Eur J Nutr (2012) 51:497–506
DOI 10.1007/s00394-011-0235-y

ORIGINAL CONTRIBUTION

Influence of extra virgin olive oil diet enriched with hydroxytyrosol in a chronic DSS colitis model

Susana Sánchez-Fidalgo · L. Sánchez de Ibargüen · A. Cárdeno · C. Alarcón de la Lastra

Received: 9 March 2011 / Accepted: 27 July 2011 / Published online: 27 August 2011

Abstract
Purpose Recent epidemiological studies have shown that habitual consumption of extra virgin olive oil (EVOO), the characteristic culinary fat of the Mediterranean area, is effective in the prevention of diverse types of digestive disorders such as inflammatory bowel disease. Many of these benefits are, in addition to its high proportion of oleic acid, due to the high content of phenolic compounds.
Methods Six-week-old mice were randomized into three dietary groups: standard, EVOO and hydroxytyrosol-enriched EVOO. After 30 days, mice that were exposed to 3% DSS for 5 days developed acute colitis that progressed to severe chronic inflammation during a regime of 21 days of water.
Results Diets enriched with EVOO significantly attenuated the clinical and histological signs of damage, improving results from disease activity index and reducing about 50% the mortality caused by DSS. Moreover, hydroxytyrosol supplement showed better results. Cytokines study showed that TNF-α was maintained near to sham control and IL-10 levels were significantly improved in EVOO and EVOO plus hydroxytyrosol diet-DSS groups. In the same way, COX-2 and iNOS were downregulated, and the activation of p38 MAPK was reduced. We also observed a higher significant reduction in iNOS in hydroxytyrosol-enriched EVOO compared with EVOO alone.
Conclusions EVOO diets exerted a noteworthy beneficial effect in chronic DSS-induced colitis by cytokine modulation and COX-2 and iNOS reduction via downregulation of p38 MAPK. In addition to the beneficial effect by EVOO, supplementation of the diet with hydroxytyrosol may improve chronic colitis through iNOS downregulation plus its antioxidant capacity.

Keywords Extra virgin olive oil · Hydroxytyrosol · Chronic colitis · Cytokines · COX-2 · iNOS

Introduction

Inflammatory bowel diseases (IBD), i.e. Crohn's disease (CD) and ulcerative colitis (UC), are chronic and inflammatory disorders of the gastrointestinal tract, with an increasing prevalence in developed countries. Although the specific causes remained to be recognized, understanding of the molecular mediators and mechanisms of tissue injury has really advanced. It is true that increase in inflammatory mediators, including reactive oxygen species such as nitric oxide, prostaglandins and inflammatory cytokines, plays an important role in immune dysregulation. Moreover, they can activate diverse downstream signalling pathways leading to the activation of transcription factors, which modulate a number of different steps in the inflammatory cascade as pro-inflammatory cytokines in different cell types, neutrophil degranulation, as well as the expression of important inducible inflammatory enzymes such as cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) [1, 2].

Currently, nutrition therapy, in addition to their dietary support, can exert therapeutic effects without undesirable effects that accompany the classical pharmacotherapy.

L. Sánchez de Ibargüen has to be considerate equal as the first author.

S. Sánchez-Fidalgo (✉) · L. Sánchez de Ibargüen · A. Cárdeno · C. Alarcón de la Lastra
Department of Pharmacology, School of Pharmacy, University of Seville, Profesor Garcia González no 2 Street, Seville, Spain
e-mail: fidalgo@us.es

In the last years, it has been highlighted that some foods with a nutritive function provide a beneficial health effects in the prevention and treatment of certain diseases [3, 4]. Oil from the olive, a typical ingredient of the Mediterranean diet, possesses many beneficial health effects, and it can be considered as a functional food of great qualities. Extra virgin olive oil (EVOO), obtained by direct pressing or centrifugation of the olives, contains major and minor components. Major components glycerides represent more than 98% of the total oil weight, where the monounsaturated oleic acid, the main component, accounting up to 80% of the total lipidic composition. Minor components, present in a small amount (about 2% of oil weight), include more than 230 chemical compounds such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds and antioxidants represented by polyphenols [5]. Most of the work has focused on the health benefits of EVOO showing important effects as antioxidant, anti-inflammatory, chemopreventive and anticancer [5–7]. Many of these benefits are due, in addition to its high proportion of oleic acid, to the high content of phenolic compounds, which have mainly antioxidant [8], anti-inflammatory and anticancer activities, among others [7, 9]. Among the olive oil phenolic compounds, hydroxytyrosol is considered one of the most abundant and representative olive oil phenols; indeed, studies have demonstrated that it has cardioprotective, anti-inflammatory, antiplatelet aggregation effects, antimicrobial and anticancer activities [10]. However, the precise molecular mechanisms responsible for this protection are still not fully understood.

In rodents, oral dextran sulphate sodium (DSS) administration in the drinking water has been found to induce colonic inflammation with clinical and histological similarity to human UC, being useful to identify and validate new therapies for the treatment of IBD [11]. The present study was designed to examine the protective/preventive effects of dietary EVOO and hydroxytyrosol-enriched EVOO intake in a chronic colitis model induced by DSS in C57BL/6 mice by macroscopic and histology parameters and to explore the anti-inflammatory mechanisms involved in its effects evaluating cytokine production, COX-2 and iNOS expression. Finally, we also studied the role of p38 MAPK signalling pathway in the effects of EVOO and hydroxytyrosol.

Materials and methods

Animals and diets

A total of 75 6-week-old female C57BL/6 mice (Charles River, Tokyo, Japan) were used in this study. They were acclimatized in our Animal Laboratory Centre under standard conditions (temperature 24–25 8 °C, humidity 70–75%, lighting regimen of 12L/12D) and were fed pellet diets and water ad libitum. Mice were randomized into three dietary groups: one group (25 animals) were fed with standard diet prepared with sunflower oil, other group (25 animals) were fed with diet enriched with EVOO and the third group (25 animals) were fed with EVOO enriched with 40 mg/kg of diet of hydroxytyrosol (Sigma-Aldrich Company Ltd, Spain) (Table 1) during all experimental period. Hydroxytyrosol-enriched EVOO group consumed an average of 3 g/day of diet, resulting in a dose of 5 mg/kg body weight of hydroxytyrosol ingested. The administered dose of hydroxytyrosol was chosen based on the analyses described in the literature [12]. Diets were formulated on the basis of the American Institute of Nutrition (AIN) standard reference diet with the modification of various sources of carbohydrate, being the percentage of oil total of 10%. All diets were prepared by mixing the respective compounds under yellow light and stored at −80 °C. Fresh diet was provided daily. Experiments followed a protocol observed 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 Counsel 86/609/EC).

