Dietary extra virgin olive oil polyphenols supplementation modulates DSS-induced chronic colitis in mice

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Abstract

We evaluated the protective effect of dietary extra virgin olive oil (EVOO) polyphenol extract (PE) supplementation in the inflammatory response associated to chronic colitis model. Six-week-old mice were randomized in four dietary groups: standard diet (SD), EVOO diet and both enriched with PE (850 ppm) (SD + PE and EVOO + PE). After 30 days, animals that were exposed to dextran sodium sulfate (DSS) (3%) followed by 3 weeks of drinking water developed chronic colitis, which was evaluated by disease activity index (DAI) and histology. Cell proliferation was analyzed by immunohistochemical and changes in monocyte chemotactic protein (MCP)-1 and tumor necrosis factor (TNF)-α mRNA expression by quantitative real-time polymerase chain reaction. Colonic expression of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, mitogen-activated protein kinases (MAPKs), iNOS inhibitory and peroxisome proliferator-activated receptor gamma (PPARγ) were determined by western blotting. SD-DSS group showed a significant increase of DAI, histological damage and cell proliferation, as well as an up-regulation of TNF-α, MCP-1, COX-2 and iNOS proteins. p38 and JNK MAPKs phosphorylation, iNOS degradation and PPARγ deactivation were also observed. However, in DSS-treated and EVOO + PE-fed mice, DAI and cell proliferation were significantly reduced, as well as MCP-1, TNF-α, COX-2 and iNOS expression levels. In addition, this dietary group, notably down-regulated JNK phosphorylation, prevented iNOS degradation and PPARγ deactivation. These results demonstrated, for the first time, that EVOO-PE supplementation possessed marked protective effects on experimental colitis through PPARγ up-regulation and nuclear transcription factor-kappa B and MAPK signaling pathway inhibition, decreasing the inflammatory cascade. We concluded that PE-enriched EVOO diet could be a beneficial functional food on ulcerative colitis.

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Keywords: Polyphenol extract; Extra virgin olive oil; Ulcerative colitis; PPARγ; iNOS

1. Introduction

Inflammatory bowel diseases (IBD) encompass at least two forms of intestinal inflammation: Crohn’s disease and ulcerative colitis (UC). Both are chronic and inflammatory disorders in the gastrointestinal tract, with an increasing prevalence associated with industrialization of nations and a rising in countries developing [1]. In the last years, it has been demonstrated that oxidative stress and chronic inflammation are the more relevant etiopathogenic factors. An increase in the inflammatory mediators, including reactive oxygen species, and up-regulation of certain proteins such as cyclooxygenase (COX)-2 or inducible nitric oxide synthase (iNOS) plays an important role in immune dysregulation [2–4]. On the other hand, signaling pathways, such as mitogen-activated protein kinases (MAPKs), are also involved in transcription factors activation. Among them, nuclear transcription factor-kappa B (NFkB) takes part in controlling pro-inflammatory cytokine genes activation that supports a critical role in the pathogenesis of UC [5]. Over the last years, peroxisome proliferator-activated receptor gamma (PPARγ), one of the major anti-inflammatory receptors in colon, also seems to be associated with experimental colitis [6–8]. Moreover, NFκB expression and activation could be modulated by PPARγ through several mechanisms, including (i) direct interactions with NFκB, (ii) nucleocytoplasmic redistribution of the p65 subunit of NFκB and (iii) interactions with transcriptional co-repressors of NFκB in the gut [9].

Although the knowledge in this pathology is progressing, new therapeutic strategies continue to be investigated. In this sense, the interest by dietary supplements without undesirable effects that accompany the classical pharmacotherapy is growing. Epidemiological studies about consumption of functional foods, particularly extra virgin olive oil (EVOO) in Mediterranean countries, have showed important beneficial effects as antioxidant, anti-inflammatory, chemopreventive and anticancer [10–13].

EVOO, obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to oil alteration, has shown anti-inflammatory, immunomodulatory, anti-proliferative and anti-apoptotic effects [14–18]. Traditionally, these
health-protective effects have been ascribed to its high monounsaturated fatty acid content [19,20]; although, nowadays, it is clear that many of the beneficial effects of EVOO intake are due to its minor highly bioactive components. Among them, the phenolic compounds have shown anti-inflammatory, antioxidant, antimicrobial, anti-proliferative, antiarrhythmic, platelet aggregant and vasodilatory effects, as well as the ability to modulate important cellular signaling pathways [21,22].

Current epidemiological and experimental studies support a beneficial role of dietary polyphenols in several gastrointestinal diseases, including IBD [23–25]. Scientific evidence supports the potential use of nutraceuticals focused in polyphenols constituents as agents capable to prevent or accelerate healing of gastrointestinal mucosal damage [26–28]. Besides, previous studies have showed protective/preventive effect of polyphenolic compounds in the progression of colorectal cancer [29–31].

In this way, our research group has previously demonstrated that a diet made with EVOO enriched with hydroxytyrosol, a polyphenol present in the oil, may improve inflammatory response [32].

