An Extra-Virgin Olive Oil Rich in Polyphenolic Compounds Has Antioxidant Effects in Of1 Mice

Maria-Jesús Oliveras-López, Genoveva Berná, Everardo M. Carneiro, Herminia López-García de la Serrana, Franz Martin, and M. Carmen López

Extra-virgin olive oil (OO) is becoming more important in daily diets due to its beneficial effects on health, most of which are because of its antioxidant content. We studied the antioxidant activity and mechanisms of an extra-virgin OO that is rich in phenolics on pancreatic islets and liver in control mice (CTL) fed a nonpurified diet and in mice supplemented with 50 µL/d sunflower oil (SO) or 50 µL/d extra-virgin OO for 4 d. Plasma hydroxytyrosol concentration was determined by HPLC-diode array detector. Plasma antioxidant capacity, enzymatic activities, and lipid peroxidation were measured by spectrophotometry. Islet function was studied by measuring insulin release. Islet cell gene expression was examined using quantitative RT-PCR. The plasma hydroxytyrosol concentration was greater in OO mice than in CTL or SO mice (P < 0.05) and was greater in SO mice than in CTL mice. The ratio of reduced:oxidized glutathione and the antioxidant capacity in plasma was greater in OO mice than in CTL or SO mice (P < 0.05) and higher in SO mice than in CTL mice. Glucose-stimulated insulin secretion was greater in OO mice than in CTL or SO mice (P < 0.05) and was also higher in SO mice than in CTL mice. Protection against liver cell and β cell membrane lipid peroxidation was greater in OO mice than in CTL or SO mice (P < 0.05) and was greater in SO mice than in CTL mice. Catalase (CAT) expression in the islet of Langerhans was higher in OO mice than in CTL mice and SO mice (P < 0.05). The CAT and glutathione peroxidase 1 activities in the islet of Langerhans were 25% greater in OO mice than in CTL mice and higher than in SO mice (P < 0.05) and they were greater in SO mice than in CTL mice. These results indicate that, in metabolic tissues, protection by extra-virgin OO against oxidative stress occurs primarily through a direct antioxidant effect as well as through an indirect mechanism that involves greater expression and activity of certain enzymes with antioxidant activities.

Introduction

Extra-virgin olive oil (OO) is becoming more important in daily diets due to its beneficial effects on health. Phenolic compounds, oleic acid, and tocopherols are thought to be related to some of these beneficial effects (1). Among these antioxidants from extra-virgin OO, phenolic compounds have received the most attention. Oleuropein derivatives, especially hydroxytyrosol, have been shown to have protective effects against markers associated with the atherogenic process (1–3) and to have an antioxidant capacity higher than that of other known antioxidants such as vitamins E and C (4). Recent studies (5) now show, however, opposite results with hydroxytyrosol administration in the atherosclerotic process.

Previous articles (6) have shown that seed oils, with low levels of polyphenols and a greater concentration of vitamin E, exhibited a smaller antioxidant capacity than the extracts obtained from extra-virgin OO.

Due to the possible health benefits derived from the consumption of polyphenols, the study of their possible mechanisms of protection is becoming a matter of great importance. In humans, several studies have investigated the bioavailability of polyphenols from extra-virgin OO after diet supplementation (1). Reported results with humans and animals show that the antioxidant activity of hydroxytyrosol is preserved after ingestion (7–8).

The studies of total antioxidant capacity (TAC) attempted to evaluate general levels of antioxidants in plasma (9), finding decreased TAC in animals exposed to oxidative stress. Tissues with low levels of the reduced form of glutathione (GSH) are...
much more sensitive to external oxidative injuries (10), as cells exposed to higher oxidation exhibit accumulation of oxidized glutathione (GSSG).

Pancreatic islets have a higher risk of developing oxidative stress (11) than other tissues. In this regard, a protective effect of polyphenol-rich diets has been studied previously in diabetes (12). In addition, a positive effect of monounsaturated fat-rich diets on insulin sensitivity has been shown in both healthy (13) and diabetic (14) subjects. Malondialdehyde (MDA) accumulation results from membrane peroxidation (15). Therefore, quantification of MDA accumulation provides insight into the integrity of lipid membranes.

Catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GTpx) are considered some of the more important antioxidant defenses of the organism against the production of free radicals (10). The low level of expression of these antioxidant enzymes has been considered the main reason for the susceptibility of the β cell to oxidative stress (16); therefore, it was predicted that increasing the expression of these enzymes would impart a beneficial effect of antioxidant protection.