Induction of colitis

During 30 days after weaning, in each diet, 17 mice received 3% DSS (DSS group; MW: 40000, ICN Pharmaceuticals, Costa Mesa, CA) in the 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 [11]. Then, animals were killed by an overdose of i.p. chloral hydrate.

Evaluation of the severity of clinical colitis

The clinical activity of colitis was evaluated during the experimentation phase in order to determine the disease activity index (DAI) as described by Melgar et al. [11] with slight modifications as shown in Table 2. 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 week when the animals were on a pure water. The average of the three values constituted the DAI [13].

Macroscopic and histopathological evaluation

At the end of the experimental period, the colons were removed, slightly cleaned in physiological saline to remove

Table 1 Composition of experimental diets

Ingredients	Standard diet (g/kg of diet)	Extra virgin olive oil diet (g/kg of diet)	Extra virgin olive oil plus hydroxytyrosol diet (g/kg of diet)
Casein	200	200	200
DL-Methionine	3	3	3
Cornstarch	150	150	150
Sucrose	449.91	449.91	449.91
Cellulose	50	50	50
Sun flower[a]	100	–	–
EVOO[b]	–	100	100
Mineral mix[c]	35	35	35
Vitamin mix[d]	10	10	10
Choline bitartrate	2	2	2
Fe (sulphate)	90×10^{-3}	90×10^{-3}	90×10^{-3}
Hydroxytyrosol	–	–	40×10^{-3}

Diet was formulated on the basis of the American Institute of Nutrition (AIN) standard reference diet with the modification of various sources of carbohydrate

[a] Sunflower oil from Ibarra SL (Spain)

[b] EVOO, extra virgin olive oil from Picual Virgin, Jaen, Spain. OLEOESTEPA (Sociedad Cooperativa Andaluza)

[c] Mineral mix provided the following (g/kg diet): calcium carbonate, 35.7; monopotassium phosphate, 25.0; sodium chloride, 7.4; potassium sulphate, 4.66; potassium citrate monohydrate, 2.8; magnesium oxide, 2.4; ferric citrate, 0.606; zinc carbonate, 0.165; manganese carbonate, 0.063; copper carbonate, 0.03; potassium iodate, 0.001; sodium selenate, anhydrous, 0.001025; ammonium molybdate–$4H_2O$, 0.000795; sodium metasilicate–$9H_2O$, 0.145; chromium potassium sulphate–$12H_2O$, 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; D-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; alpha tocopherol powder (250 U/g), 300 mg; vitamin A palmitate (250,000 U/mg), 16 mg; vitamin D3 (400,000 U/g), 2.5 mg; phylloquinone, 0.75 mg

Table 2 Assessment of inflammation by means of clinical parameters and macroscopic score

Score	Bleeding	Weight loss (% of initial wt)	Stool consistency	Inflammatory score
0	Normal	<1	Normal pellets	Normal
1	Slightly bloody	1–4.99	Slightly loose faeces	Slight inflammation
2	Bloody	5–10	Loose faeces	Moderate inflammation and/or oedema
3	Blood in whole colon	>10	Watery diarrhoea	Heavy inflammation and/or ulcerations and/or oedema

faecal residues, weighed and measured in order to evaluate the variations in the weight/length as an inflammation index. The macroscopic appearance of the colon (inflammatory score), based on the degree of inflammation and the presence of oedema and/or ulcerations, was also evaluated (Table 2).

Samples of three 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; 7-μm-thick slices from paraffin sections were stained with haematoxylin and eosin in accordance with the standard procedures for the histological evaluation of colonic damage. Histological study was representative of 3 animals per group, and its evaluation was determined by a pathologist who was unaware of the experimental protocol. A histological score, as described by Melgar et al. [11] with some modifications, was established in an scale of 0–4, where 0 = no signs of damage; 1 = few inflammatory cells, no signs of epithelial degeneration; 2 = mild inflammation, few signs of epithelial degeneration; 3 = moderate inflammation, few epithelial ulcerations; 4 = moderate to severe inflammation, ulcerations in more than 25% of the tissue section.

Assessment of TNF-α, IL-1β and IL-10

Colon samples were weighed and homogenized in phosphate-buffered saline solution (PBS, pH = 7.2) containing

a proteases cocktail at 4 °C and centrifuged at 12,000×g (10 min). Mucosal cytokine levels were assayed twice with quantitative ELISA kits (eBioscience Inc., San Diego, USA). TNF-α, IL-1β and IL-10 values were measured as pg/mg tissue and expressed as percentage compared with sham-standard diet group.

Western blot analysis

Frozen colonic tissues were weighed and homogenized in ice-cold lysis buffer. Homogenates were centrifuged (12,000×g, 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. Aliquots of supernatants containing equal amounts of protein (50 mg) were evaluated to determine COX-2, iNOS and p38 MAPK proteins by western blot as described by Sánchez-Fidalgo et al. [13]. The figures shown are representative of colonic mucosa from 4 animals per group.

Statistical analysis

All values in the figures and text are expressed as arithmetic means ± standard error (SEM). Data were evaluated using Graph Pad Prism® Version 2.01 software. The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA) and using Tukey–Kramer multiple comparison test as post hoc test. Inflammatory and histological score was evaluated by non-parametric Kruskal–Wallis test. p values of <0.05 were considered statistically significant.