Taken this background into account, the present study was designed to evaluate the influence of dietary EVOO-polyphenol extract (PE) enrichment on the protective/preventive effects of EVOO intake in a chronic colitis model induced by dextran sodium sulfate (DSS) in mice by macroscopic and histology parameters, exploring the anti-inflammatory mechanisms involved in its effects.

2. Materials and methods

2.1. Extraction of EVOO-PE

EVOO picual variety (Aceite de las Valdesas, Córdoba, Spain) was used as the matrix to carry out phenolic extract (PE) enrichments. PE was obtained using the method described by Vazquez Roncero et al. [33] with some modifications. Fifty grams of oil was extracted with methanol:water (80:20, v/v, 125 ml). The mixture was mixed with a vortex at 5000 g for 1 min and sonicated for 15 min. After decantation, the methanolic extract was concentrated in vacuum under a stream of nitrogen at <35°C until it reached a syrupy consistency; finally, it was lyophilized and stored at −80°C until its use for oil enrichment.

2.2. Chemical characterization of PE

Quantitative and qualitative analysis of PE was performed according to the COI/T20/29doc (International Olive Council) for olive oil. The method is based on direct extraction of the phenolic minor polar compounds from olive oil by means of a methanol solution and subsequent quantification by high-performance gradient liquid chromatograph (HPLC). After direct extraction of the phenolic minor polar compounds by means of a methanol solution, an aliquot of the supernatant phase was taken and filtered through a 0.45-mm PVDF filter, injected into the HPLC system equipped with a C18 reverse-phase column (4.6 mm×25 cm), type Spherisorb ODS-2 (5 mm), 100 Å, with spectrophotometric UV detector at 280 nm and integrator. The polar compounds by means of a methanol solution, an aliquot of the supernatant phase was

<table>
<thead>
<tr>
<th>Phenol composition</th>
<th>ppm (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxytyrosol</td>
<td>45.0</td>
</tr>
<tr>
<td>Tyrosol</td>
<td>44.4</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>6.1</td>
</tr>
<tr>
<td>P-Coumaric acid</td>
<td>4.0</td>
</tr>
<tr>
<td>Decarboxymethyl oleylpyruv aglycone (dialdehyde)</td>
<td>23.4</td>
</tr>
<tr>
<td>Tyrosyl acetate</td>
<td>6.4</td>
</tr>
<tr>
<td>Decarboxymethyl ligstroside aglycone (dialdehyde)</td>
<td>24.5</td>
</tr>
<tr>
<td>Pinoselinol</td>
<td>15.7</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>6.8</td>
</tr>
<tr>
<td>Acetoxyl-pinoserinol</td>
<td>18.4</td>
</tr>
<tr>
<td>Oleuropein aglycone, aldehyde form</td>
<td>88.2</td>
</tr>
<tr>
<td>Ligstroside aglycone, dialdehyde form</td>
<td>56.6</td>
</tr>
<tr>
<td>Luteolin</td>
<td>8.7</td>
</tr>
<tr>
<td>Apigenin</td>
<td>1.7</td>
</tr>
<tr>
<td>Total phenols expressed in tyrosol</td>
<td>350</td>
</tr>
</tbody>
</table>

2.3. Animals and diets

A total of 80 six-week-old female C57BL/6 mice were provided by Charles River (Tokyo, Japan) and maintained in our Animal Laboratory Center under standard conditions (temperature, 24–25°C; humidity, 70–75%; lighting regimen, 12L:12D). They were fed pellet diets and water ad libitum. Mice were randomized into four dietary groups (20 animals per group) during all experimental period: (i) standard diet (SD) group received a diet elaborated with a marketable sunflower oil (Koipesol, DEOLEO, Spain), (ii) EVOO group were fed with a diet made with a marketable EVOO picual variety (Aceite de las Valdesas, Córdoba, Spain) and (iii and iv) both sunflower oil and EVOO enriched with 850 ppm of PE (SD+PE and EVOO+PE, respectively) (Table 2). The PE dose has been selected based in the literature [35,36]. Diets were formulated on the basis of the American Institute of Nutrition (AIN) standard reference diet with the modification of various sources of carbohydrate, at 10% of oil total. All diets were prepared by mixing the respective compounds under yellow light and stored at −80°C. Fresh diets were provided daily. Experiments followed a protocol approved by the Animal Ethics Committee of the University of Seville, and all experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 86/609/EC).

2.4. Induction of colitis

Colitis was induced according to the procedure described by Melgar et al. [37]. This colitis model induced by DSS administration in the drinking water has been found to induce colonic inflammation with clinical and histological similarities to human UC, being useful to identify and validate new therapies for the treatment of IBD. Thirty days after weaning, animals from each dietary groups (n=20 per group) were divided in sham group (n=8) and DSS group (n=12). Animals from DSS groups received 3% DSS (DSS group, MW: 40,000; ICN Pharmaceuticals, Costa Mesa, CA, USA) in drinking water for 5 days followed by a regime of 21 days of water, reflecting chronic inflammation. Control healthy mice (8 mice) were allowed to drink only water. Then, animals were killed by an overdose of ip chloral hydrate.