In the present study, some of the mechanisms by which polyphenols from extra-virgin OO impart protective and beneficial effects as antioxidants were proposed. The in vivo biological effects of a diet rich in extra-virgin OO phenolics, especially hydroxytyrosol, were studied in mice.

Materials and Methods

Mice and diets. Twelve to 16-wk-old male OF1 mice purchased from Charles River were used throughout the experiments. The mice were allowed free access to food and water and were maintained on a 12-h light-dark cycle at 24°C and constant humidity. Mice were fed with a standard nonpurified diet containing 145 g/kg protein, 35 g/kg fat, 3.2 kcal/g (13.4 kJ/g), and 45 g/kg fiber (Teklad Global 14% Protein Maintenance Diet 2014, Harlan Interfarma Iberica S.A.). Three groups of mice with the same mean body weights (31–33 g) were established: 1) control (CTL) group (n = 21), mice fed a standard rodent nonpurified diet; 2) sunflower oil (SO) group (n = 46), mice fed with standard rodent nonpurified diet plus SO for 4 d; and 3) OO group (n = 49), fed a standard rodent nonpurified diet plus extra-virgin OO for 4 d. Mice were dosed daily (at 0900–1000) with 50 μL of oil. The extra-virgin OO used had a high total phenol concentration (>1500 mg/kg) with an elevated hydroxytyrosol concentration (10 μg/g), as determined by Mateos et al. (17). The SO used had very low levels of total polyphenols (6,18). Mice received the oil by oral feeding using a sounding line connected to a syringe to control the dosage employed. Extra-virgin OO or SO supplementation did not modify food or water intake (data not shown). The review boards of animal ethics at our institutes (Andalusian Center of Molecular Biology and Regenerative Medicine of Seville and Institute of Biology; State University of Campinas) approved this study. We followed the requirements regarding the protection of animals used for experimental and other scientific purposes (council directive of 24 November 1986; 86/609/EEC).

Sample collection and processing. Twenty-four hours after the 4th oil dose, mice were anesthetized with 50 mg/kg of sodium pentobarbital, and 1 mL of blood was obtained by intracardiac puncture. Plasma was separated by centrifugation at 1000 × g; 12 min at 4°C in nonsilicon-coated tubes with sodium heparin. All samples were stored at −80°C until analysis.

Plasma hydroxytyrosol determination. We performed polyphenol plasma extraction and quantitative plasma hydroxytyrosol determination as reported by Ruiz-Gutiérrez et al. (19). All analytical determinations were performed in triplicate. The plasma hydroxytyrosol concentration was determined in CTL, OO, and SO mice after 3 and 4 d of supplementation with 50 μL/d and 100 μL/d of oil.

Measurements of plasma antioxidant capacity. Antioxidant capacity and the GSH/GSSG relationship were measured by colorimetric assays using the Antioxidant Potential assay kit (Deltacron) and the GSH/GSSG-412 assay kit (Oxid International), respectively. Regarding the GSH/GSSG measurements, the plasma was immediately frozen at −80°C until the determination day to maintain the reduct state of the GSH. All analytical determinations were conducted in triplicate.

Insulin measurements. Insulin measurements were performed as previously described (20). Briefly, fresh collagenase-isolated islets were incubated for 30 min at 37°C in fresh modified Krebs ringer buffer (KRB) containing 115 mmol/L NaCl, 10 mmol/L NaHCO3, 5 mmol/L KCl, 2 mmol/L Na2HPO4, 1.1 mmol/L MgCl2, 25 mmol/L HEPES acid, and 2.56 mmol/L CaCl2 and supplemented with 5.6 mmol/L glucose and 1% bovine serum albumin (Sigma), pH 7.4. Then, one-half of the islets were incubated for 30 min at 37°C in the same KRB buffer plus 10 μmol/L hydrogen peroxide, whereas the rest were incubated in the same KRB buffer alone. Finally, islets were incubated in groups of 4 in 1 mL of KRB with 1% bovine serum albumin plus the different glucose concentrations (3 and 22.2 mmol/L) for 60 min at 37°C. Insulin content and secretion were measured as previously described (20). In addition, 15 μL aliquots of the same samples were stored at −80°C for protein determination by Bradford assay. Insulin was assayed by radioimmunossay using a kit from Diagnostic Products. Standard curves and experimental points were conducted in triplicate.

