Basic nutritional investigation

Influence of dietary fat on oxidative stress and inflammation in murine macrophages

Rocío de la Puerta, Ph.D.\textsuperscript{a,}\textsuperscript{*}, Ana Marquez-Martin, Ph.D.\textsuperscript{b}, Angeles Fernandez-Arche, Ph.D.\textsuperscript{a}, and Valentina Ruiz-Gutierrez, Ph.D.\textsuperscript{b}

\textsuperscript{a} Pharmacology Department, Faculty of Pharmacy, University of Seville, Seville, Spain
\textsuperscript{b} Instituto de la Grasa (C.S.I.C.), Seville, Spain

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Abstract

Objective: Many studies have shown that the nature of the lipid consumed in the diet significantly affects the development of inflammatory diseases. In this study, we compared the effect of diets supplemented with 15\% by weight of fish oil (FO), refined olive oil (ROO), and pomace olive oil (POO) with that of a low-fat diet, 2\% by weight of corn oil, considered as the basal diet (BD), on the ability to modify reactive oxidative species and proinflammatory mediator generation by stimulated murine macrophages.

Methods: Mice were fed the different oil-enriched diets for 8 wk. Peritoneal macrophages were isolated from these mice and subsequently stimulated. Reactive oxygen species and proinflammatory mediators were measured in the corresponding supernatants. Data were statistically treated by one-way analysis of variance and Tukey’s multiple comparison post hoc test.

Results: The ROO and POO significantly reduced the hydrogen peroxide production compared with BD, whereas FO stimulated its production. Moreover, the generation of nitric oxide was significantly prevented in all the experimental oil-enriched dietary groups. The ROO and FO groups showed significantly reduced cytokine (tumor necrosis factor-\alpha, interleukin-1\beta, interleukin-6) and prostaglandin E\textsubscript{2} production.

Conclusion: These results confirm the prevention action on proinflammatory mediator generation exerted by FO and demonstrate the protective antioxidant properties not only of olive oil but also of POO. The consumption of these olive oils may help to prevent cellular oxidative stress and inflammation. © 2009 Published by Elsevier Inc.

Keywords: Orujo olive oil; Refined olive oil; Reactive oxygen species; Prostaglandin; Cytokine; Nitric oxide

Abbreviations: FO, fish oil; ROO, refined olive oil; POO, pomace olive oil; BD, basal diet; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ROS, reactive oxygen species; PG, prostaglandin; PUFA, polyunsaturated fatty acid

Introduction

Epidemiologic and experimental data have indicated that changes in the source of lipid consumed in the diet may modify the fatty acid composition of many cell types, including macrophages, involved in the development of many inflammatory and immunologic diseases.

In this regard, the \(\omega-3\) polyunsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), found primary in fish and fish oil, have been extensively investigated. Thus, many studies have demonstrated the beneficial properties of EPA and DHA on cardiovascular [1,2] and autoimmune [3–5] diseases. However, since the Seven Country Study [6], the Mediterranean diet, rich in monounsaturated fatty acids, especially oleic acid from olive oil, has been proposed as a healthy standard due to its association with a low rate of cardiovascular mortality [7,8]. More recently, it has been demonstrated that other specific non-fatty constituents have an important role in these beneficial effects of olive oil [9,10].
Pomace olive oil (POO) is obtained by chemical processes from the mechanical extraction of virgin olive oil. Although this byproduct of virgin olive oil is traditionally commercialized in Spain, the nutritional similarities and differences between POO and other olive oils, such as virgin and refined olive oils, have not been studied yet. The new improved procedures for POO extraction allow the presence of a number of unsaponifiable components from the skin of the olive that are present in low concentration in virgin olive oil [11]. Thus, the unsaponifiable fraction of POO contains elevated amounts of sterols, tocopherols, waxes, and triterpenic acids and alcohols, such as oleanolic acid and erythrodiol [12], with important biological activities. These latter molecules possess a wide range of interesting properties [13]. From our experience, triterpenes from orujo olive oil have been demonstrated to modulate cytokine secretion [14] and reduce oxidative species release [15] by different cell systems. In addition, long-chain fatty alcohols, as a mix of tetracosanol, hexacosanol, and octacosanol, have been shown to be effective in downregulating nitric oxide (NO) and tumor necrosis factor-α (TNF-α) generation [16] by murine macrophages.