2.5. Evaluation of the severity of clinical colitis

The clinical activity of colitis was evaluated by an independent observer who was blinded to the treatment in order to determine the disease activity index (DAI) as described by Melgar et al [37], with slight modifications as shown in Table 3. The presence of diarrhoea, rectal bleeding and weight loss were registered at the beginning (Day 0), in the middle (Day 3) and at the end of the DSS treatment (Day 5), as well as during the follow-up of 3 weeks when the animals received ordinary tap water. The average of the three values comprised the DAI.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>SD</th>
<th>SD+PE</th>
<th>EVOO</th>
<th>EVOO+PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>α-Methionine</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Sucrose</td>
<td>449.91</td>
<td>449.82</td>
<td>449.91</td>
<td>449.82</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Sunflower</td>
<td>100</td>
<td>100</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EVOO</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Fe (sulfate)</td>
<td>90×10⁻³</td>
<td>90×10⁻³</td>
<td>90×10⁻³</td>
<td>90×10⁻³</td>
</tr>
<tr>
<td>PE</td>
<td>–</td>
<td>85×10⁻³</td>
<td>–</td>
<td>85×10⁻³</td>
</tr>
</tbody>
</table>

Diet was formulated on the basis of the AIN standard reference diet with the modification of various sources of carbohydrate. a Sunflower oil from Koipesol (Spain); b EVOO from picual variety, Las Valdesas, Jaen, Spain. c Mineral mix provided the following (g/kg diet): calcium carbonate, 35.7; monopotassium phosphate, 25.0; sodium chloride, 7.4; potassium sulfate, 4.66; potassium citrate monohydrate, 2.8; magnesium oxide, 2.4; ferric citrate, 0.066; zinc carbonate, 0.165; manganese carbonate, 0.063; copper carbonate, 0.03; magnesium iodate, 0.001; sodium selenate, anhydrous, 0.0001025; ammonium molybdate-4H₂O, 0.000795; sodium metasilicate-H₂O, 0.145; chromium potassium sulfate-12H₂O, 0.0275; boric acid, 0.00815; sodium fluoride, 0.00635; nickel carbonate, 0.00318; lithium chloride, 0.00174; ammonium vanadate. d Vitamin mix provided the following (g/kg diet): nicotinic acid, 30 mg; α-calcium pantothenate, 16 mg; pyridoxine HCl, 7 mg; thiamine HCl, 6 mg; riboflavin, 6 mg; folic acid, 2 mg; D-biotin, 0.2 mg; vitamin B₁₂, 25 mg; α-tocopherol powder (250 U/g), 300 mg; vitamin A palmitate (250,000 U/mg), 16 mg; vitamin D₃ (400,000 U/mg), 2.5 mg; phylloquinone, 0.75 mg. e PE from EVOO picual variety, Las Valdesas, Jaen, Spain.
2.6. Macroscopic and histopathological evaluation

At the end of the experimental period, colons were removed, slightly cleaned in physiological saline solution to remove fecal residues, weighed and measured in order to evaluate the variations in the weight/length as an inflammation index. The results were expressed in percentage respect to the control sham group. Samples of three colonic regions (proximal, middle and rectum) were excised out of every segment, fixed in 4% buffered formaldehyde, dehydrated by increasing concentrations of ethanol and embedded in paraffin; 4-μm-thick slices from paraffin sections were stained with hematoxylin and eosin in accordance with the standard procedures for the histological evaluation of colonic damage. The rest of the pieces from the colon were collected and frozen in liquid nitrogen to measure biochemical parameters.

Three colonic sections from each animal were scored by a pathologist who was unaware of the experimental protocol using a colitis score as previously described by Dieleman et al. [38]. In brief, for each category of the score (inflammation, extent, crypt damage), points were multiplied by a factor of involvement of the visible epithelium (Table 4). The sum of the three categories scores adds up to the total score of each section (0–40 points).

2.7. Immunohistochemistry for proliferating cell nuclear antigen

Proliferating cell nuclear antigen (PCNA) staining was carried out using a streptavidin-biotin-peroxidase method [39]; 4-μm-thick tissue sections were dried in an oven at 37°C for 24 h and then deparaffinized in two changes of xylene and hydrated through graded alcohols to water. A pressure-cooking antigen retrieval system was used. Histological slides were boiled in citrate buffer (pH 6.0) at maximum pressure for 3 min, followed by cooling at room temperature for 20 min. The endogenous peroxidase activity was inhibited with hydrogen peroxide and then the sections were incubated in normal horse serum (Vectastain Kit; Vector Laboratories, Burlingame, CA, USA) for 20 min to reduce nonspecific staining and successively incubated with monoclonal mouse anti-PCNA (DakoCytomation, Denmark) at dilution 1:1000 overnight at 4°C. Later on, slides were treated with anti-mouse IgG antibody (Vectastain Kit; Vector Laboratories, Burlingame, CA, USA) for 30 min and incubated with the streptavidin–biotin–peroxidase complex (Vectastain Kit; Vector Laboratories, Burlingame, CA, USA) for 30 min, both steps were carried out at room temperature. The enzymatic study was developed using 3,3′-diaminobenzidine, and the sections were counterstained with hematoxylin. Negative control sections were treated as the same way omitting the primary antibody. For immunohistochemical analysis, the pathologist quantified PCNA expression by means of the observation of three arbitrary high-power fields from four animals’ colons for each dietary DSS group. The number of positive cells from each field was counted, and the average of these values was calculated. Therefore, PCNA staining was expressed as the percentage of cells in each group showing nucleus expression in mucosa, submucosa and interstitial region [40].