Lipid peroxidation determination. Fresh collagenase-isolated islets and 30 mg of disaggregated liver were incubated for 30 min at 37°C in KRB buffer plus 10 μmol/L hydrogen peroxide. Afterwards, both tissues were washed with PBS, sonicated at 4°C in lysis buffer (50 mmol/L Tris-HCl (pH 7.5), 0.9% NaCl, 1 mmol/L EDTA, 100 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L leupeptin, 1 g/L pepstatin, and 100 g/L aprotinin), and centrifuged at 20,800 × g; 15 min at 4°C to obtain supernatants. In addition, 15 μL aliquots of the same samples were stored at −80°C for protein determination by Bradford assay. Lipid peroxidation was assessed using an assay based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA and 4-hydroxyalkenals at 45°C, as described by Esterbauer and Cheeseman (21).

Quantitative PCR. cDNA was extracted with a specific RNA protocol. RNA was prepared using Trizol (Invitrogen) reagent according to the instructions, and 2 μg of total RNA was reverse transcribed using TaqMan Universal PCR Master mix (Applied Biosystems). The purity of the RNA extracted was measured in a spectrophotometer at 260 nm. The cDNA was amplified by quantitative real-time PCR using an ABI prism 7700 Sequence Detection system (Applied Biosystems). Quantitative real-time PCR were performed in triplicate using 5 μL of cDNA, 10 μL of Taqman Universal PCR Master mix 2×, and 1 μL of mix 20× of probe and primers in a final volume of 20 μL. Primers and probes for genes were from Applied Biosystems Assay on-Demand Gene expression products: Catalase (Mm 00437992_m1), Glutathione peroxidase 1 (Mm 00657676_g1), Superoxide dismutase 2 (Mm 00449726_m1), and Insulin (Mm 00448751_m1). Insulin measurements were performed as previously described (20).

Measurement of antioxidant enzyme activities. The activities of SOD, CAT, and GTpx were measured by spectrophotometric assay kits from Cayman Chemicals. Antioxidant enzyme activities were measured in freshly isolated islets incubated for 60 min at 37°C in the presence of 5.6 mmol/L glucose. Islets were homogenized in cold buffer containing 50 mmol/L potassium phosphate and 1 mmol/L EDTA at pH 7.4. Total SOD activity was measured by utilization of a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. CAT activity was measured using the peroxidatic function of CAT.

Antioxidant effects of extra-virgin olive oil 1075
CAT. The GT\textsubscript{PX} activity was measured indirectly by a coupled reaction with glutathione reductase. All analytical determinations were conducted in triplicate.

**Statistical analysis.** Results are expressed as means ± SEM. Differences in means were tested for significance by 1-way ANOVA and by the following post hoc tests when necessary for multiple comparisons: Bonferroni, Fisher least significant difference tests, and the Tukey-Kramer test when the group size was the same. Analyses were performed using the SPSS statistical software package (version 15.0). Differences were considered significant for P < 0.05.

**Results**

Mice from the OO and SO groups reached their maximum plasma hydroxytyrosol levels after 3 d of supplementation with 50 μL/d of oil. The plasma hydroxytyrosol concentration was greater in OO mice than in CTL or SO mice (P < 0.05) and was greater in SO mice than in CTL mice for all the doses and time points assayed. There were, however, no significant differences within each group. After 4 d of 50 μL/d oil supplementation, the plasma hydroxytyrosol concentration in OO mice (2.3 ± 0.19 μmol/L) was 10-fold higher than that of the CTL group (0.21 ± 0.06 μmol/L) and 0.9-fold higher than in the SO group (1.21 ± 0.09 μmol/L) (P < 0.05). The hydroxytyrosol concentration from the SO group, however, was 4.7-fold higher than that of the CTL group (P < 0.05). The plasma GSH:GSSG ratio was greater in the OO group than in the SO group (P < 0.05) and CTL group (P < 0.05) (Table 1). In addition, plasma antioxidant capacity differed in mice from the OO group compared with the SO (P < 0.05) and CTL groups (P < 0.05). The CTL and SO groups did not differ (Table 1).

Extra virgin OO or SO supplementation did not modify basal (3 mmol/L) or glucose-stimulated (22 mmol/L) insulin secretion. The CTL group did not differ from the OO or SO groups in glucose-stimulated insulin secretion. In contrast, after 10 μmol/L hydrogen peroxide incubation, islets from extra-virgin OO-supplemented mice had a higher (P < 0.05) glucose-stimulated insulin secretion (0.1 ± 0.006 pmol-μg protein\textsuperscript{-1}·h\textsuperscript{-1}) by 22 mmol/L glucose compared with the CTL group (0.05 ± 0.007 pmol-μg protein\textsuperscript{-1}·h\textsuperscript{-1}) and the SO group (0.08 ± 0.007 pmol-μg protein\textsuperscript{-1}·h\textsuperscript{-1}). SO supplementation, however, resulted in an improvement (P < 0.05) of 22 mmol/L glucose-stimulated insulin secretion compared with CTL mice.

