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Francesco Visioli
Donatella Caruso
Simona Grande
Raffaella Bosisio
Marco Villa
Giovanni Galli
Cesare Sirtori
Claudio Galli

Virgin Olive Oil Study (VOLOS): vasoprotective potential of extra virgin olive oil in mildly dyslipidemic patients

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F. Visioli · D. Caruso · S. Grande · G. Galli · C. Sirtori · C. Galli
Dept. of Pharmacological Sciences
University of Milan
Milan, Italy

R. Bosisio · C. Sirtori
Grossi Paoletti Center for Dismetabolic Diseases
Niguarda Hospital
Milan, Italy

M. Villa
CNR-ITB
Milan, Italy

Dr. Francesco Visioli (✉)
Via Balzaretti 9
20133 Milan, Italy
Fax: +39-027/0042-6106
E-Mail: francesco.visioli@unimi.it

Summary *Background* In vitro, olive phenols exert potent antioxidant and enzyme-modulating activities. *Aim of the study* We comparatively evaluate, in mildly dyslipidemic patients, the vasoprotective potential of extra virgin olive oil. *Methods* 22 patients were administered 40 mL/day of either extra-virgin, i. e. phenol rich, or refined, i. e. phenol poor, olive oils (EVOO or ROO, respectively, with nearly identical fatty acid composition), with a crossover design. Each treatment was carried out for seven weeks, with four weeks of washout in between. Plasma antioxidant capacity, serum thromboxane B_2 (TXB_2) formation, and urinary isoprostane excretion were evaluated as surrogate markers of cardioprotective potential and vascular function. *Results* No effects on plasma lipid/lipoprotein profile were observed. Conversely, EVOO consumption was associated with favorable effects on circulating markers. Namely, decreased serum TXB_2 production and increased plasma antioxidant capacity were observed when EVOO was administered in both treatment arms. Neither treatment had any significant effect on isoprostane excretion. *Conclusions* EVOO consumption by mildly dyslipidemic patients is associated with favorable changes in circulating markers of cardiovascular condition. Based on current knowledge, these effects may be associated with cardioprotection.

Key words atherosclerosis – antioxidants – coronary disease – free radicals – olive oil – isoprostanes

Introduction

The incidence of major risk factors for coronary heart disease (CHD), e. g. high blood pressure and elevated serum cholesterol concentrations, is similar between populations of the Mediterranean area and those of other North European and Western countries, despite remarkable differences in the prevalence of CHD in these areas [1, 2]. This observation, together with experimental evidence of the involvement of lipid, namely low density lipoprotein (LDL), oxidation [3–5] in the onset of atherosclerosis, led investigators to propose a protective role for dietary antioxidants. Accordingly, the consumption of diets rich in antioxidants, e. g. vitamins and polyphenols, is associated with decreased oxidative stress [6] and lower incidence of CHD [7–9].

Olive oil is the principal source of fat in the Mediterranean area, where cardiovascular mortality is remarkably low [10–12]. Thus far, most of the cardioprotective effects of olive oil have been ascribed to its high content of the monounsaturated fatty acid (MUFA) oleic acid (18:1n-9). Whether the beneficial effects of olive oil on the cardiovascular system are exclusively due to oleic acid is, however, questionable. Indeed, intake of MUFAs lowers LDL susceptibility to oxidation compared to sup-

plementation with polyunsaturated fat [13–15]. Furthermore, oleic acid has been shown to exert endothelial vasomodulatory and antioxidant effects [16, 17]. Yet, the overall proportion of MUFAs in the Mediterranean diet is not much different from that of other diets, like the North American one, which are associated with higher cardiovascular mortality [18]. The high proportion of MUFAs, namely oleic acid, in food items such as poultry, pork, and recently-marketed seed oils accounts for this similarity. Moreover, the direct effects of MUFAs on serum lipid/lipoprotein profiles are, at present, equivocal [19]. Replacement of saturated fat with MUFA is, in fact, associated with favorable changes in plasma lipoprotein profile (reduction of LDL cholesterol and increase in HDL cholesterol concentrations) but this effect may partly be attributed to the concomitant reduction of saturates. Further, equicalorical replacement of carbohydrates with MUFAs leads to modest modifications of serum cholesterol and lipoprotein concentrations [20].