Results

Effect of EVOO and EVOO supplemented with hydroxytyrosol diets on clinical signs

Two phases were observed in this experimental model: one acute phase when animals were exposed to 3% DSS (5 days), characterized by the loss of body weight, not formed stool and rectal bleeding, and other phase after DSS removal, where disease progressed to a severe chronic colitis (21 days). Significant loss of body weight was observed from 5th day after DSS treatment ($p < 0.01$) and at 1 and 2 weeks ($p < 0.001$ and $p < 0.05$) after DSS removal in standard diet group versus sham animals. Signs of diarrhoea were clear from day 3 ($p < 0.01$) until final treatment ($p < 0.001$) and rectal bleeding was significant at day 5 with respect to sham group ($p < 0.001$), although gradually decreased as the chronic inflammation progressed. In the same line, DAI showed a significant increase at 5th day of DSS exposure and 1 week after DSS removal ($p < 0.01$; Fig. 1). By contrast, dietary EVOO and EVOO plus hydroxytyrosol counteracted all these clinical signs, although there were no significant differences between both groups. Although the animals also showed weight loss, it was significantly lower in relation to that observed in the standard diet at 1 and 3 weeks after DSS removal ($p < 0.001$ and $p < 0.05$, respectively). Stool consistency showed better results at day 5 of DSS administration versus standard diet ($p < 0.01$), although it was elevated in the chronic phase. Finally, rectal bleeding also improved at day 5 versus standard diet group ($p < 0.01$). Accordingly, results from DAI in animals fed with diets enriched with EVOO improved significantly at 5th day of

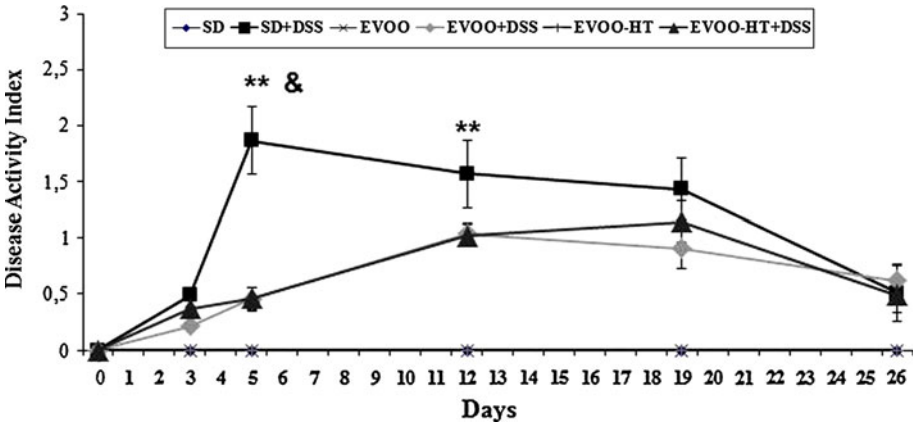

Fig. 1 Disease activity index after extra virgin olive oil (EVOO) and supplemented with hydroxytyrosol (EVOO-HT) diets during acute and chronic colitis by dextran sodium sulphate (DSS) in C57BL/6 mice. Disease activity index was evaluated as average of score of clinical parameters as body weight changes, rectal bleeding and stool consistency or diarrhoea (25 animals per diet groups: 8 per sham group and 17 per DSS group). Data are expressed as the means ± SEM. $^{**}p < 0.01$ versus sham group, $^{\&}p < 0.05$ versus DSS-standard diet (SD) group

DSS treatment, although in the chronic phase showed similar results versus standard diet group (Fig. 1). Moreover, animals fed with standard diet and treated with DSS showed a mortality of 41.2% (7/17); however, the groups fed with EVOO and hydroxytyrosol-enriched EVOO diets showed a mortality of 17.6 (3/17) and 23.5% (4/17), respectively, showing a diminution of mortality about 50%.

Effect of EVOO and EVOO supplemented with hydroxytyrosol diets on colon weight/length and macroscopic inflammation

The weight/length ratio is a morphological parameter useful for assessing colonic inflammation [14]. Control DSS-treated group showed a relationship between weight and length of 118.32 ± 15 mg/cm ($p < 0.01$ vs. sham group) that was significantly decreased to 80.82 ± 7.05 mg/cm in the DSS-EVOO plus hydroxytyrosol group ($p < 0.05$) versus DSS-standard group (Fig. 2). Moreover, macroscopic inflammatory score in group fed with EVOO and hydroxytyrosol-enriched EVOO was significantly lower (0.95 ± 0.12 and 0.55 ± 0.09, respectively, $p < 0.05$) versus DSS-standard group (1.65 ± 0.18) at the end of treatment; however, there were no significant changes between both groups.

Histopathological analysis of DSS-induced chronic colitis after dietary EVOO and EVOO supplemented with hydroxytyrosol

Histological sections of colonic tissue from healthy animals, in our study, fed with different diets showed a normal structure without histological changes (Fig. 3). By contrast, the administration of DSS in animals fed with standard diet caused injuries that affected most of the mucosa of the three colonic segments (proximal, middle and rectum), with the loss of histological structure and alteration in glandular epithelium. Epithelial cells had practically disappeared, showing desquamation, ulceration and loss of mucosal crypts. 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. 3; Table 3). However, slides from the group of animals treated with DSS but fed with both EVOO diets showed, to a minor extent, affected mucosa reducing the histological signs of damage of the three colonic segments. Shedding epithelial cells was detected throughout the mucosa, although no ulceration or total loss of crypts of mucosa was observed, while other areas had good preservation of the glandular structure or a regeneration of crypts and reepithelialization even could be observed. Also, there was a decrease in inflammatory infiltrate in all samples (Fig. 3; Table 3). Moreover, slides from the rectum section of animals fed with hydroxytyrosol supplement showed significantly better histological score.