Oil supplementation protected against cell membrane lipid peroxidation. Liver tissue from the SO and OO groups incubated with 10 μmol/L hydrogen peroxide displayed significant differences in MDA production compared with the CTL group (n = 4) (Table 2). Pancreatic islets from the SO and OO groups incubated with 10 μmol/L hydrogen peroxide displayed the same significant difference in MDA production compared with the CTL group (n = 4). In addition, the MDA concentration in the SO group was higher than that of the OO group (P < 0.05) in both tissues (Table 2).

In islets of Langerhans from mice supplemented with extra-virgin OO, CAT expression was 98% greater than in the CTL group (P < 0.05) (Table 3). SO supplementation, however, elicited a 40% decrease relative to the CTL group. By contrast, SO and OO groups showed a decrease of 22% in SOD 2 compared with the CTL group, with no significant differences (Table 3). Neither SO nor extra-virgin OO supplementation modified GT\textsubscript{PX} 1 expression compared with the CTL group (Table 3).

Though SO and extra-virgin OO supplementation did not modify SOD 2 activity compared with the CTL group (Table 4), CAT and GT\textsubscript{PX} 1 had greater activity in the OO group (P < 0.05) compared with the CTL and SO groups (Table 4).

**Discussion**

To establish the relationship between polyphenols from foods and their biological effects related to disease prevention, it is essential to understand the factors influencing the absorption of these compounds (22). Mechanisms of absorption, bioavailability, and distribution of hydroxytyrosol in human tissues are currently being studied by several authors (8,23), because this compound is actually considered one of the polyphenols with the highest antioxidant activity (7,8). Bioavailability assays performed with animals and humans reveal that phenolics from extra-virgin OO are absorbed in a proportion of 55–60% (24), but most of these tests are generally achieved with polyphenol amounts higher than those usually seen in the common daily diet.

In this article, we tested different periods of time and different oil doses (up to 100 μL) and found the same levels of plasma hydroxytyrosol content after 3 d of oil administration. Thus, our results reveal the first day at which we observed a significant increase in the hydroxytyrosol levels. We have shown that with the consumption of an extra-virgin OO with a high phenolic content (0.494 μg of hydroxytyrosol), the plasma levels for this compound increases up to 10-fold greater. Although this compound has been shown to be absorbed in a dose-dependant manner by mammals (7), we revealed that increased oil ingestion

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### Table 1

<table>
<thead>
<tr>
<th>mRNA relative concentration</th>
<th>SO</th>
<th>104 \textsuperscript{-fold of CTL}</th>
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</thead>
<tbody>
<tr>
<td>CAT</td>
<td>0.6 ± 0.1</td>
<td>1.98 ± 0.69</td>
</tr>
<tr>
<td>SOD2</td>
<td>0.78 ± 0.1</td>
<td>0.77 ± 0.12</td>
</tr>
<tr>
<td>GT\textsubscript{PX}</td>
<td>1.16 ± 0.26</td>
<td>0.85 ± 0.25</td>
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\(^{1}\) Values are means ± SEM, n = 4. Means in a row without a common letter differ, P < 0.05.

### Table 2

<table>
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<th>Hydrogen peroxide treatment</th>
<th>CTL</th>
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<th>00</th>
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</thead>
<tbody>
<tr>
<td>Liver</td>
<td>19.68 ± 2.39(^{a})</td>
<td>11.98 ± 1.56(^{b})</td>
<td>8.32 ± 0.89(^{b})</td>
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<tr>
<td>Pancreatic islets</td>
<td>31.81 ± 3.5(^{c})</td>
<td>21.92 ± 2.98(^{b})</td>
<td>14.62 ± 1.33(^{c})</td>
</tr>
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</table>

\(^{1}\) Values are means ± SEM, n = 4. Means in a row without a common letter differ, P < 0.05.

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### Table 3

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and more days of oil administration do not lead to further increases in the plasma levels.

The results of our study suggest that the differences among the 3 mice groups studied were a result of the total phenol content of their respective diet. In this sense, our results are in accordance with the absorption studies of Vissers et al. (24), but other authors (7,25) have found higher levels of phenols in plasma and urine than expected, suggesting the formation of metabolites from breakdown by colonic microflora transformation and tissue degradation. This hypothesis could also explain the concentrations detected in the CTL and SO mice groups from our study.