Extra virgin olive oil (EVOO) is rich in phenols; specifically, the catecholic compounds hydroxytyrosol (HT) and oleuropein (OE) act as potent antioxidants, free radical scavengers, and modulators of various oxygen-dependent enzymes [21–23]. Although most of these properties have been demonstrated *in vitro*, published data show that HT is dose-dependently absorbed after oral ingestion by rats [24–28] and humans [29–31] and that it retains its antioxidant properties *in vivo* [28, 32–34].

Based on such data, we sought to comparatively investigate the effects of extra virgin (phenol-rich) olive oil supplementation on oxidative and enzymatic markers of processes currently considered relevant for CHD, in mildly dyslipidemic patients. The Virgin Olive Oil Study (VOLOS) is a randomized, single-blind, crossover study aimed at comparing the effects of EVOO with those of refined (phenol-poor) olive oil, thus maintaining identical MUFA supplementation.

Materials and methods

Olive oils

Extra virgin olive oil (EVOO), naturally rich in phenolic compounds, and refined olive oil (ROO), very low in phenolics, were provided by Carapelli s. p. a. (Tavarnelle Val di Pesa, Italy) in unlabeled 500 mL dark glass bottles. A dietician instructed study subjects to consume 40 mL of raw olive oil/day, subdivided between lunch and dinner, to be eaten with pasta or vegetables. This amount is within the range of habitual consumption in Mediterranean populations [35]. Olive oil was provided to study subjects in large excess for consumption by the rest of their family.

EVOO had a total hydroxytyrosol content (as free HT + HT esterified in oleuropein), of 166 mg/L, as assessed by gas-chromatography/mass spectrometry analysis of the phenolic extracts [29, 36], while the phenolic concentration of ROO was 2 mg/L. Estimated daily intakes of total HT were therefore 6.64 and 0.08 mg, respectively.

Fatty acid analysis of EVOO and ROO was performed by gas liquid chromatographic separation of the methyl esters [37] and revealed nearly identical fatty acid (FA) profiles. The predominant FAs in both oils were oleic (70.9 % in EVOO vs. 72.7 % in ROO), palmitic (11.5 % vs. 10.7 %), and linoleic (8.5 % vs. 7.6 %) acids.

All reagents were from Sigma (Milan, Italy) and were of the highest purity available.

Patient selection and study design

This study conforms with the principles outlined in the "Declaration of Helsinki", and has been approved by the Niguarda Hospital Internal Review Board.

Study subjects were selected among those who were diagnosed for mild dyslipidemia at the Grossi Paoletti Center, University of Milan, before initiation of pharmacological therapy. Inclusion criteria were: total cholesterol 220–280 mg/dL, low-density lipoprotein cholesterol (LDL-C) 150–170 mg/dL, and triglycerides (TG) 130–180 mg/dL.

Twenty-two subjects (12 males and 10 females, aged 18 to 65 years, seven smokers and 15 nonsmokers, BMI < 25 kg/m^2) were enrolled and gave informed consent to the study.

The study followed a crossover design. Three weeks (T –21 days, "run-in period") before initiation of the two treatments, all subjects consumed 40 mL/day of ROO, in order to minimize potential body storage of olive oil phenolic compounds. This run-in period was followed by treatments with either one of the two oils, which lasted for a period of seven weeks (T49 days). During this period, patients were randomly divided into two groups. The first one, consisting of 13 individuals, was randomly assigned to the administration of EVOO. The second group (nine subjects) was initially given ROO. The treatment period was followed by four weeks of washout (WO), during which all subjects consumed ROO. After the washout period, subjects were switched treatment, which lasted for an additional seven weeks. Compliance was monitored by frequent interviews with the dietician, who also controlled the diet for other polyphenol-rich foods, e. g. tomato.