2.8. Total RNA extraction and reverse transcription

Total RNA was extracted from samples by a modification of Chomczynski and Sacchi’s method using TriPure Isolation Reagent (Roche, Mannheim, Germany) as a denaturing solution and appropriate chloroform volume. After cell lysis and RNA extraction, RNA was precipitated with isopropanol, and the pellet was washed in 75% ethanol. The RNA samples were recovered by centrifugation at 12,000 rpm for 15 min and then dried. Each RNA pellet was dissolved in 500 μl RNase-free water, and quantity and quality of RNA was assessed spectrophotometrically, in the NanoDrop 2000 (Thermo Scientific NanoDrop Products, Wilmington, DE, USA) at 260 and 260/280 nm, respectively. In an attempt to discard DNA contamination before cDNA synthesis, the RNA sample underwent a purification process by means of QuantisTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA); 1 μg of RNA was mixed with the genomic DNA elimination reaction and incubated for 10 min at 42°C. Single-strand cDNA was then synthesized adding to template RNA a reverse transcription (RT) reaction components from Transcriptor Kit and incubating for 15 min at 42°C. The reaction components was terminated by placing it on ice after deactivation at 95°C for 5 min and stored at −80°C until use.

2.9. mRNA analysis by real-time polymerase chain reaction

Real-time quantitative polymerase chain reaction (PCR) was performed using SYBR Green I kit for the LightCycler 480 instrument (Roche Diagnostics, Indianapolis, IN, USA). The DNA was amplified in a reaction containing RT product as template DNA (100 ng), FastStartTag DNA Polymerase, SYBR Green I and 0.4 μM sense and antisense primers of the gene under study or 0.4 μM for the housekeeping gene (β-actin), in a final volume of 20 μl. The sequences of primers are shown in Table 5.

The PCR started by a 5-min activation of hot-start DNA polymerase at 95°C followed by 49 cycles of target cDNA amplification. The template was initially denatured at 95°C for 15 s followed by 49 cycle program with 5 s annealing at 65°C (monocyte chemotactic protein (MCP)-1) or at 66°C (tumor necrosis factor (TNF)-α) and 10 s elongation at 72°C and acquisition of SYBR Green fluorescence. In addition, the ubiquitously expressed β-actin mRNA was used to monitor the quality of RNAs and the efficiency of the RT and PCR processes. During PCR, the intercalate agent is separated resulting in increased fluorescence. The LightCycler 480 instrument reads each well every few seconds and measures the increase in fluorescence that is a direct consequence of the target amplification during PCR. The results were reported as threshold cycle (Ct) values, the cycle at which fluorescence readings exceed the mean baseline readings. The mathematical model of 2−ΔΔCt (Livak’s method) was used for relative quantification of MCP-1 and TNF-α to β-actin. The mean and SD were then determined from samples (n=4) in triplicate at each group. The value of the mean fold change at sham control group was very close to one (20 ± 1).

2.10. Western blot analysis

Frozen colonic tissues were weighed and homogenized in ice-cold lysis buffer (50 mM Tris–HCl pH 7.5, 8.5 mM MgCl2, 5 mM EDTA, 0.5 mM EDTA, 0.01 mg/ml leupeptin, 0.01 mg/ml papain, 1 mM phenylmethylsulfonyl fluoride and 250 μM NaCl). Homogenates were centrifuged (12,000g, 15 min, 4°C), and the supernatants were collected and stored at −80°C. Protein concentration of the homogenate was determined following Bradford colorimetric method [41]. Aliquots of supernatants containing equal amounts of protein (50 μg) were separated on 10% acrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In the next step, the proteins were electroblotted transferred onto a nitrocellulose membrane and incubated with specific primary antibodies: rabbit anti-COX-2 and rabbit anti-iNOS (Cayman Chemical, Ann Arbor, MI, USA) at a dilution of 1:2500 and 1:1000, respectively; rabbit anti-β-actin (Cell Signalling, Danvers, MA, USA) (1:1000); mouse anti-pERK and mouse anti-ERK (Cell Signalling, Danvers, MA, USA) (1:2500); and P-PiNOS (Cell Signalling, Danvers, MA, USA) (1:1000), overnight at 4°C. After rinsing, the membranes were incubated with a horseradish-peroxidase-labeled secondary antibody anti-rabbit (Cayman Chemical, Ann Arbor, MI, USA) at a dilution of 1:2500 and 1:10000, respectively; rabbit anti-β-actin, MCP-1 and TNF-α to β-actin (Sigma Aldrich, St. Louis, MO, USA). Immunodetection was performed using enhanced chemiluminescence light-detecting kit (Pierce, Rockford, IL, USA). Then, the immunosignals were captured using LAS-3000 Imaging System from Fujifilm Image Reader (Mac™ and Windows) and densitometric data were studied following normalization to the control (housekeeping gene). The signals were analyzed and quantified by an Image Processing and Analysis in Java (ImageJ, Softonic, USA) and expressed in percentage respect to control sham group.