When considering the GSH-GSSG relationship, our results agree with those found previously for healthy mice fed red wine (26), assuming that a positive ratio is related to a better antioxidant status. Previous bioavailability studies with chocolate in humans (27) have shown both increased polyphenols levels and TAC in plasma. As reported previously, metabolites also contribute to increasing antioxidant capacity (25). The difficulty of comparing our results with previous reports is based on the fact that a single approach for quantifying TAC in plasma or serum does not exist (28). Based on our observations, the results in this study reveal a relationship between the consumption of oil with high polyphenol content and the moderate but significant increase in the antioxidant capacity of plasma measured by 2 different methods.

The data regarding glucose-stimulated insulin secretion indicates that pancreatic islets from the extra-virgin OO group better preserved their physiologic function after an exposure to hydrogen peroxide. The importance of this approach is enhanced if we consider that diabetic patients have higher oxidative stress (12), which is also a common mechanism involved in diabetic complications. In addition, hyperglycemia causes enhanced free radical concentration (11), so islets of Langerhans are exposed to higher levels of oxidation products. The results from our study show that, in this tissue, supplementation of mice with extra-virgin OO potentially has a direct protective effect against oxidation.

It is important to investigate the relationship between free radical production and membrane damage combined with the subsequent production of toxic products from peroxidation. Most of the studies regarding MDA formation describe the mechanisms involved in the in vitro degradation of proteins, DNA, RNA, and other biomolecules (15). Previous articles confirm a reduction of MDA concentrations when epicatechins, polyphenolic antioxidants from tea, were added to cell cultures exposed to hydrogen peroxide (10). This study, however, corroborates the direct antioxidant effect of extra-virgin OO against cell lipid peroxidation. We performed in vitro assays of 2 metabolic tissues easily subjected to oxidative stress, confirming that the group fed with extra-virgin OO was protected over a longer duration.

An increase in expression of antioxidant enzymes is an important defense against oxidative stress (10). Previous studies report that overexpression of CAT, SOD, or GT_PX reduces oxidative damage (29,30). In all cases, damage had previously been induced in cell cultures. More recently, pure antioxidant compounds have also been tested in diverse in vitro and in vivo (31) assays and have been shown to promote gene expression of these antioxidant enzymes.

Still, few articles have reported (32,33) the influence of diet supplementation on antioxidant enzyme expression. This is a novel approach, in which gene expression from antioxidant enzymes was measured in healthy mice fed extra-virgin OO as a part of their diet. Pancreatic islets from assays had not previously been exposed to an oxidizing agent and the supplementation of mice with extra-virgin OO was performed in doses lower than that in current articles. The CAT enzyme exhibited a higher response in mRNA synthesis modulated by extra-virgin OO ingestion. The 3 studied enzymes operate in a coordinated system, so antioxidant protection would not involve the overexpression of all of them (34). In addition, the enzymatic activity of CAT and GT_PX 1 increased significantly in the OO group. Thus, we suggest that extra-virgin OO probably also has an indirect effect against oxidative stress by modulating gene expression and enzyme activity, which enhances enzymatic antioxidant defenses.

Our results show that extra-virgin OO can have high nutritional value. These findings indicate that a moderate daily ingestion of extra virgin OO for 3 d reduces oxidative stress in the pancreas, so the use of this oil could have advantages for use in enteral formulas. These findings could have significant consequences for human health and thus deserve further investigation.

Acknowledgments
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Literature Cited

TABLE 4 Antioxidant enzymes activity in pancreatic islets in OF1 mice fed different oils for 4 d

<table>
<thead>
<tr>
<th>Antioxidant enzyme activity</th>
<th>CTL</th>
<th>S0</th>
<th>OO</th>
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<tbody>
<tr>
<td></td>
<td>μmol·min⁻¹·mg protein⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAT</td>
<td>6.2 ± 0.2ₚ</td>
<td>6.4 ± 0.3ₚ</td>
<td>7.8 ± 0.2ₚ</td>
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<tr>
<td>SOD-2</td>
<td>115 ± 12</td>
<td>103 ± 10</td>
<td>107 ± 13</td>
</tr>
<tr>
<td>GT_PX</td>
<td>4.3 ± 0.2ₚ</td>
<td>4.6 ± 0.3ₚ</td>
<td>5.6 ± 0.3ₚ</td>
</tr>
</tbody>
</table>

¹ Values are means ± SEM, n = 4. Means in a row without a common letter differ, P < 0.05.