Blood analyses

Blood was drawn in the morning from the antecubital vein of fasting subjects at the following time points: T-

21 (recruitment), T0 (initiation of treatment), T28 and T49 (28 and 49 days, respectively, middle and end points of first treatment), washout, T28 and T49 of the second treatment.

One test tube was employed for lipid analysis (by routine laboratory methods), while another tube was placed in a 37 °C water bath for one hour, to allow for clotting [38]. Serum was then separated by centrifugation at 2000 x g for 10 min and subsequently stored at –80 °C. Thromboxane B_2 (TXB_2) in serum was quantified by immunoassay (Cayman Chemical, Ann Arbor, MI), following the manufacturer's instructions.

Blood was also collected into a third tube, containing Li^+ heparin as anticoagulant. Total antioxidant capacity of plasma was evaluated by an established method based on the reduction, by antioxidants, of Cu^{++} to Cu^+, as previously described (Oxis Research, Portland, OR) [28, 39].

Urine analyses

24-hour urine was collected at the beginning and at the end of each treatment (including washout). Total collected volumes per individual (which varied very little from one collection to the other) were measured and 40 mL aliquots were stored at –80 °C. The F_2 isoprostane, namely 8-iso-$PGF_{2\alpha}$ ($iPF_{2\alpha}$-III), concentration was evaluated by a mass spectrometry-validated immunoassay, as previously described [34, 40].

Laboratory personnel were blinded to treatments.

Statistical analyses

Data were analyzed for distribution using frequency histograms. Two separate analyses were performed: 1) a three periods crossover analysis with two treatments and two sequences and 2) repeated measures analysis of variance. The latter was performed to confirm the results of the crossover analysis and to identify potential trends in the outcomes. Software: Stata version 7 and SAS version 8.01. A p value less than 0.05 was considered statistically significant.

Results

Treatments with either EVOO or ROO were not associated with marked variations of serum lipid and lipoprotein parameters, such as total, LDL-, and HDL-cholesterol, and triacylglycerols (Table 1). Other indexes such as BMI, mean blood pressure, and glycemia were also unaffected by the administration of either oil (data not shown).

Serum TXB_2 concentrations, a sensitive index of thromboxane production by maximally activated platelets, were decreased (~21 %) by EVOO supplementation. This effect was noted both when EVOO was administered in the first and in the second arm of the study (Fig. 1). In particular, seven weeks of EVOO intake were associated with a reduction of serum TXB_2 of approximately 20 % (Fig. 1a). This effect was reverted upon subsequent ROO administration. These data were confirmed by those obtained following administration of EVOO in the second arm of the study, which led to a ~30 % reduction of serum TXB_2 production (Fig. 1b). On the other hand, ROO as first treatment did not affect TXB_2 production.

The antioxidant capacity of plasma increased after EVOO administration (from 210 to 291 μM Cu^{++} reduced, i. e. by approximately 40 %), returning to basal levels after washout (Fig. 2a). A further reduction was observed after treatment with ROO. When ROO was administered during the first arm of treatment, a reduction of antioxidant capacity from 221 to 161 μM Cu^{++} reduced (–28 %) was observed (Fig. 2b). Subsequent consumption of EVOO increased antioxidant capacity up to ~12 % over basal levels.

Table 1 Serum lipid profile of patients administered extra virgin olive oil or refined olive oil

	T0	T28EVOO	T49EVOO	WO	T28ROO	T49ROO
CHO	249.8±23.6	240.2±69.6	247.9±28.6	260.4±21.9	257.6±15.3	261.6±23.3
TG	129.1±62.1	117.0±49.3	101.0±29.8	121.1±53.7	118.0±43.3	135.8±62.3
HDL	53.4±15.1	54.7±15.6	53.3±14.4	57.5±16.6	52.9±10.8	63.8±21.9
LDL	170.4±19.7	177.5±27.2	175.7±28.8	178.9±22.2	180.5±10.1	170.3±28.1
	T0	T28ROO	T49ROO	WO	T28EVOO	T49EVOO
CHO	223.4±22.6	238.4±34.7	256.3±44.9	246.0±36.6	238.3±23.3	253.6±37.9
TG	140.3±42.8	164.5±54.7	213.3±78.5	123.5±43.3	148.9±88.9	162.3±81.4
HDL	51.1±13.2	53.1±18.9	51.0±18.8	57.4±18.4	54.8±15.4	60.3±18.4
LDL	144.2±32.8	152.3±35.5	170.4±49.3	162.9±30.8	153.2±28.6	160.4±42.1