Table 3

<table>
<thead>
<tr>
<th>Score</th>
<th>Bleeding</th>
<th>Weight loss (% of initial wt)</th>
<th>Stool consistency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
<td>&lt;1</td>
<td>Normal pellets</td>
</tr>
<tr>
<td>1</td>
<td>Slightly bloody</td>
<td>1–4.99</td>
<td>Slightly loose feces</td>
</tr>
<tr>
<td>2</td>
<td>Bloody</td>
<td>5–10</td>
<td>Loose feces</td>
</tr>
<tr>
<td>3</td>
<td>Blood in whole colon</td>
<td>&gt;10</td>
<td>Watery diarrhea</td>
</tr>
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</table>

Table 4

<table>
<thead>
<tr>
<th>Score</th>
<th>Inflammation</th>
<th>Extent</th>
<th>Crypt damage</th>
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<tr>
<td>0</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Slight</td>
<td>Mucosa</td>
<td>Basal 1/3 damaged</td>
</tr>
<tr>
<td>2</td>
<td>Moderate</td>
<td>Mucosa and submucosa</td>
<td>Basal 2/3 damaged</td>
</tr>
<tr>
<td>3</td>
<td>Severe</td>
<td>Transmural</td>
<td>Only surface epithelium intact</td>
</tr>
</tbody>
</table>

Table 5

<table>
<thead>
<tr>
<th>Sequence of β-actin, MCP-1 and TNF-α primers used in the RT-PCR study</th>
<th>Sense</th>
<th>Primer sequence</th>
<th>Amplicon size (bp)</th>
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</thead>
<tbody>
<tr>
<td>j-Actin</td>
<td>Forward</td>
<td>GGCCTGATATCCCCTCCAT</td>
<td>141</td>
</tr>
<tr>
<td>Reverse</td>
<td>ATGGCATCCTGGATTTGTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>Forward</td>
<td>ACCACGATCAGCTGCTGCAC</td>
<td>75</td>
</tr>
<tr>
<td>Reverse</td>
<td>AGGACGAACACAATGCGTCC</td>
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<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward</td>
<td>CAAGGGACACATGGCTCCGC</td>
<td>109</td>
</tr>
<tr>
<td>Reverse</td>
<td>CACGGCGGCTGTCGCGCG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.11. Statistical analysis

All values in the figures and text are expressed as arithmetic means±S.E.M. Data were evaluated using GraphPad Prism Version 5.01 software. The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance and using Tukey-Kramer multiple comparison test as post hoc test. P values of <.05 were considered statistically significant. In the experiment involving histology and western blot, the figures shown are representative of at least 4–5 experiments performed on different days.

3. Results

3.1. Effect of EVOO polyphenol supplementation on DSS-induced chronic colitis

This experimental model leads to two well-differentiated phases: (i) an initial acute phase characterized by loss of body weight, not formed stool and rectal bleeding when animals were exposed to 3% DSS for 5 days, and (ii) another phase where disease progressed to a severe chronic colitis for 21 days after DSS removal. SD-DSS animals underwent severe anorexia with a marked body weight loss compared with the sham animals during acute and chronic phase. Signs of diarrhea were clear from the 3rd day until final treatment although gradually decreased as the chronic inflammation progressed. Rectal bleeding also showed a maximal peak at Day 3 in group fed with SD and treated with DSS.

On the contrary, both dietary groups, EVOO+PE and EVOO, showed a significant reduction in weight loss at second week when compared with SD and SD+PE groups (EVOO+PE: P<.001 vs. SD+PE-DSS group, EVOO: P<.05 vs. SD and P<.001 vs. SD+PE-DSS groups). Moreover, after 3 weeks of DSS removal, all studied diets showed a significant weight gain respect to SD-DSS group (P<.001). On the other hand, both stool consistency and rectal bleeding were significantly reduced with all diets in study in comparison with DSS group fed with SD.

Similarly, DAI showed a significant increase from the 5th day of DSS exposure until 3 weeks after DSS removal in SD-DSS group (Fig. 1B). DAI
score was significantly improved at the end of chronic phase in both diets supplemented with PE (SD+PE and EVOO+PE) and EVOO diet (P<.001 vs. SD-DSS group); moreover, dietary EVOO+PE significantly showed the lowest DAI score during all the experimental period.

A significant increase of weight/length ratio of the mice colon, an indicator of inflammation [42], was observed in all DSS-treated groups when compared with its respective sham control diet groups (P<.001). However, this relationship significantly decreases in EVOO+PE- and EVOO-fed animals (P<.001 vs. SD and SD+PE), although there were no significant differences among them (Fig. 1C).