Macrophages play a significant role in the host defense mechanism [17]. Several functions of macrophages depend on their lipid composition, such as signal reception and transduction, phagocytosis, and synthesis of inflammatory mediators [18]. The aim of this study was to compare the effect of diets supplemented with fish oil (FO), refined olive oil (ROO), and POO with that of a low-fat diet, considered the basal diet (BD), on the ability to modify reactive oxidative species (ROS) and proinflammatory mediator generation by stimulated murine macrophages.

**Materials and methods**

**Experimental animals**

Weaned male Swiss albino mice (Centro de Instrumentación Científica, Granada, Spain) weighing 5–10 g were placed in cages with wire-net floors and maintained in a humidity- and temperature- (20–22°C) controlled environment with a 12-h light/dark cycle. Mice were distributed randomly into groups of six animals and given free access to food and water throughout the experimental period. All experiments were performed according to the European Union guidelines for the ethical treatment of animals.

**Diets**

Diets were made according to American Institute of Nutrition (AIN) recommendations [19]. The animals were fed the entire 8 wk of the experimental period with a basal diet, containing 2% by weight of corn oil (BD), considered a low-fat diet, or high-fat diets containing 15% by weight of ROO, POO, or menhaden (fish) oil (FO), as previously described [20]. Diets were prepared weekly by the addition of the appropriate amount of oil to a mix of the other dietary components listed in Table 1 and stored in darkness at 4°C under N₂ atmosphere to minimize oxidation. Body weight and food intake were recorded each week. The dietary components (Table 1) used in the diets were corn oil (Asua, Koipe S.A., Córdoba, Spain), sucrose, casein corn starch, α-cellulose, dl-methionine, and choline chloride purchased from Musal & Chemical (Granada, Spain); minerals (AIN-93 mineral mix) and vitamins (AIN-93 vitamin mix) were purchased from Biolink 2000 S.A. (Barcelona, Spain); and ROO, POO, and FO, were supplied by the Instituto de la Grasa (Seville, Spain). The fatty acid composition of the oils was determined as previously described [21] and is presented in Table 2.

**Chemicals**

RPMI 1640 medium was purchased from Biochrom AG (Berlin, Germany); thioglycolate from Scharlau Microbiology

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### Table 1

<table>
<thead>
<tr>
<th>Components used to elaborate assayed diets</th>
<th>Low-fat diet (%)</th>
<th>High-fat diets (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>50.0</td>
<td>43.5</td>
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<tr>
<td>Casein</td>
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<td>Corn starch</td>
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<td>α-Cellulose</td>
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</tr>
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<td>Minerals</td>
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<td>3.5</td>
</tr>
<tr>
<td>Vitamins</td>
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<td>1.0</td>
</tr>
<tr>
<td>dl-Methionine</td>
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<td>0.3</td>
</tr>
<tr>
<td>Choline chloride</td>
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<td>0.2</td>
</tr>
<tr>
<td>BD</td>
<td>2.0</td>
<td>—</td>
</tr>
<tr>
<td>ROO</td>
<td>—</td>
<td>15</td>
</tr>
<tr>
<td>POO</td>
<td>—</td>
<td>15</td>
</tr>
<tr>
<td>FO</td>
<td>—</td>
<td>15</td>
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**Table 2**

<table>
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<th>Fatty acids</th>
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<th>ROO</th>
<th>POO</th>
<th>FO</th>
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</thead>
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<td>0.02</td>
<td>0.02</td>
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<tr>
<td>16:0</td>
<td>10.36</td>
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<td>19.56</td>
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<td>0.82</td>
<td>0.76</td>
<td>8.13</td>
</tr>
<tr>
<td>18:0</td>
<td>2.36</td>
<td>3.53</td>
<td>2.95</td>
<td>4.10</td>
</tr>
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<td>77.80</td>
<td>74.27</td>
<td>9.83</td>
</tr>
<tr>
<td>18:2/9</td>
<td>50.89</td>
<td>4.52</td>
<td>8.07</td>
<td>2.35</td>
</tr>
<tr>
<td>18:3/9</td>
<td>0.71</td>
<td>0.62</td>
<td>0.70</td>
<td>2.63</td>
</tr>
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<td>—</td>
<td>0.42</td>
<td>0.45</td>
<td>2.85</td>
</tr>
<tr>
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<td>0.25</td>
<td>0.33</td>
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</tr>
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<td>20:5/3</td>
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<td>22:5/6</td>
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<td>—</td>
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</tr>
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<td>22:6/3</td>
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<td>—</td>
<td>—</td>
<td>10.49</td>
</tr>
<tr>
<td>24:0</td>
<td>—</td>
<td>0.05</td>
<td>0.07</td>
<td>—</td>
</tr>
</tbody>
</table>