CHO total cholesterol; *TG* triacylglycerols; *HDL* high density lipoprotein; *LDL* low density lipoprotein; *EVOO* extra virgin olive oil; *ROO* refined olive oil; *T0* beginning of trial; *T28* 28 days of supplementation; *T49* 49 days of supplementation; *WO* washout (four weeks). n = 22. Data are means ± S. D. and are expressed as mg/dL

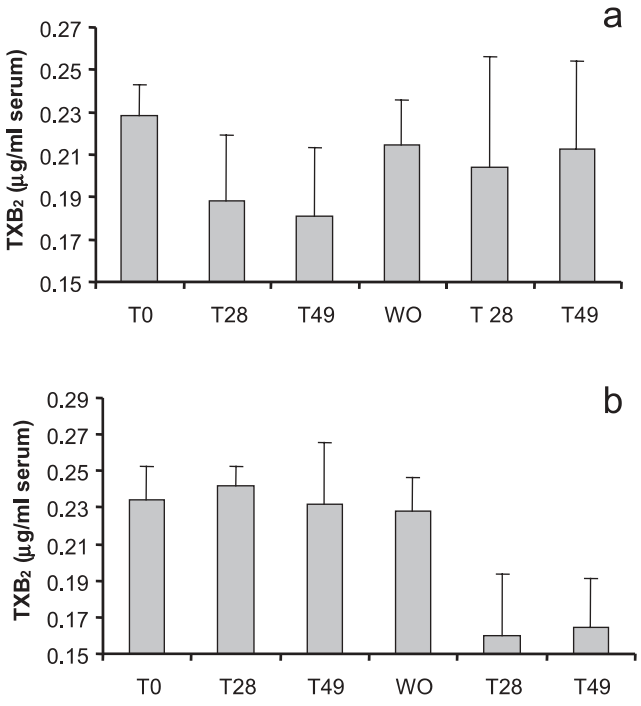

Fig. 1 Serum TXB_2 concentrations (µg/ml) in subjects administered EVOO or ROO for 49 days. **a** Subjects were first given EVOO and then, after washout, ROO. **b** Subjects were first given ROO and then, after washout, EVOO. Data are means ± S. D., n = 22

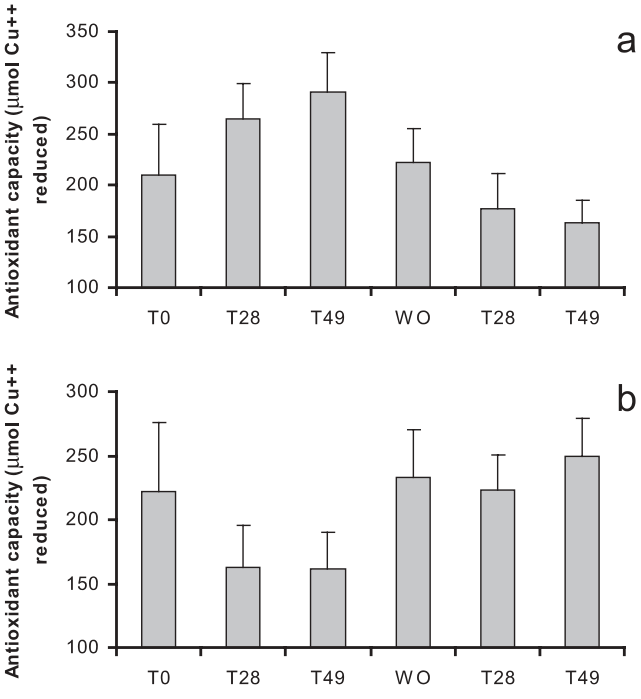

Fig. 2 Plasma antioxidant capacity (as µM Cu^{++} reduced) in subjects administered EVOO or ROO for 49 days. **a** Subjects were first given EVOO and then, after washout, ROO. **b** Subjects were first given ROO and then, after washout, EVOO. Data are means ± S. D., n = 22