3.2. Histopathological analysis of DSS-induced chronic colitis after EVOO polyphenol supplementation

Histological sections of colonic tissue from healthy animals fed with different diets showed a normal structure without histological changes. By contrast, the administration of DSS in animals fed with SD caused injuries that affected most of the mucosa from the three colonic segments (proximal, middle and rectum), with the loss of histological structure, and a strong epithelial disintegration, immune cell infiltrates, edema and epithelial denudation. Epithelial cells had practically disappeared, showing ulceration and loss of mucosal crypts especially in middle and rectum sections. There were also characteristic signs of chronic inflammation as the presence of a lymphocytic infiltrate with granulocytes, monocytes and macrophages in the mucosa and submucosa (Fig. 2A). In contrast, colonic slides from animals treated with DSS and fed with the different diets under study revealed reduced signs of inflammation into the colonic tissue and a minor extent affected mucosa with moderate loss of epithelial cells, especially from proximal and middle colonic segments. Moreover, a preservation of the glandular structure or a regeneration of crypts and reepithelialization in some areas could be observed (Fig. 2D–F).

Quantification of the results by histological scoring revealed a significant increased score in SD-DSS group, which reached 9.5±3.6 (proximal), 36.5±6.0 (middle) and 16±1.8 (rectum) points, whereas the mean score in SD+PE, EVOO and EVOO+PE-treated mice was significantly lower in proximal and middle sections. However, no significant differences between rectums sections were observed (Fig. 2G).

3.3. Effect of dietary EVOO polyphenol supplementation on PCNA in DSS-induced chronic colitis

PCNA is a nuclear protein necessary for cell proliferation and is expected to play a key role in the inflammatory processes [43]. In order to explore whether PE-enrichment EVOO diet could interfere with inflammatory and enterocyte cells proliferation, we evaluated anti-PCNA positive cells in colon tissue in DSS-induced chronic.

Tissue sections from sham control mice exhibited specific staining for PCNA in epithelial cells at the bottom layer of crypts. In contrast, the highest PCNA-labeling index was observed in colon tissue from SD-DSS group, mainly in the inflammatory cells in the submucosal layer and in the interstitial region (Fig. 3A and C), demonstrating that hyper proliferation was associated with chronic inflammation. On the other hand, this index was significantly reduced by all diets under study when compared with SD-DSS being more evident after PE-enrichment EVOO consumption (Fig. 3B and C).

3.4. Effect of dietary EVOO polyphenol supplementation on MCP-1 and TNF-α in DSS-induced chronic colitis by quantitative RT-PCR

RNA of colon tissue from sham and DSS animals fed with the different diets were isolated and RT and PCR amplification assays were performed. Quantitative RT-PCR assays were established for β-actin (housekeeping gene), MCP-1 and TNF-α (Fig. 4A and B). For each PCR run, a negative control was systematically added, in which R-Nase-free water replaced cDNA. In addition, a single peak was observed for each of the products by melting curve analysis, performed routinely in all samples after amplification. Product Tm values were as follows: β-actin, 86.3°C; MCP-1, 85.7°C; TNF-α, 85.9°C. No product was detected in the absence of RT in any assay for any of the products, thus indicating that there was no genomic contamination. Data for each diet-DSS group samples were normalized by those obtained for each diet-sham group.

As shown in Fig. 4, significant levels of MCP-1 and TNF-α mRNA were found in colon tissues from DSS group fed with SD (P<.001). The relative quantification assay revealed notably differences in both MCP-1 and TNF-α mRNA expression in colon tissue from DSS animals fed with each diets when compared with values obtained in SD-DSS group (Fig. 4A). EVOO+PE animal group showed significantly the highest reduction in both inflammatory mediators in comparison with those values obtained from group fed only with EVOO (P<.05) (Fig. 4B).

3.5. Effect of dietary EVOO polyphenol supplementation on colonic expression of inflammatory proteins in DSS-induced chronic colitis

Cytosolic COX-2 and iNOS protein expression levels were measured by western blotting from colon mucosa. As shown in Fig. 5, exposure of colon to DSS and treated with SD caused strong iNOS and COX-2 protein expression (P<.001). Dietary treatments were able to diminish significantly the up-regulation of these pro-inflammatory proteins. In addition, animals fed with EVOO and those supplemented with PE showed a higher down-regulation of iNOS and COX-2 compared with SD+PE-DSS group (P<.001). Furthermore, a notably iNOS reduction was observed in animals groups fed with EVOO+PE vs. EVOO group (P<.001).

3.6. Effect of dietary EVOO polyphenol supplementation on the activation of MAPK signaling in DSS-induced chronic colitis

We also examined the expression and activation of MAPKs by western blot analysis using phosphospecific MAPK antibodies. To standardize protein loading in each line, blots were stripped and reproven with the corresponding antibodies against non-activated MAPKs proteins. In the present study, administration of DSS was found to activate p38 and JNK phosphorylation proteins (P<.001), indicating that both MAPK proteins activation could be induced at the chronic stage of colonic lesion in animals fed with SD. Data from animals fed with PE-supplemented diets (SD and EVOO) and EVOO diet and treated with DSS showed a significant reduction of MAPKs activation (Fig. 6). Moreover, down-regulation of JNK activation was higher in those animals fed with PE-supplemented EVOO diet (P<.01 vs. SD+PE-DSS group and P<.001 vs. EVOO-DSS group) (Fig. 6B). In addition, ERK1/2 activation protein expression was modified neither after DSS administration nor with any diets used in our study (Fig. 6C).

