

Effects of dietary virgin olive oil polyphenols: hydroxytyrosyl acetate and 3,4-dihydroxyphenylglycol on DSS-induced acute colitis in mice

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Received 21 April 2014; received in revised form 26 November 2014; accepted 2 December 2014

Abstract

Hydroxytyrosol, a polyphenolic compound from extra virgin olive oil (EVOO) has exhibited an improvement in a model of DSS-induced colitis. However, other phenolic compounds present such as hydroxytyrosyl acetate (HTy-Ac) and 3,4-dihydroxyphenylglycol (DHPG) need to be explored to complete the understanding of the overall effects of EVOO on inflammatory colon mucosa. This study was designed to evaluate the effect of both HTy-Ac and DHPG dietary supplementation in the inflammatory response associated to colitis model. Six-week-old mice were randomized in four dietary groups: sham and control groups received standard diet, and other two groups were fed with HTy-Ac and DHPG, respectively, at 0.1%. After 30 days, all groups except sham received 3% DSS in drinking water for 5 days followed by a regime of 5 days of water. Acute inflammation was evaluated by Disease Activity Index (DAI), histology and myeloperoxidase (MPO) activity. Colonic expression of iNOS, COX-2, MAPKs, NF- κ B and FOXP3 were determined by western blotting. Only HTy-Ac-supplemented group showed a significant DAI reduction as well as an improvement of histological damage and MPO. COX-2 and iNOS protein expression were also significantly reduced. In addition, this dietary group down-regulated JNK phosphorylation and prevented the DSS-induced nuclear translocation level of p65. However, no significant differences were observed in the FOXP3 expression. These results demonstrated, for the first time, that HTy-Ac exerts an antiinflammatory effect on acute ulcerative colitis. We concluded that HTy-Ac supplement might provide a basis for developing a new dietary strategy for the prevention of ulcerative colitis.

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Keywords: Hydroxytyrosyl acetate; 3,4-dihydroxyphenylglycol; Inflammation; Ulcerative colitis; NF- κ B

1. Introduction

Epidemiological data suggest an association between inflammatory bowel disease (IBD) and a number of environmental factors, such as antibiotic use, microbial exposure both early and late in life and, possibly, diet. Although the etiology of IBD remains largely unknown, it involves a combination of a patient's genetic, microbiome, immune response and the environment that result in an excessive and abnormal immune response against commensal flora in genetically susceptible individuals [1]. Various components of the mucosal immune system are implicated in the pathogenesis of IBD and include intestinal epithelial cells, innate lymphoid cells, cells of the innate (macrophages/monocytes, neutrophils and dendritic cells) and adaptive (T-cells and B-cells) immune system and their secreted mediators (cytokines and chemokines) [2]. Besides, evidences have been accumulated regarding the essential roles of regulatory T cells (Tregs), which are characterized

by the expression of Foxp3, regulating and suppressing potentially aggressive immune responses toward autoantigens and commensal enteric antigens in IBD [3,4].

Increase in inflammatory mediators and up-regulation of certain proteins, that is, cyclooxygenase (COX)-2 or inducible nitric oxide synthase (iNOS), also plays an important role in immune dysregulation in IBD [5]. Signaling pathways such as mitogen-activated protein kinases (MAPKs) are also implicated by leading to the activation of nuclear transcription factors. Among them, nuclear factor-kappa B (NF- κ B) takes part controlling the activation of various proinflammatory cytokine genes supporting a critical role in the pathogenesis of ulcerative colitis [6].

Although the knowledge of this pathology is in progress, new therapeutic strategies continue being investigated. In this sense, the interest by dietary supplements and nutraceuticals without undesirable effects that accompany the classical pharmacotherapy is growing. In fact, current epidemiological and experimental studies support a beneficial role of dietary polyphenols in several gastrointestinal diseases, including IBD [7–9].

