peritoneal macrophage oxidative stress could be noted. Furthermore, the addition of GTPPs to the olive oil did not improve the effect of olive oil on oxidative stress in the peritoneal macrophages of mice as opposed to the inhibitory effect shown in serum. This may be related to the different lipid packaging (and, hence, to different susceptibility to oxidation) in the lipoprotein unilamellar membrane versus the cellular bilamellar membrane. Furthermore, the peritoneal macrophages from E^0 mice were also shown to be under increased oxidative stress [9], and it could have been that the concentration of olive oil polyphenols that the peritoneal macrophages were exposed to in the peritoneal cavity was not high enough to decrease macrophage oxidative stress, to increase cellular antioxidants levels or to affect oxidative-related genes.

The most significant effect in vivo of EVOO and EVOO-GTPP consumption was on macrophage cholesterol metabolism and mostly on HDL-mediated cholesterol efflux from the macrophages of mice. Stimulation of HDL-mediated macrophage cholesterol efflux after EVOO or more so after EVOO-GTPP consumption is probably ABCA-1 mediated since similar results to those obtained with HDL were also observed upon using ApoA-I (a known ligand for the ABCA-1 transporter; [59]), instead of HDL. To our knowledge, there are no other studies that analyzed the effect of olive oil consumption on the mouse macrophage's ability to cause cholesterol efflux from the cells to HDL. It was shown, however, that serum or HDL_3 fractions isolated from the blood of healthy subjects after consumption of olive oil induced enhanced cholesterol efflux from cells in culture [60,61]. These effects could be related to physicochemical changes that occur to the HDL particles after interaction with olive oil fatty acids [60]. Similarly, in the present study, olive oil consumption by E^0 mice could affect the macrophage plasma membrane properties such as increased fluidity and changes in the composition of the phospholipids, which could stimulate cellular cholesterol efflux by HDL. Removal of cholesterol from arterial wall cells to the liver by HDL (reverse cholesterol transport) is a most important anti-atherogenic process, which leads to an attenuation in atherosclerotic lesion development [62]. Thus, the significant decrement in the atherosclerotic lesion size of E^0 mice after consumption of EVOO could be possibly related to the increased cholesterol efflux rate from their macrophages. The further stimulatory effect of EVOO-GTPP versus EVOO consumption on HDL-mediated macrophage cholesterol efflux rate was three times higher than the effect of EVOO, indicating that the GTPPs, which are probably bound to the cells' plasma membrane, contributed to the stimulation of HDL-mediated cholesterol efflux from the cells. Indeed, it was recently demonstrated that the major green tea polyphenol epigallocatechin gallate increased cholesterol efflux from human liver hepatocytes [63]. This effect could be secondary to the ability of epigallocatechin gallate to form a complex with cellular free cholesterol [63]. In several studies with RAW or THP-1 macrophage-like cell line, cholesterol efflux rates by HDL were lower than those shown in the present study upon using MPMs. In these studies, however [64,65], the methodology used by the authors was somewhat different from the present one and they used cell lines (which proliferate during the experiment) in contrast to the nonproliferating peritoneal macrophages used in the present study. On using MPMs [66] that were incubated for 4 h with HDL (100 μg of protein/ml), a 15% cholesterol efflux was shown, just like the results presented in the current study (see data for

MPM that were harvested from the placebo-treated mice). The further increased cholesterol efflux rates were observed in MPM from mice that consumed EVOO or EVOO-GTPP, and this additional increase could be related to a direct effect of olive oil phenolics and GTPPs. The increased cholesterol efflux, together with an increase in serum HDL levels, could be helpful antiatherogenic properties of olive oils in humans.

Our studies on the effects of specific antioxidants and their capabilities to retard the progression of atherosclerotic plaque in the E^0 mouse model were conducted on relatively young mice, beginning at 6 weeks and run for a limited period of 8 weeks of treatment. This is in light of our extensive experience in this animal model with large number of antioxidants (red wine, licorice, pomegranate), which have been previously published [67–69]. The differences in atherosclerosis development between antioxidant-treated and placebo-treated mice were always the greatest when using mice at the early stages of atherogenesis. At 20 weeks of age, the extent of the lesions in placebo-treated mice is so extensive that it is difficult to see any beneficial effect at this late stage of atherogenesis. In the above mouse model, there is no need for any addition of cholesterol-rich diet since these mice develop spontaneously elevated serum cholesterol levels and atherosclerotic plaque.

5. Conclusion

We conclude that the antiatherogenic effects of EVOO or more so of EVOO-GTPP could be the underlying mechanism for the significant attenuation in atherosclerosis development, and thus, their consumption is recommended in subjects with a high risk for atherosclerosis development.

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