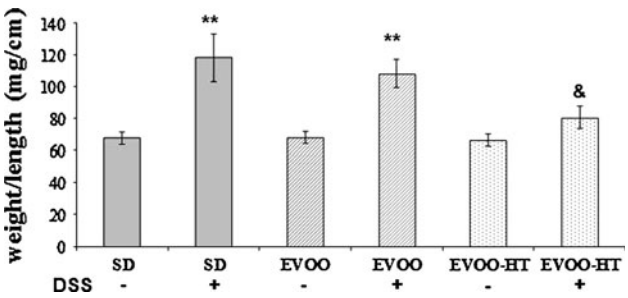

Fig. 2 Effect of extra virgin olive oil (EVOO) and supplemented with hydroxytyrosol (EVOO-HT) diets on weight/length of the colon in the experimental animal model of colitis by dextran sodium sulphate (DSS) (8 animals per each sham group, 10 animals per DSS-SD group, 14 animals per DSS-EVOO group and 13 animals per DSS-EVOO-HT group). Data are expressed as the means ± SEM. $^{**}p < 0.01$ versus sham group. $^{\&}p < 0.05$ versus DSS-standard diet (SD) group

Effect of dietary EVOO and EVOO supplemented with hydroxytyrosol on colonic cytokine levels in DSS-induced chronic colitis

In animals fed with standard diet, chronic colonic injury induced by DSS administration was characterized by an increase in the pro-inflammatory cytokines TNF-α and IL-1β ($p < 0.05$) and a reduction in the anti-inflammatory cytokine IL-10 ($p < 0.01$) compared with sham control group. In contrast, although IL-1β levels were not modified, the levels of TNF-α were maintained near to sham control in the groups fed with EVOO and EVOO plus hydroxytyrosol diets. Moreover, IL-10 levels were significantly augmented in these groups compared with the effects observed for DSS-standard diet group ($p < 0.05$; Fig. 4).

Effect of dietary EVOO and EVOO supplemented with hydroxytyrosol on colonic expression of inflammatory proteins in DSS-induced chronic colitis

The levels of protein expression were measured by western blotting of cytosolic extracts from colonic mucosa. As shown in Fig. 5a, DSS exposure caused significant expression of COX-2 in animals fed with standard diet ($p < 0.001$); on the contrary, the groups fed with EVOO and hydroxytyrosol-enriched EVOO diets induced the downregulation of the protein ($p < 0.05$ vs. DSS-standard diet group). In the same way, iNOS was significantly expressed in DSS group fed with standard diet ($p < 0.001$) and both diets with EVOO and EVOO enriched with hydroxytyrosol significantly reduced its expression ($p < 0.001$ vs. DSS-standard diet). Moreover,

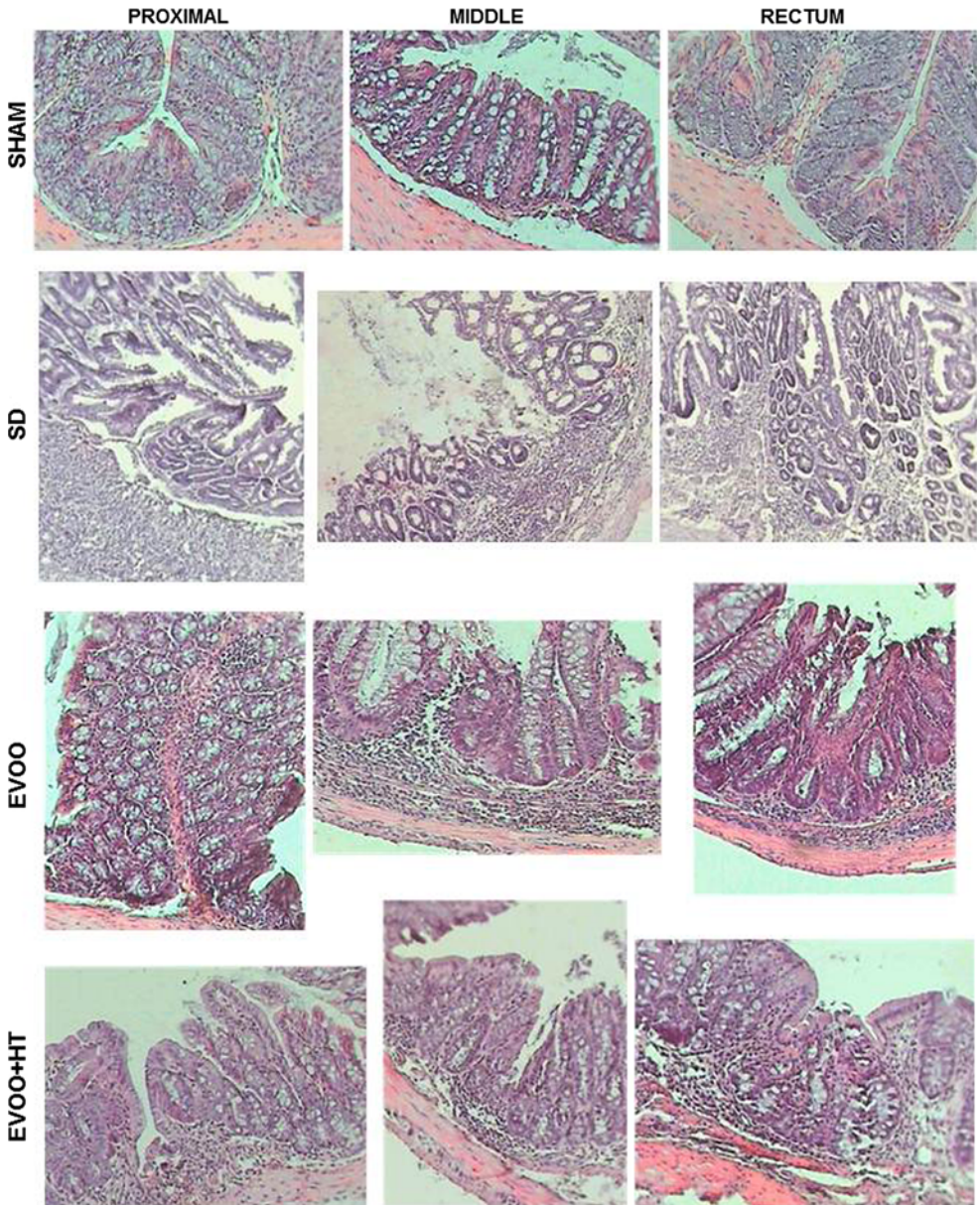

Fig. 3 Histopathology sections of proximal, middle and rectum of colonic lesions of mice fed with standard diet (SD), with extra virgin olive oil diet (EVOO) or supplemented with hydroxytyrosol diet (EVOO + HT) and treated with 3% dextran sodium sulphate (DSS) for 5 days followed by 3 weeks of water. Histology sections of sham animals fed with standard diet (SHAM); 3 animals per DSS-SD, EVOO and EVOO-HT groups, respectively. Haematoxylin and eosin stain. Original magnification ×20

animals fed with EVOO and EVOO plus hydroxytyrosol showed a higher and significant reduction in iNOS compared with EVOO alone in DSS group ($p < 0.01$; Fig. 5b).