Actually, the phenolic compounds from virgin olive oils have shown antiinflammatory, antioxidant, antimicrobial, antiproliferative,

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antiarrhythmic, platelet antiaggregant and vasodilator effects, as well as the ability to modulate important cellular signaling pathways [10–13].

Extra virgin olive oil is rich in a variety of phenolic compounds, mainly constituted by secoiridoid derivatives of 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol, HTy) and of 2-(4-hydroxyphenyl)ethanol (tyrosol), and hydroxytyrosyl acetate (HTy-Ac), along with minor amounts of free HTy [14]. Partial hydrolysis of secoiridoid derivatives and HTy-Ac during gastric and intestinal digestion has been extensively studied [15,16], which increases HTy concentration at the colonic level. Therefore, extensive investigation has focused on HTy as a chronic disease preventive agent. In fact, HTy has been extensively studied demonstrating cardioprotective, antiinflammatory, antiplatelet aggregation, antimicrobial and anticancer activities [17]. More concretely, dietary HTy supplement has exhibited an improvement in an *in vivo* model of DSS-induced colitis [18]. However, other phenolic compounds present in virgin olive oil such as HTy-Ac and 3,4-dihydroxyphenylglycol (DHPG) need to be explored in depth to complete the understanding of the overall effects of virgin olive oil in the colon.

To date, HTy-Ac has shown protection effects against oxidative DNA damage in blood cells [19] as well as against iron-induced oxidative stress in human cervical cells (HeLa) [20]. In addition, a study from González-Correa et al. [21] showed a neuroprotective effect of HTy-Ac in a model of hypoxia–re-oxygenation in rat brain slices, both *in vitro* and after oral administration. More recently, a greater antiplatelet aggregating activity than HTy has also been demonstrated [22]. On the other hand, DHPG presented higher antioxidant and antiradical capacities and reducing power than HTy and prevented lipid peroxidation to a degree that was comparable with vitamin E [23]. Furthermore, DHPG is bioavailable and has antioxidant properties in vitamin E-deficient rats [24]. It may also protect against platelet activation and adhesion and possibly has antiinflammatory properties [25].

Taken this background into account, the present study was designed to evaluate the effects of both HTy-Ac and DHPG dietary supplementation, on an acute colitis model induced by DSS in mice. Inflammatory response was assessed by histology and myeloperoxidase (MPO) activity, as an index of quantitative inflammation and neutrophil infiltration in the mucosa. Protein expression of COX-2 and iNOS was evaluated by western blot. Moreover, we studied the role of MAPKs, NF- κ B and FOXP3 signaling pathways involved in the beneficial effects after dietary treatments in colonic mucosa under early acute inflammatory conditions.

2. Material and methods

2.1. General chemical procedures

^1H (300MHz) and ^{13}C (75.5MHz) NMR spectra were recorded on Bruker Avance-300 spectrometer. Mass spectra (EI, CI) were recorded on a Micromass AutoSpec-Q mass spectrometer. IR spectra were recorded on Jasco FT/IR-4100 spectrometer. TLC was performed on silica-coated aluminium sheets (E. Merck Silica gel 60 F₂₅₄), spots were visualized by UV light and by staining with vanillin/H₂SO₄ in EtOH (1.5 g vanillin in 100 ml 95% EtOH/conc. H₂SO₄ 100:1). Column chromatography was performed using E. Merck Silica Gel 60 (63–200 μm). All reactions were carried out in dry solvents, under argon and in darkness. HTy used as starting material was provided as an ethanol/water 1:4 solution at 6.3% (p/v) (purity degree 97%, HPLC) obtained from olive mill wastewaters [26].

2.2. Hydroxytyrosyl acetate, HTy-Ac (2)

To a solution of HTy (1) (540 mg, 3.50 mmol) in EtOAc (50 ml), Amberlite IR-120(H+) resin (2.0 g) was added. The mixture was refluxed under Ar in the dark, for 11 h, filtered and concentrated to dryness. Column chromatography (hexane→1:2 EtOAc–hexane) gave two (510 mg, 74%). HRCI-MS *m/z* calcd for C₁₀H₁₂O₄ [M]⁺: 196.0736, found 196.0742.