**BD, basal diet; FO, fish oil; POO, pomace olive oil; ROO, refined olive oil**
(Barcelona, Spain); chloroform methanol from Romil Pure Chemistry (Cambridge, United Kingdom); sodium methyolate, H₂SO₄, and KOH from Panreac Química (Barcelona, Spain); hexane from Prolabo (Paris, France); and enzyme-linked immunosorbent assay kits from Endogen Searchlight (Rockford, IL, USA). The other reagents were purchased from Sigma-Aldrich (Madrid, Spain).

Isolation and culture of murine peritoneal macrophages

Mice were used to obtain highly purified peritoneal macrophages that were isolated and cultured as follows: cells were harvested by peritoneal lavage 4 d after an intraperitoneal injection of 1 mL of 10% thioglycolate broth. Cells were then resuspended (10⁶ cells/mL) in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin, and incubated at 37°C in a 5% CO₂ humidified incubator for 2 h. The adherent cells were used to perform the experiments.

Measurement of ROS: superoxide and hydrogen peroxide from stimulated murine macrophages

Superoxide secretion by macrophages in response to phorbol-12-myristate-13 acetate (PMA) was measured by the oxidation of reduced cytochrome c [22]. Macrophages (10⁶ cells/mL), once adhered to the plate, were preincubated in 1 mL of Hank’s balanced saline solution containing 0.2% glucose (w/v) and cytochrome c (80 µmol/L) at 37°C for 30 min. Stimulation was performed adding 1 µM of PMA. After 2 h the medium was removed and the absorbance was read at 550 nm in a Biochrom Asys Hitech Expert 96 (Eugendorf, Austria) microplate reader. Superoxide concentrations were calculated using the molar extinction coefficient of oxidized cytochrome c (2.1 × 10⁴ mol·L⁻¹·cm⁻¹).

Hydrogen peroxide secretion by macrophages in response to stimulation by PMA was measured by oxidation of phenol red in the presence of peroxidase [22,23]. Macrophages (10⁶ cells/mL), once adhered to the plate, were preincubated in 1 mL of Hank’s balanced saline solution containing 0.2% glucose (w/v), phenol red (28 mmol/L), and horseradish peroxidase (50 µg/mL) at 37°C for 30 min. Stimulation was performed adding 1 µmol/L of PMA. After 2 h the medium was removed and the absorbance was read at 610 nm in the microplate reader. Hydrogen peroxide concentrations were calculated by comparison with a standard curve.

Measurements of nitrite, prostaglandin E₂, and cytokines (TNF-α, interleukin-1β, interleukin-6) from stimulated murine macrophages

Murine peritoneal macrophages (10⁶ cell/mL) were cultured in 24-well plates and stimulated with 10 µg/mL of lipopolysaccharide (LPS) for 18 h. As an indicator of NO production, nitrite (NO₂⁻) concentration was measured in the supernatants. Equal volumes of culture supernatants and Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-phenylphenylenediamine-HCl) were mixed and the absorbance was read at 450 nm in the microplate reader [24]. The amount of nitrite was obtained by an extrapolation from a standard curve with sodium nitrite. Prostaglandin E₂ (PGE₂) and the cytokines TNF-α, interleukin (IL)-1β, and IL-6 were measured in the culture supernatants by immunoassay.

Extraction of lipids and fatty acid analysis of mice murine macrophages

Extraction of lipids from macrophages was performed according to the method of Folch et al. [25] in the presence of butylated hydroxytoluene (2%, w/v) as an antioxidant. The fatty acid composition of macrophages from each dietary oil-supplemented group was determined by gas chromatography as previously described [26]. Briefly, the samples were saponified by heating them for 25 min with 5 mL of sodium methyolate 0.2 mol/L and heating again at 80°C for 25 min with 6% H₂SO₄ in anhydrous methanol (v/v). The fatty acid methyl esters formed were recovered with hexane and analyzed in a Chrompack (Middelburg, The Netherlands) CP900 gas chromatograph equipped with an SGL-5 capillary column 30 m long, 0.25 mm inner diameter, and 0.25-µm film thickness of 5% phenyl methyl polysiloxane stationary phase. The carrier gas was hydrogen, with a head pressure of 110 KPa and 1:60 split ratio. The injector temperature was 280°C and the detector temperature was 290°C. The oven temperature was programmed from 215°C to 290°C at a rate of 2°C/min.