We also examined the expression and activation of the p38 MAPK by western blot analysis using phosphospecific MAPK antibodies. To standardize protein loading in each line, blots were stripped and reprobed with the corresponding antibodies against p38 MAPK. Administration of DSS resulted in a significant increase in the phosphorylation of p38 MAPK protein ($p < 0.001$), indicating that the p38 MAPK protein activation could be induced at the

Table 3 Histological evaluation of DSS-induced colitis animals fed with standard diet (SD), extra virgin olive oil diet (EVOO) or supplemented with hydroxytyrosol diet (EVOO + HT)

Diet groups	Histological score		
	Proximal	Middle	Rectum
SD	1.0 ± 1.0	1.67 ± 0.67	3.67 ± 0.33
EVOO	0.33 ± 0.33	0.67 ± 0.33	1.33 ± 0.33
EVOO + HT	0 ± 0	0.33 ± 0.33	0.67 ± 0.33*

* $p < 0.05$ versus standard diet-DSS group

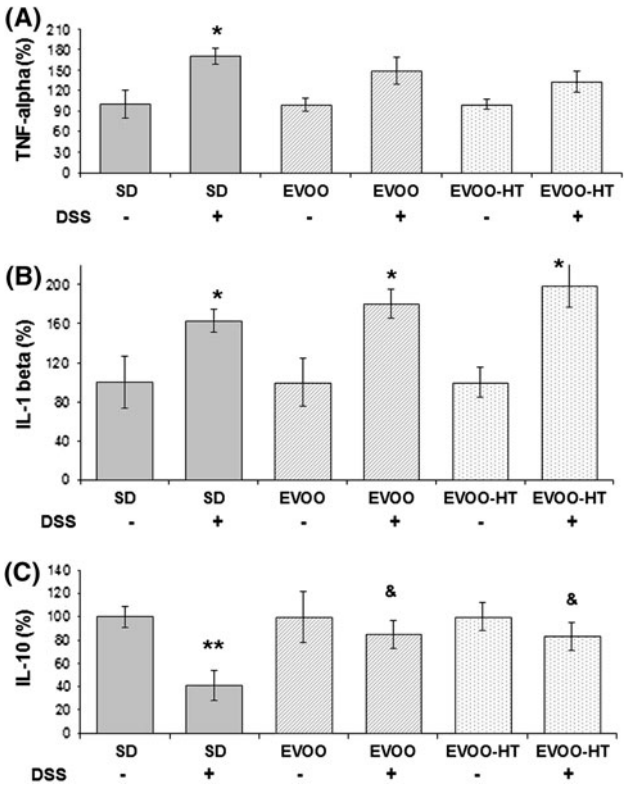

Fig. 4 Effect of extra virgin olive oil (EVOO) and supplemented with hydroxytyrosol (EVOO-HT) diets on TNF-α (**a**), IL-1β (**b**) and IL-10 (**c**) in the colon tissue after 3% of dextran sodium sulphate (DSS) for 5 days followed by 3 weeks of water (8 animals per each diet, 10 animals per DSS-SD group, 14 animals per DSS-EVOO group and 13 animals per DSS-EVOO-HT group). Data are expressed as percentage compared with sham-standard diet group and as the means ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus sham groups; $^{\&}p < 0.05$ versus DSS-standard diet (SD) group

chronic stage of colonic lesion caused by DSS. Our results from animals fed with EVOO and EVOO plus hydroxytyrosol and treated with DSS demonstrated a significant reduction in this activation versus animals fed with standard diet ($p < 0.01$; Fig. 5c).

Discussion

Recent epidemiological and clinical studies have confirmed that regular consumption of EVOO within the context of the Mediterranean diet is effective in preventing and treating certain diseases related to oxidative stress, inflammation and the immune system [15–19]. Numerous studies have demonstrated that the beneficial effects of EVOO consumption can be ascribed to minor phenol components such as flavonoids and secoiridoids oleuropein and their hydrolysis products such as hydroxytyrosol, which has demonstrated to be dose dependently absorbed in animals and humans [20]. Moreover, current knowledge denotes that hydroxytyrosol can fortify health [10].

Results from our study indicate that dietary administration of EVOO and EVOO enriched with hydroxytyrosol 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. There were no/few clinical signs and the mortality was reduced. An improvement in colon weight/length relationship and a reduced inflammatory response were also observed, especially with hydroxytyrosol supplement. This supplement showed significantly better results, demonstrating an anti-inflammatory effect in vivo by the polyphenol.

Our study is in agreement with those from other authors that documented the beneficial effect of olive oil diets in different experimental colitis models [21–23]. Our results are also coherent with studies from our investigation group that demonstrated that EVOO prevents the development of dysplasia and cancer after chronic inflammation [7]. In addition, although there are few designs of phenolic compounds at intestine level, the anti-inflammatory effects observed may be due to its antioxidant properties, since reactive oxygen species are significantly involved in this pathology. In this way, numerous studies on the biological activity of this phenol have demonstrated its great antioxidant and inherent radical scavenging activity [24–26]. Martin et al. [25] concluded that this molecule modulates signalling pathways involved in antioxidant/detoxifying enzyme regulation contributing to prevent oxidative stress-associated cell damage. More recently, a study has showed that hydroxytyrosol reduces DNA, lipid and protein damages and strengthens the intracellular antioxidant enzymes in protecting the peripheral blood mononuclear cells against oxidative stress [26].