2.3. (3,4-Dihydroxyphenyl)glycol, DHPG (7)

A suspension of (6) (204 mg, 0.60 mmol) in 1-M aqueous H₂SO₄ (10 ml) was stirred at rt for 24 h. After neutralization with solid NaHCO₃, the mixture was concentrated to

dryness. Column chromatography (30:1→10:1 CH₂Cl₂–MeOH) gave seven (56 mg, 55%) as a syrup. HREI-MS *m/z* calcd for C₈H₁₀O₄ [M]⁺: 170.0579, found: 170.0587.

2.4. Animals and diets

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 of 12 L/12 D). After weaning mice were randomized into three dietary groups: standard diet (SD) and SDs supplemented with HTy-Ac and DHPG, respectively, at 0.1%. Animals were fed with the standard and supplemented diets during all the experimental study until sacrifice. Treatments with HTy-Ac and DHPG were administered for all study as a syrup form included in a SD, supplemented with the concentration indicated above. Fresh diets were provided daily and protected from light. Animals from dietary groups consumed an average of 4 g/day of diet, resulting in an estimated intake of about 4 mg of HTy-Ac or DHPG per mice. This dosage is about 20 μM of both polyphenols, and it is in accordance with the literature consulted [27]. Body weight, food and water intake were evaluated weekly. The protocol for animal handling and experimentation was in accordance with the European Union European Community guidelines for the ethical treatment of animals (2010/63/UE) and was approved by the Ethical Committee for Animal Research of the University of Seville.

2.5. Induction of colitis

Colitis was induced according to the procedure described by Melgar et al. [28]. After 4 weeks of dietary treatments, 12 animals from each dietary group received 3% DSS (DSS group; MW: 40000, ICN Pharmaceuticals, Costa Mesa, CA, USA) in drinking water for 5 days followed by a regime of 5 days of water, reflecting acute inflammation. Control healthy mice (sham group from each diet) were allowed to drink only water. Then, animals were sacrificed by an overdose of ip chloral hydrate.

2.6. 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. [28] with slight modifications [18] (Table 1). The presence of diarrhea, rectal bleeding and weight loss was 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 following 5 days when the animals were on a pure water. The average of the three values constituted the DAI.

2.7. Macroscopic and histopathological evaluation

At the end of the experimental period, the colons were removed, slightly cleaned in physiological saline to remove fecal residues, weighed and measured in order to evaluate variations in the weight/length as an inflammation index. The results were expressed in percentage respect to control sham group.

Samples of rectum were excised out of every segment, fixed in 4% buffered formaldehyde, dehydrated by increasing concentrations of ethanol and embedded in paraffin. Four-micrometer-thick slices from paraffin sections were stained with haematoxylin and eosin in accordance with the standard procedures for histological evaluation of colonic damage. The rest of the pieces of the colon were collected and frozen in liquid nitrogen to measure biochemical parameters.

For DSS colitis, distal colonic section of each animal were scored by a pathologist who was unaware of the experimental protocol using a colitis score as previously was described [29]. 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 2). The sum of the 3 category scores adds up to the total score.

2.8. Assessment of leukocyte involvement

MPO activity was assessed as a marker of neutrophil infiltration according to the methods of Grisham et al. [30] with slight modifications. In all animals, one sample from the distal colon was obtained. Samples were excised from each animal and rapidly rinsed with ice-cold saline, blotted dry and frozen at 70°C. The tissue was thawed, weighed and homogenized in 10 volumes of 50-mM PBS, pH 7.4. The homogenate was centrifuged at 20,000 g 4°C. The pellet was again homogenized in 10 volumes of 50-mM PBS, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide (HETAB) and 10-mM ethylenediamine tetraacetic acid (EDTA). This homogenate was subjected to one cycle of freezing/thawing and a brief period of sonication. The homogenate were diluted in 50 volumes of 50mM PBS, pH6. Then it was added consecutively 50 ml of odianisidine dihydrochloride (0.067%), of HETAB (0.5%) and of hydrogen peroxide (0.003%). Each well containing the complete reaction mixture was incubated for 5 min in darkness. The changes in absorbance at 450 nm were measured with a microplate reader (Labsystem Multiskan EX, Helsinki, Finland). Results were quantified as U MPO/mg tissue.