Statistical analysis

Results are presented as mean ± SEM from data of cells from six animals fed each diet. Data were analyzed using one-way analysis of variance followed by Tukey’s multiple comparison post hoc test. Statistically significant differences are denoted as *P < 0.05, **P < 0.01, and ***P < 0.001 versus BD; +P < 0.05, ++P < 0.01, and ++++P < 0.001 versus FO; and −P < 0.05, −−P < 0.01, and −−−−P < 0.001 versus POO.

Results

No differences were found on the amounts of food consumed by the four dietary groups of animals (data not shown). The means of final body weight were 34.2 ± 0.8 g for the BD group, 30.4 ± 1.4 g for the ROO group, 34.7 ± 0.8 g for the POO group, and 31.6 ± 1.0 g for the FO group. We observed only a short significant difference (P < 0.05) between the ROO and FO groups.
Measurement of ROS: superoxide and hydrogen peroxide from stimulated murine macrophages

The modification of macrophage ROS generation by enriched diets in response to PMA-stimulation is shown in Figure 1. The BD production of these species by 10^6 cells/mL was 58.12 nmol of hydrogen peroxide and 33.80 nmol of superoxide. The ROO and POO groups showed significantly reduced (\(P < 0.01\) and \(P < 0.001\) versus BD) hydrogen peroxide production compared with the BD group, whereas FO stimulated its production (Fig. 1A).

Effect of dietary oil supplementation on murine macrophage fatty acid composition

The macrophage fatty acid composition, as a percentage of total fatty acids, is presented in Table 3. As expected, oleic acid (18:1 \(\omega-9\)) was the main fatty acid incorporated in the cells isolated from mice fed the ROO and POO diets. Moreover, \(\omega-3\) polyunsaturated fatty acids were mainly incorporated into macrophages from the FO-fed animals, because these are characteristic fatty acids of this oil. In contrast, more arachidonic acid (20:4 \(\omega-6\)) was synthesized in cells from animals fed the POO diet, likely due to the larger amount of its precursor, linoleic acid (18:2 \(\omega-6\)), present in POO.

Discussion

The relation between cell membrane fatty acid composition and biologic function has extensively been examined [27,28]; therefore, dietary lipid manipulation has an important contribution to prevent and improve many inflammatory and immunologic diseases [29–31]. Stimulated macrophages secrete a variety of substances including ROS, NO, eicosanoids, and cytokines that play important roles in key macrophagocytic/phagocytic functions, such as the killing of bacteria and the regulation of the immune–inflammatory response.

Even though the generation of ROS by macrophages is tightly controlled and plays an important role in host defense against microbial infection [32], under adverse circumstances, these oxidizing agents can react with a wide range of biological targets, causing cellular damage.

Our results demonstrated that olive oils (ROO and POO) significantly reduce hydrogen peroxide production after PMA stimulation in the BD and FO groups. In addition, POO even reduced something the level of superoxide anion radical. Although the effect produced by ROO was not in this way, no decrease in the release of superoxide was detected by this group, as would have been expected. In contrast, FO seemed to weakly increase hydrogen peroxide but did not modify superoxide production in our experiments.

Macrophage production of ROS is linked to activation of reduced nicotinamide adenine dinucleotide phosphate oxidase, which is usually dormant but can be rapidly activated by a variety of stimuli associated with phagocytic functions. Cherny et al. [33] described the activation of reduced nic-
otinamide adenine dinucleotide phosphate oxidase and the subsequent ROS production as consequence of the free arachidonic acid content. Thus, diets rich in monounsaturated and ω-3 polyunsaturated fatty acids, by replacing arachidonic acid with oleic acid and EPA and DHA in phospholipid membranes, respectively, impair the release of arachidonic acid from membranes and thus modify in a different way the release of free radicals [34].