The urinary excretion of 8-iso-$PGF_{2\alpha}$ (Fig. 3) was not affected by the two different treatments (see later), although a trend toward an enhancement of total isoprostane excretion by ROO was recorded in both arms of the crossover study (Fig. 3a and b).

The statistical analyses of the crossover design excluded any carry-over effect.

As far as TXB_2 production was concerned, the crossover analysis showed two separate effects of treatment ($p < 0.0001$) and period ($p < 0.01$). The analysis of variance confirmed the treatment and period effects and highlighted the presence of a linear trend in the outcome, both overall ($p < 0.001$) and within treatments ($p < 0.001$).

The antioxidant capacity exhibited similar statistical significance. The crossover analysis showed again two separate effects of treatment ($p < 0.0001$) and period ($p < 0.0001$). The analysis of variance confirmed the treatment effect ($p < 0.0001$). A linear trend in the outcome was found only within treatments ($p < 0.001$).

Finally, the crossover analysis of 8-iso-$PGF_{2\alpha}$ excretions showed both treatment ($p < 0.05$) and period (0.0001) effects. The analysis of variance confirmed a period effect ($p < 0.01$) but did not reveal any effect due to the treatment.

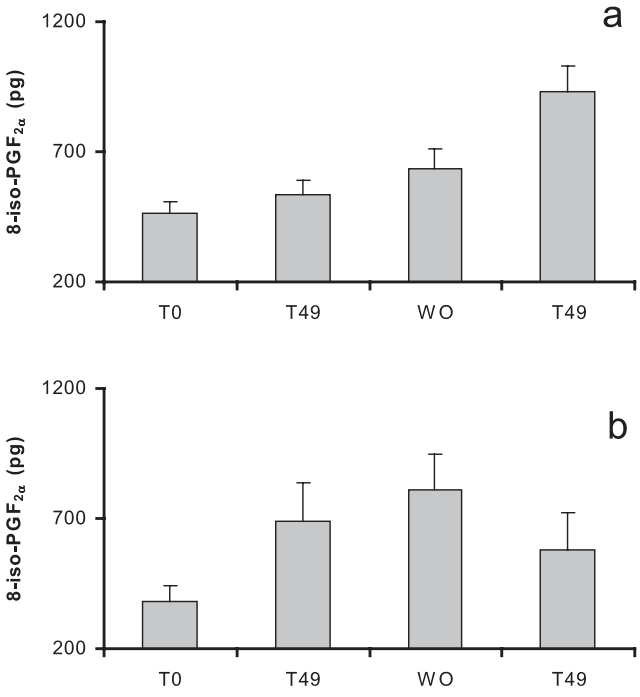

Fig. 3 Total, 24-hour urinary 8-iso-$PGF_{2\alpha}$ excretion (pg) in subjects administered EVOO or ROO for 49 days. **a** Subjects were first given EVOO and then, after washout, ROO. **b** Subjects were first given ROO and then, after washout, EVOO. Total volume of 24-hour urine was measured and 8-iso-$PGF_{2\alpha}$ concentration was evaluated by immunoassay. Data are means ± S. D., n = 22

Discussion

Interest in the biological activities of olive oil phenolics has been steadily growing over recent years, as their "pharmacological" properties might partly explain the low CHD mortality in the Mediterranean area. In fact, the low CHD mortality associated with olive oil consumption appears not to be exclusively related to its high monounsaturated fatty acid content. Currently, in vivo studies of the biochemical effects of natural and synthetic antioxidants are scarce, mostly due to the lack of appropriate markers of antioxidant effect [41, 42]. In the VOLOS study, we focused on the evaluation of biochemical indexes, either related to the presence of the compounds under investigation in the analyzed biological samples (antioxidant capacity and serum TXB_2) or direct markers of in vivo oxidative processes and their prevention (urinary isoprostanes).