2.9. Isolation of cytoplasmic and nuclear proteins and immunoblotting detection

Frozen colonic tissues were processed as described by Sánchez-Hidalgo *et al.* [31] in order to isolate cytoplasmic or nuclear proteins. Protein concentration of the homogenate was determined following Bradford's colorimetric method [32]. Aliquots of supernatant that contain equal amount of protein (50 µg) were separated on 10% acrilamide gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In the next step, the proteins were electrophoretically transferred onto a nitrocellulose membrane and incubated with specific primary antibodies: rabbit polyclonal anti-COX-2 and anti-iNOS (Cayman Chemical, USA) at dilution of 1:3000, rabbit polyclonal anti-IκBα (Cell Signalling Technology, USA) at dilution of 1:1000, rabbit polyclonal p65 (Santa Cruz Biotechnology, Inc.) at dilution of 1:200, mouse polyclonal anti-JNK and anti-pJNK (Santa Cruz Biotechnology, Inc.) at dilution of 1:1000, and mouse polyclonal anti-p38 and anti-p38 (Santa Cruz Biotechnology, Inc.) at dilution of 1:1000 and 1:1000, respectively, and rabbit polyclonal anti-FOXP3 (Santa Cruz Biotechnology, Inc.) at dilution 1:500, overnight at 4°C. After that, each filter was washed three times for 15 min and incubated with a horseradish peroxidase-labeled secondary antibody antirabbit (Pierce Biotechnology, IL, USA) or antimouse (Dako Cytomation, USA) containing blocking solution for 1–2 h at room temperature. To prove equal loading, the blots were analyzed for *b*-actin expression using an anti-*b*-actin antibody (Sigma–Aldrich, MO, USA). Immunodetection was performed using enhanced chemiluminescence light-detecting kit (SuperSignal West Femto Chemiluminescent Substrate, Pierce, IL, USA). 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 (Image J, Softonic, USA).

2.10. Statistical analysis

All values in the figures and text are expressed as arithmetic means ± standard error (S.E.M.). Data were evaluated with 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, using Tukey–Kramer multiple comparisons test as post hoc test. *P* values of <0.05 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. Chemistry

The synthesis of HTy-Ac (2) has been carried out in a 74% yield by regioselective acid catalyzed acetylation of HTy (1) by refluxing in EtOAc in the presence of Amberlite IR-120(H⁺) resin (Fig. 1), a proprietary procedure [33]. A previous report describes the use of TsOH in AcOEt, followed by neutralization [34].

We also describe the first synthesis of naturally occurring DHPG (7). We have used commercial and unexpensive phenacyl chloride (3) as starting material (Fig. 1). Treatment of (3) with a mixture of NaOAc in Ac₂O at 140°C yielded (4) in a slight modification of the procedure described by Tozuka *et al.* [35]. The catalytic hydrogenation of (4) in the presence of Ac₂O afforded a mixture of a mixture of tri and tetra-*O*-acetylated derivatives (5) and (6). Conventional acetylation of this mixture gave (6) in 80% yield from (3). Deacetylation of (6) carried out in diluted sulfuric acid, and afforded glycol (7) after chromatographic purification in 44% overall yield from (3).