Three weeks after DSS removal, an increase in proinflammatory cytokine production could be seen. Cytokines, such as IL-1 and TNF-α, share a multitude of pro-inflammatory properties and are crucial in the amplification of mucosal inflammation in IBD [1, 2]. In the last years, TNF-α has been the target of clinical investigations aimed at blocking its activity as a novel form of therapy for UC [27]. Our data showed that in DSS groups fed with EVOO diets, TNF-α values were maintained near to sham controls, which is in line with the aforementioned previous reports, where blocking TNF-α can inhibit the pro-inflammatory response. In addition, IL-10 is an immunoregulatory cytokine that influences the immunological system, both on the innate and on cell-mediated response. It affects the gastrointestinal mucosal homoeostasis through the down-regulation of colon inflammation and the inhibition of both antigen presentation and release of proinflammatory cytokines, and it is related to the activity of regulatory cells

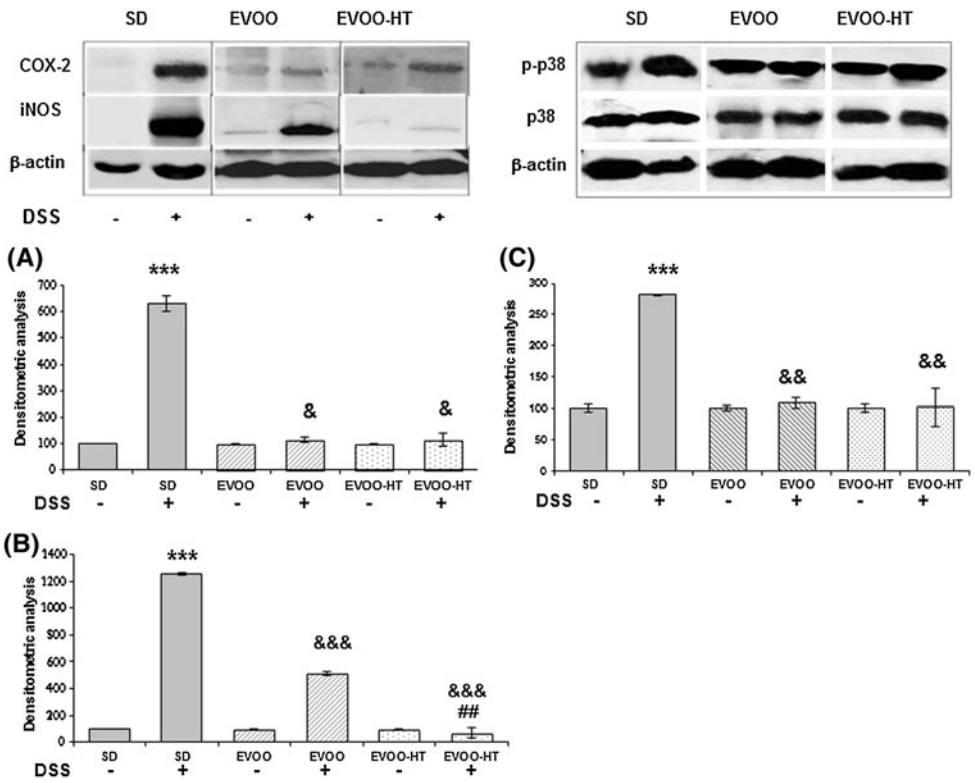

Fig. 5 Effect of extra virgin olive oil (EVOO) and supplemented with hydroxytyrosol (EVOO-HT) diets on the expression of COX-2 (**a**), iNOS (**b**) and p38 MAPK (**c**) using phosphospecific MAPK antibodies in the colon tissue after 3% of dextran sodium sulphate (DSS) for 5 days followed by 3 weeks of water; 4 animals per sham group; 4 animals per DSS-SD, EVOO and EVOO-HT groups, respectively. Data are expressed as the means ± SEM. $^{***}p < 0.001$ versus sham group; $^{\&}P < 0.05$, $^{\&\&}p < 0.01$ and $^{\&\&\&}p < 0.001$ versus DSS-standard diet (SD) group; $^{\#\#}p < 0.01$ versus DSS-EVOO diet group

[28]. In our model, the degree of inflammation and damage induced by DSS were paralleled to low levels of the anti-inflammatory cytokine IL-10 in standard diet group and significant high levels in EVOO diet groups. Therefore, increasing IL-10 in the groups fed with EVOO and EVOO supplemented with hydroxytyrosol may contribute to the improvement of the progression of colitis. Accordingly, the ability of EVOO to partially reduce the inflammatory process in the colon could be in part explained by the observed modulation in the levels of these cytokines, although a greater effect after supplementation with hydroxytyrosol could not be observed [13, 29].

COX-2 and iNOS are inflammatory proteins that play a pivotal role in mediating inflammation and contribute to DSS-induced inflammation [7, 13]. In this regard, COX-2 and iNOS activation produces excessive inflammatory mediators that may be detrimental to the integrity of the colon and contribute to the development of intestinal damage. Additionally, iNOS acts in synergy with COX-2 to promote the inflammatory reaction [30]. Our data support the concept that inhibitors of both inducible enzymes are effective anti-inflammatory agents, being an important mechanism to improve the UC development. After 3 weeks of DSS removal, EVOO diets reduced significantly both COX-2 and iNOS expression versus standard diet, where these proteins were appreciably increased compared to sham group. These results are also consistent with other experimental models where, for example, olive oil inhibited COX-2 immunostaining in IL-10 knockout mice [27], endogenously synthesized $n-3$ PUFAs downregulated COX-2 expression in DSS-induced colitis in *fat*-1 mice [31] or $n-3$ PUFA-rich diet showed beneficial effect via inhibition of COX-2 and iNOS expression, among other inflammatory parameters, in TNBS-induced colitic rats [32].

Moreover, a very interesting result has been a major downregulation of iNOS expression by hydroxytyrosol-enriched diet, being an important anti-inflammatory mechanism not described before in vivo. This result supports other in vitro studies, where hydroxytyrosol has showed an anti-inflammatory effect downregulating iNOS in human monocytic (THP-1) cells and in J774 murine macrophages stimulated with lipopolysaccharide [33, 34]. This effect suggests that hydroxytyrosol may represent a potential non-toxic agent for the control of inflammation in UC, strengthening the current knowledge concerning the main biological properties attributed to hydroxytyrosol.