Moreover, our results do not totally agree with data from other experimental results from animal models, where it has been described how a FO-rich diet increased not only hydrogen peroxide release but also superoxide production compared with a diet rich in oleic acid and EPA and DHA in phospholipid membranes, respectively, impair the release of arachidonic acid from membranes and thus modify in a different way the release of free radicals [34].

Nitric oxide is involved in phagocytosis at a physiologic level. However, when produced in a large amount, NO can enhance inflammation and tissue injury [36]. In the supernatants from the LPS-stimulated macrophages, we could observe that NO production was prevented by dietary lipid manipulation, although there was no variability in the values from mice fed the three highly oil-enriched diets. In this regard, different results have been obtained by several investigators depending on the experimental animal models, time of feeding, and percentage of oil supplementations. In previous studies, it has been reported that 20% supplementation with olive, safflower, and menhaden oils in mouse diets produced and enhanced NO release after LPS-stimulation compared with a low-fat diet. However, in the present investigation, the results showed a decrease in NO. The mechanism by which the lipid-enriched diets inhibited NO could be due to a decrease...
that an olive oil diet significantly reduced arachidonic acid (analysis of variance and Tukey’s multiple comparison test).

of variance and Tukey’s multiple comparison test).

IL-6 are part of a larger network of inflammatory regulation though not to the same extent.

PGE2 was significantly lower in macrophages from mice of two dietary groups. POO was able to decrease PGE2, a long-chain fatty acids contained in ROO and POO that might have an influence on the variable response of both dietary groups.

Table 3

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>BD</th>
<th>ROO</th>
<th>POO</th>
<th>FO</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>27.3 ± 1.1</td>
<td>28.4 ± 0.4&lt;sup&gt;11&lt;/sup&gt;</td>
<td>25.5 ± 0.4</td>
<td>26.2 ± 0.6</td>
</tr>
<tr>
<td>16:1</td>
<td>3.4 ± 0.7</td>
<td>5.6 ± 0.9&lt;sup&gt;11&lt;/sup&gt;</td>
<td>3.9 ± 0.2</td>
<td>5.9 ± 0.3&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:1 ω-9</td>
<td>21.7 ± 0.3</td>
<td>28.5 ± 0.4&lt;sup&gt;1&lt;/sup&gt;</td>
<td>27.5 ± 2.1&lt;sup&gt;11&lt;/sup&gt;</td>
<td>20.0 ± 0.5</td>
</tr>
<tr>
<td>18:2 ω-6</td>
<td>5.1 ± 0.4</td>
<td>3.3 ± 0.6</td>
<td>4.8 ± 0.1</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>18:3 ω-3</td>
<td>0.2 ± 0.2</td>
<td>0.5 ± 0.0</td>
<td>1.0 ± 0.2</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>20:4 ω-6</td>
<td>3.8 ± 0.2</td>
<td>3.4 ± 0.8&lt;sup&gt;1&lt;/sup&gt;</td>
<td>7.7 ± 0.5&lt;sup&gt;1&lt;/sup&gt;</td>
<td>3.7 ± 0.5</td>
</tr>
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<td>0.8 ± 0.2</td>
<td>1.9 ± 0.5&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1.2 ± 0.1&lt;sup&gt;1&lt;/sup&gt;</td>
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<td>4.1 ± 0.8</td>
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<td>1.6 ± 0.0</td>
<td>1.9 ± 0.4</td>
</tr>
</tbody>
</table>

BD, basal diet; FO, fish oil; POO, pomace olive oil; ROO, refined olive oil

* Values represent mean ± SEM from six determinations.
<sup>1</sup> P < 0.05, <sup>2</sup> P < 0.01, <sup>3</sup> P < 0.001 versus BD, statistically significant (analysis of variance and Tukey’s multiple comparison test).
<sup>4</sup> P < 0.05, <sup>5</sup> P < 0.01, <sup>6</sup> P < 0.001 versus FO, statistically significant (analysis of variance and Tukey’s multiple comparison test).
<sup>7</sup> ** P < 0.05, <sup>11</sup> P < 0.01, versus ROO, statistically significant (analysis of variance and Tukey’s multiple comparison test).

Conclusions

These results confirm the prevention action on proinflammatory mediator generation exerted by FO and provide important additional data about the protective antioxidant properties not only of olive oil but also of POO. It could be concluded that the consumption of these olive oils may help to prevent cellular oxidative stress and inflammation.

References