3.7. Effect of dietary EVOO polyphenol supplementation on NFκB transcription factors activation in DSS-induced chronic colitis

NFκB is a family of highly regulated dimeric transcription factors that play pivotal roles in inflammatory responses and immunological reactions. Its activity is controlled by a family of regulatory proteins, called inhibitors of NFκB, such as IκBα, that mediate binding to NFκB dimers. After IκBα is degraded, the NFκB–IκBα complex is free to be translocated into the nucleus, where it can induce the expression of pro-inflammatory genes contributing to the damage. As shown in Fig. 7A, intestinal
inflammation of DSS group induced a significant \( \text{I} \kappa \text{B} \alpha \) degradation in the animals fed with SD (\( P < .05 \) vs. sham SD group), which is consistent with an up-regulation in the NF-\( \kappa \)B-binding activity, whereas this degradation was lesser and no significant respect to sham groups in the animals fed with SD+PE and EVOO diets. Moreover, the PE-enriched EVOO diet showed an interesting result up-regulating \( \text{I} \kappa \text{B} \alpha \) expression significantly respect to the other dietary DSS groups (\( P < .001 \) vs. SD-DSS group, \( P < .01 \) vs. SD+PE-DSS group and \( P < .05 \) vs. EVOO-DSS group).

3.8. Effect of dietary EVOO polyphenol supplementation on PPAR\( \gamma \) in DSS-induced chronic colitis

Given that NF-\( \kappa \)B expression and activation and the expression of genes encoding cytokines could be modulated by PPAR\( \gamma \), its expression was evaluated. In this sense, our results demonstrated that DSS treatment down-regulated PPAR\( \gamma \) expression in all diet groups (Fig. 7B). However, PPAR\( \gamma \) expression increased significantly in those animals fed with EVOO and EVOO+PE diet when compared...
with SD-DSS (P<.05 and P<.001, respectively) and SD+PE-DSS groups (P<.01 and P<.001, respectively). Once again, animals fed with PE-enriched EVOO diet showed a higher PPARγ activation than those fed with EVOO diet (P<.001).

4. Discussion

It is well known that EVOO is rich in phenolic compounds, which other vegetable oils do not contain [44,45]. In terms of healthful qualities, numerous evidences propose that the intake and interaction of several “micronutrients” provided by a healthy diet, such as the EVOO’s polyphenols, can probably be the link that affords protection from several pathologies [46]. Although the beneficial role of olive oil in the diet in different experimental colitis models have already been documented [47–49], no reports are available regarding the evaluation of the EVOO’s polyphenols in inflammation and particularly in UC.

Based on this background, our results revealed, for the first time, that dietary supplementation of PE and mainly EVOO enriched with PE reduced the severity and extent of progressive chronic colonic damage induced by a short 5-day (acute phase) exposure of DSS followed by a 3-week rest period in C57BL/6 mice. These results were corroborated by histological findings.

Since our results show that EVOO+PE diet was more effective in reducing colitis severity than those from SD enrichment with PE, we could suggest that the responsibility for such beneficial properties could be assigned to both an adequate fatty acid profile of EVOO and the presence of a high proportion of phenolic compounds.
Moreover, these improved effects observed could be due to a possible synergistic effect among EVOO constituents, since it’s not clear whether all the possible beneficial mechanisms act independently of each other or whether they have a synergistic or competitive action [45,50].

Although the etiology of UC remains essentially unknown, data from many studies in humans and animal models suggest that it is related to an abnormal immune response in the gastrointestinal tract. Among others, the abnormal presence of inflammatory cells within the mucosa produces increased concentrations of inflammatory cytokines such as TNF-α [51] and chemokines, for example, MCP-1 [52], and a selective blockade of both inflammatory mediators significantly decreases severity of colitis and neutrophil/macrophage migration [53,54]. Our results demonstrate that the dietary treatment was able to significantly reduce MCP-1 and TNF-α mRNA levels, being the animals group fed with EVOO+PE that showed the lowest levels. Considering these data, it is possible to suggest that a higher PE proportion in the diet could modulate macrophage activation and, consequently, decrease cytokine production in UC. In other inflammatory pathologies, dietary polyphenols have shown similar effects. For example, in a study developed by Mukai and Sato, polyphenol-containing azuki bean (Vigna angularis) seed coats attenuated vascular oxidative stress and inflammation during the progression of hypertension, reducing MCP-1 levels among others [55]. Beside, polyphenols from the red propolis have shown to reduce atherosclerotic lesions through mechanisms including inflammatory factors modulation, as MCP-1, in initial atherosclerotic lesions in mice [56]. Other PE from acacia decreased TNF-α expression in white adipose tissue using a metabolic syndrome model [57].

PCNA is a fundamental protein involved in cell proliferation [43]. Chronic inflammation seems to be associated to a higher proliferative activity, and it has been reported an increase of its expression under this condition [58]. In our study, diet enrichment with PE from EVOO notably reduced PCNA immunostaining and protein activation in colonic tissue, suggesting that PE ameliorates chronic colitis, at least in part, by reducing cell proliferation.