Finally, p38 MAPK has emerged as a key modulator of several target genes controlling the infiltration of monocytic cells, acute intestinal inflammation and intestinal electrolyte and water secretion. Recent experiments have evidenced the importance of p38 MAPK in UC, since they have demonstrated the use of p38 MAPK inhibitors for abrogated colitis [13, 35, 36]. MAPKs regulate cytokine production in response to a variety of stimuli [37] and upregulate COX-2 and iNOS expression in intestinal

epithelial cells [38, 39]. Interestingly, our study reports, for the first time, a reduction in the expression of p-p38 MAPK by EVOO. In line with this finding, Corona et al. [40] showed that olive oil polyphenolic extract exerted chemopreventive effects by interacting with signalling pathways as the inhibition of p38 phosphorylation on human colon adenocarcinoma cells.

In summary, we have demonstrated that EVOO diet exerted a significant beneficial effect in chronic DSS-induced colitis. The anti-inflammatory effects seem to be related to a cytokine modulation and a reduction in COX-2 and iNOS expression in colonic mucosa, by downregulation of p38 MAPK pathway in addition to other possible mechanisms. Moreover, supplementation with the phenolic compound contributed to improve inflammatory response through iNOS downregulation plus its antioxidant capacity. We concluded that besides the beneficial effect by EVOO, supplementation of the diet with hydroxytyrosol may improve chronic colitis.

Acknowledgments The research was supported in part by grants from Ministerio de Ciencia y tecnología (AGL 2008-02475) and Junta de Andalucía (Spain). The authors are grateful to Teresa Lopez-Soto (Department of English Language, University of Seville) for revision of grammar and linguistic style.

Conflict of interest The authors have declared no conflict of interest.

References

1. Braus NA, Elliott DE (2009) Advances in the pathogenesis and treatment of IBD. Clin Immunol 132:1–9
2. Wei J, Feng J (2010) Signaling pathways associated with inflammatory bowel disease. Recent Pat Inflamm Allergy Drug Discov 4:105–117
3. Ortega RM (2006) Importance of functional foods in the Mediterranean diet. Public Health Nutr 9:1136–1140
4. Sirtori CR, Galli C, Anderson JW, Arnoldi A (2009) Nutritional and nutraceutical approaches to dyslipidemia and atherosclerosis prevention: focus on dietary proteins. Atherosclerosis 203:8–17
5. Servili M, Esposto S, Fabiani R, Urbani S, Taticchi A, Mariucci F, Selvaggini R, Montedoro GF (2009) Phenolic compounds in olive oil: antioxidant, health and organoleptic activities according to their chemical structure. Inflammopharmacol 17:76–84
6. Quiles JL, Ramirez-Tortosa MC, Yaqoob P (2006) Olive oil and health. CABI Publishing, Oxford
7. Sánchez-Fidalgo S, Villegas I, Cárdeno A, Talero E, Sánchez-Hidalgo M, Motilva V, Alarcón de la Lastra C (2010) Extra-virgin olive oil-enriched diet modulates DSS-colitis-associated colon carcinogenesis in mice. Clin Nutr 29:663–673
8. Vissers MN, Zock PL, Katan MB (2004) Bioavailability and antioxidant effects of olive oil phenols in humans: a review. Eur J Clin Nutr 58:955–965
9. Visioli F, Galli C (2002) Biological properties of olive oil phytochemicals. Crit Rev Food Sci Nutr 42:209–221
10. Granados-Principal S, Quiles JL, Ramirez-Tortosa CL, Sanchez-Rovira P, Ramirez-Tortosa MC (2010) Hydroxytyrosol: from laboratory investigations to future clinical trials. Nutr Rev 68:191–206
11. Melgar S, Karlsson L, Rehnström E, Karlsson A, Utkovic H, Jansson L, Michaëlsson E (2008) Validation of murine dextran sulfate sodium-induced colitis using four therapeutic agents for human inflammatory bowel disease. Int Immunopharmacol 8:836–844
12. González-Correa JA, Navas MD, Muñoz-Marín J, Trujillo M, Fernández-Bolaños J, de la Cruz JP (2008) Effects of hydroxytyrosol and hydroxytyrosol acetate administration to rats on platelet function compared to acetylsalicylic acid. J Agric Food Chem 56:7872–7876
13. Sánchez-Fidalgo S, Cárdeno A, Villegas I, Talero E, de la Lastra CA (2010) Dietary supplementation of resveratrol attenuates chronic colonic inflammation in mice. Eur J Pharmacol 633:78–84
14. Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R (1990) A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology 98:694–702
15. Motilva V, Sánchez-Fidalgo S, Calvo JR, Talero E (2008) Mechanism of increased gastric protection after NSAID-administration in rats consuming virgin olive oil diets. ESPEN 3:e9–e16
16. Perona JS, Montero E, Sánchez-Domínguez JM, Cañizares J, Garcia M, Ruiz-Gutiérrez V (2009) Evaluation of the effect of dietary virgin olive oil on blood pressure and lipid composition of serum and low-density lipoprotein in elderly type 2 diabetic subjects. J Agric Food Chem 57:11427–11433
17. Razquin C, Martinez JA, Martinez-Gonzalez MA, Mitjavila MT, Estruch R, Marti A (2009) A 3 years follow-up of a Mediterranean diet rich in virgin olive oil is associated with high plasma antioxidant capacity and reduced body weight gain. Eur J Clin Nutr 63:1387–1393
18. Konstantinidou V, Covas MI, Muñoz-Aguayo D, Khymenets O, de la Torre R, Saez G, Tormos Mdel C, Toledo E, Marti A, Ruiz-Gutiérrez V, Ruiz Mendez MV, Fito M (2010) In vivo nutrigenomic effects of virgin olive oil polyphenols within the frame of the Mediterranean diet: a randomized controlled trial. FASEB J 24:2546–2557
19. Barceló F, Perona JS, Prades J, Funari SS, Gomez-Gracia E, Conde M, Estruch R, Ruiz-Gutiérrez V (2009) Mediterranean-style diet effect on the structural properties of the erythrocyte cell membrane of hypertensive patients: the prevencion con dieta Mediterranea study. Hypertension 54:1143–1150
20. Khymenets O, Fitó M, Touriño S, Muñoz-Aguayo D, Pujadas M, Torres JL, Joglar J, Farré M, Covas MI, de la Torre R (2010) Antioxidant activities of hydroxytyrosol main metabolites do not contribute to beneficial health effects after olive oil ingestion. Drug Metab Dispos 38:1417–1421
21. Nieto N, Torres MI, Ríos A, Gil A (2002) Dietary polyunsaturated fatty acids improve histological and biochemical alterations in rats with experimental ulcerative colitis. J Nutr 132:11–19
22. Camuesco D, Gálvez J, Nieto A, Comalada M, Rodríguez-Cabezas ME, Concha A, Xaus J, Zarzuelo A (2005) Dietary olive oil supplemented with fish oil, rich in EPA and DHA ($n-3$) polyunsaturated fatty acids, attenuates colonic inflammation in rats with DSS-induced colitis. J Nutr 135:687–694
23. Hegazi RA, Saad RS, Mady H, Matarese LE, O'Keefe S, Kandil HM (2006) Dietary fatty acids modulate chronic colitis, colitis-associated colon neoplasia and COX-2 expression in IL-10 knockout mice. Nutrition 22:275–282
24. Angelino D, Gennari L, Blasa M, Selvaggini R, Urbani S, Esposto S, Servili M, Ninfali P (2011) Chemical and cellular antioxidant activity of phytochemicals purified from olive mill waste waters. J Agric Food Chem 59:2011–2018