These markers are currently considered relevant to the onset and development of CHD: it is therefore of interest that the administration of extra virgin olive oil (EVOO), naturally abundant in phenolic compounds, was associated with improvement of these indexes. The crossover design of VOLOS further strengthens these observations. In particular, consumption of EVOO increased plasma antioxidant capacity, consistent with previous data obtained in the rat but in disagreement with what was reported by Visser et al. [43], who reported no antioxidant effects after three weeks of EVOO administration (providing 18 mg/day of total phenols with an unknown proportion of catechols). Also consistent with in vitro studies [44] reporting antithrombotic potential of olive oil phenols is the observation of reduced production of TXB_2 by maximally activated platelets, such as those in serum allowed to clot at 37 °C for one hour. Other in vitro and in vivo studies suggested antiinflammatory activities of olive oil phenols [45–49], reinforcing the notion that these compounds can modulate enzymatic activities related to eicosanoid metabolism. Possibly, olive oil phenolics are able to maintain reduced intracellular environment, thus inhibiting the activities of peroxide tone-dependent enzymes [50, 51].

The prolonged administration of olive oil appears to increase 8-iso-$PGF_{2\alpha}$ excretion, regardless of the kind of olive oil supplied to the patients. This finding is apparently in contrast with results obtained after a single administration of a phenol-enriched (providing higher doses of phenol) EVOO to humans [33] or of pure HT to sidestream smoke-exposed rats [34]. Yet, the significant period effect does not allow any inference on the effects of olive oil phenolics on this parameter. Moreover, it should be noted that, unlike blood sampling, collection of urine was performed only twice for each arm of the treatment, making it impossible to statistically assess trends. Thus, the period effect might be masking a possible treatment effect.

The lack of differences in the effects of both oils on the patients' lipid/lipoprotein profiles – except for the non-significant favorable changes in TG concentrations after EVOO intake during the first arm of the treatment – was expected, due to the relatively limited duration of the study and to the nearly identical fatty acid compositions of the two oils. Relevant to these results, it is noteworthy that the Lyon Diet Heart Study observed a dramatic reduction in CHD mortality following dietary intervention that did not affect cholesterol concentrations [52, 53].

One of the most interesting aspects of the results obtained in the VOLOS study is that they were obtained with a daily ingestion of a food item (EVOO) in amounts (40 ml/day) and with a phenolic content (providing 6.6 mg/day) that are comparable to those currently consumed by many population groups in the Mediterranean area [54]. In contrast, available data on the biological effects of other antioxidants, namely vitamins, in animals and humans have been obtained by administering high doses of such molecules, up to 100-fold the average dietary intakes, i. e. with a pharmacological approach [5, 55].

As opposed to epidemiological studies of antioxidant intake and CHD mortality, recent reports of large-scale clinical trials have shown that administration of antioxidant vitamins, namely vitamin E and β-carotene, does not reduce coronary events in CHD patients [56]. Proposed interpretations include correct selection of individuals at risk for oxidative stress [57], administration of right antioxidants at right dosages for the right time [5, 58], and lack of *in vivo* markers of antioxidant effect [5, 42]. Furthermore, the possibility that dietary rather than pharmacological intake of antioxidants is accompanied by higher absorption [59–61] and ingestion of "cooperating" antioxidants [62] should be taken into account. To date, human studies based on the administration of oligonutrients from foods, e. g. from cocoa and tea, are still scarce [51, 63–66].

In conclusion, data from VOLOS demonstrate that inclusion of practical quantities of extra virgin, phenol-rich olive oil in the diet can positively modify surrogate biological markers – associated with lower oxidative stress and thrombotic potential – for vascular function and cumulative cardiovascular risk, in mildly dyslipidemic patients. Based on current knowledge, these modifications may be associated with cardioprotective effects.

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