As we have already shown, COX-2 and iNOS are pro-inflammatory proteins that play a pivotal role in mediating inflammation and contribute to DSS-induced inflammation in mice [24,32]. In the present study, PE enrichment was able to down-regulate significantly both proteins expression, although iNOS inhibition was higher in those animals fed with EVOO+PE-enriched diet. This inflammatory proteins reduction has been reported for other polyphenols. Recently D’Argenio et al. [59] have documented the efficacy of a PE from apple in a rat colitis model by reducing COX-2 expression and TNF-α level. Similarly, our group has also demonstrated that resveratrol and polyphenols present in pomegranate decreased COX-2 and iNOS overexpression in an experimental colitis model [24,25,60].

The regulation of those key inflammatory molecules can be controlled by different signaling pathways, including the MAPKs [61,62]. Accumulative evidences suggest a strong support for a role in the MAPKs mediating IBD, in fact, preclinical studies with MAPK inhibitors have demonstrated significant efficacy repeatedly in experimental colitis models [63–65] suggesting a possible application in IBD treatment. Our study revealed that dietary PE enrichment was able to diminish the p38 and JNK MAPKs up-regulation; moreover, PE-enriched EVOO showed an efficiently higher reduction of JNK activation than other diets studied. This is an interesting finding that has not been shown, and revising the literature, there are few studies...
where this mechanism has been proved in experimental UC by other polyphenols. Concretely, this beneficial effect has been reported after dietary administration of resveratrol, curcumin, ellagic acid, hydroxytyrosol or pomegranate extract [24,25,29,32,60]. Palmieri et al. also demonstrated that PE from olive pomace restored the endothelial functions impaired by anoxia regulating implicated genes expression such as MAPKs, more efficiently than its single purified components [66].

One of the well-studied transcription factors downstream of MAPKs signaling is the nuclear factor NF-κB. The NF-κB family of transcription factors consists of five mammalian members (p50, p52, p65, cRel and RelB), which can form either homodimers or heterodimers. Multiple lines of evidence suggest that NF-κB activation actively contributes to the development and maintenance of intestinal inflammation since this nuclear transcription factor binds to the promoter of many genes that are essential for the activation of immune responses including iNOS, COX-2 and TNF-α among others [67–69]. Moreover, NF-κB was found to be activated in mucosal cells from IBD patients [70,71] as well as in experimental colitis models [72]. Our results showed that chronic inflammatory conditions induced by DSS were accompanied by the inhibition in the IκBα kinase protein expression in the colon. Enrichment of EVOO with PE blocked the IκBα degradation most significantly in comparison to the other dietary groups. These data provide further evidence in the role of NF-κB pathway in the beneficial effects of PE in UC. This result is in accordance with a previous study where an EVOO extract, particularly rich in phenolic compounds, was investigated — in vitro — on NFκB translocation in monocytes and monocyte-derived macrophages isolated from healthy volunteers. The EVOO extract inhibited p50 and p65 NFκB translocation in both unstimulated and phorbolmyristate-acetate-challenged cells in a dose-dependent manner, being particularly effective on the p50 subunit [73].

PPARγ is a member in the superfamily of nuclear receptors implicated in the regulation of intestinal inflammation [8,74,75]. There are studies demonstrating that its activation could potentially reduce the severity of IBD by inhibiting excessive immunoinflammatory responses [76,77]. Our results showed that the anti-inflammatory effects of PE-enriched EVOO diet could be correlated with PPARγ activation in the colon. Although few studies have regarded polyphenols as PPARγ ligands, this study shows that it may affect PPARγ protein expression, activating the PPARγ pathway and then inhibiting NFκB activation.
Fig. 6. Effect of EVOO and EVOO+PE diet on p-p38, p-JNK and p-ERK1/2 activation in the colon tissue after 3% of DSS for 5 days followed by 3 weeks of water. Densitometry was performed following normalization to the control (p38, JNK and ERK1/2 housekeeping genes, respectively). Data are expressed as the means±S.E.M. *P<.05, **P<.01 and ***P<.001 vs. control sham group; +P<.05, ++P<.01 and +++P<.001 vs. SD-DSS group; ##P<.01 vs. SD+PE-DSS group; &&&P<.001 vs. EVOO DSS group.
On the other hand, it has been demonstrated that PPARγ inhibits inflammatory response via blocking transcription factor AP-1 activation by interfering with the JNK cascade activation [78]. Hsu et al. [79] suggested that JNK phosphorylation might be mediated by PPARγ activity. In accordance with our results, PPARγ interacts with the JNK phosphorylation-responsive part of c-Jun and then inhibits the JNK phosphorylation.

In summary, the mechanisms underlying the protective effects of dietary EVOO-PE supplementation in chronic colitis involve a cell proliferation inhibition and a PPARγ up-regulation as well as NFκB and MAPK pathways inhibition, which decreases the gene expressions of TNF-α and MCP-1 that was accompanied by a significant iNOS protein down-regulation. We concluded that PE-enriched EVOO diet could be a beneficial functional food on UC.

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