25. Martín MA, Ramos S, Granado-Serrano AB, Rodríguez-Ramiro I, Trujillo M, Bravo L, Goya L (2010) Hydroxytyrosol induces antioxidant/detoxificant enzymes and Nrf2 translocation via extracellular regulated kinases and phosphatidylinositol-3-kinase/protein kinase B pathways in HepG2 cells. Mol Nutr Food Res 54:956–966
26. Ilavarasi K, Kiruthiga PV, Pandian SK, Devi KP (2011) Hydroxytyrosol, the phenolic compound of olive oil protects human PBMC against oxidative stress and DNA damage mediated by 2,3,7,8-TCDD. Chemosphere 84:888–893
27. Oussalah A, Danese S, Peyrin-Biroulet L (2010) Efficacy of TNF antagonists beyond one year in adult and pediatric inflammatory bowel diseases: a systematic review. Curr Drug Targets 11:156–175
28. Lindsay JO, Sandison A, Cohen P, Brennan FM, Hodgson HJF (2004) IL-10 gene therapy is therapeutic for dextran sodium sulfate-induced murine colitis. Dig Dis Sci 49:1327–1334
29. Martín AR, Villegas I, Sánchez-Hidalgo M, de la Lastra CA (2006) The effects of resveratrol, a phytoalexin derived from red wines, on chronic inflammation induced in an experimentally induced colitis model. Br J Pharmacol 147:873–885
30. Itzkowitz SH (2006) Molecular biology of dysplasia and cancer in inflammatory bowel disease. Gastroenterol Clin North Am 35:553–571
31. Gravaghi C, La Perle KM, Ogrodwski P, Kang JX, Quimby F, Lipkin M, Lamprecht SA (2010) Cox-2 expression, PGE(2) and cytokines production are inhibited by endogenously synthesized $n-3$ PUFAs in inflamed colon of fat-1 mice. J Nutr Biochem. doi: 10.1016/j.jnutbio.2010.03.003
32. Hassan A, Ibrahim A, Mbodji K, Coëffier M, Ziegler F, Bounoure F, Chardigny JM, Skiba M, Savoye G, Déchelotte P, Marion-Letellier R (2010) An α-linolenic acid-rich formula reduces oxidative stress and inflammation by regulating NF-κB in rats with TNBS-induced colitis. J Nutr 140:1714–1721
33. Maiuri MC, De Stefano D, Di Meglio P, Irace C, Savarese M, Sacchi R, Cinelli MP (2005) Carnuccio R Hydroxytyrosol, a phenolic compound from virgin olive oil, prevents macrophage activation. Naunyn Schmiedebergs Arch Pharmacol 371:457–465
34. Zhang X, Cao J, Zhong L (2009) Hydroxytyrosol inhibits pro-inflammatory cytokines, iNOS, and COX-2 expression in human monocytic cells. Naunyn Schmiedebergs Arch Pharmacol 379:581–586
35. Camacho-Barquero L, Villegas I, Sánchez-Calvo JM, Talero E, Sánchez-Fidalgo S, Motilva V, Alarcón de la Lastra C (2007) Curcumin, a Curcuma longa constituent, acts on MAPK p38 pathway modulating COX-2 and iNOS expression in chronic experimental colitis. Int Immunopharmacol 7:333–342
36. Docena G, Rovedatti L, Kruidenier L, Fanning A, Leakey NA, Knowles CH, Lee K, Shanahan F, Nally K, McLean PG, Di Sabatino A, MacDonald TT (2010) Down-regulation of p38 mitogen-activated protein kinase activation and proinflammatory cytokine production by mitogen-activated protein kinase inhibitors in inflammatory bowel disease. Clin Exp Immunol 162:108–115
37. Kyriakis JM, Avruch J (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 81:807–869
38. Kim JM, Jung HY, Lee JY, Youn J, Lee CH, Kim KH (2005) Mitogen-activated protein kinase and activator protein-1 dependent signals are essential for Bacteroides fragilis enterotoxin-induced enteritis. Eur J Immunol 35:2648–2657
39. Kim YA, Lim SY, Rhee SH, Park KY, Kim CH, Choi BT, Lee SJ, Park YM, Choi YH (2006) Resveratrol inhibits inducible nitric oxide synthase and cyclooxygenase-2 expression in beta-amyloid-treated C6 glioma cells. Int J Mol Med 17:1069–1075
40. Corona G, Deiana M, Incani A, Vauzour D, Dessì MA, Spencer JP (2007) Inhibition of p38/CREB phosphorylation and COX-2 expression by olive oil polyphenols underlies their anti-proliferative effects. Biochem Biophys Res Commun 362:606–611