3.2. Effects of HTy-Ac and DHPG supplementation on DSS-induced acute colitis

In this experimental model, loss of body weight, not formed stool and rectal bleeding were observed in all animals DSS treated. Significant loss of body weight was observed from fifth day of DSS treatment and after DSS removal. However, mice fed with HTy-Ac-supplemented diet, but no DHPG, were protected from marked body weight loss (*P*<0.001 vs. DSS control group) (Fig. 2A). In the same way, DAI was improved significantly by dietary HTy-Ac since fifth day of DSS treatment until the sacrifice of the animals (*P*<0.001 vs. DSS group); however, it was aggravated in dietary DHPG group (*P*<0.001 vs. DSS group) (Fig. 2B).

After animals were sacrificed, a significant decrease of length of the mice colon (a morphological parameter useful for assessing colonic inflammation) was observed in DSS-treated group versus respective

sham control diet group (*P*<0.05). HTy-Ac-supplemented diet, but no DHPG, significantly prevented the colon shortening, since there was no significant difference with sham group (Fig. 2C).

In view of the results obtained, the histological and biochemical determinations only were carried out with the group of animals treated with the dietary HTy-Ac supplement, which presented the best clinical and macroscopic data after DSS treatment.

3.3. Histopathological analysis of DSS-induced acute colitis after HTy-Ac supplementation

The histological evaluation of colonic tissue from healthy animals revealed a normal structure without histological changes. By contrast, mice with DSS-induced colitis exhibited disruption of the epithelial barrier, a pronounced decrease in the number of crypts and marked infiltration of inflammatory cells into the mucosa and submucosa, overcoat in rectum sections (Fig. 2E). In contrast, slides from the group of animals treated with DSS but fed with HTy-Ac supplement revealed reduced signs of inflammation into the colonic tissue and a minor extent affected mucosa with moderate loss of epithelial cells. Moreover, a preservation of the glandular structure or a regeneration of crypts and reepithelialization in most of the area could be observed (Fig. 2F).

In addition, quantification of the results by histological scoring revealed a significant increased score in DSS group, which reached 27.5 ± 5.75, whereas the mean score in HTy-Ac-treated mice was significantly lower (*P*<0.01 vs. DSS control group) (Fig. 2G).

3.4. MPO was decreased after dietary HTy-Ac supplementation

As shown in Fig. 3, a marked increase in MPO activity, as an indicator of colonic infiltration with polymorphonuclear leukocytes, also characterized the colitis caused by DSS in control group (*P*<0.001 vs. sham group). Fed DSS-treated mice with HTy-Ac-supplemented diet significantly reduced the degree of polymorphonuclear neutrophil infiltration (*P*<0.05 vs. DSS control group). These results are consistent with histological findings.

3.5. COX-2 and iNOS protein expression inhibition in colon tissue after HTy-Ac-supplemented diet in DSS-induced acute colitis

The levels of inflammatory proteins expression were measured by western blotting of cytosolic extracts from colonic mucosa. As shown in Fig. 4, COX-2 and iNOS were significantly expressed in colonic samples from control group (*P*<0.05) after acute colitis induced by DSS. Interestingly, preventive treatment with HTy-Ac-supplemented diet significantly blocked the up-regulation of both iNOS and COX-2 immunosignals in colonic tissue (*P*<0.05 vs. control DSS group).

3.6. Effects of dietary HTy-Ac supplementation on activation of MAPK signaling in DSS-induced acute colitis

MAPKs transmit extracellular inflammatory signals into intracellular responses. These molecules play a key role in inducing gene expression which initiates inflammatory responses. Effects of HTy-Ac supplementation on the DSS-induced colitis activation of MAPKs were studied by western blot analysis using phosphospecific MAPKs antibodies in order to determinate their potential implications. To standardize protein loading in each line, blots were stripped and reprobed with the corresponding antibodies against nonactivate MAPKs proteins. DSS was found significantly to activate p38 and JNK proteins (*P*<0.05 vs. sham group), indicating that both MAPKs protein activation are induced at the acute stage of colonic lesion. After preventive dietary treatment HTy-Ac, our results demonstrated that both MAPKs were reduced, although significant changes were only

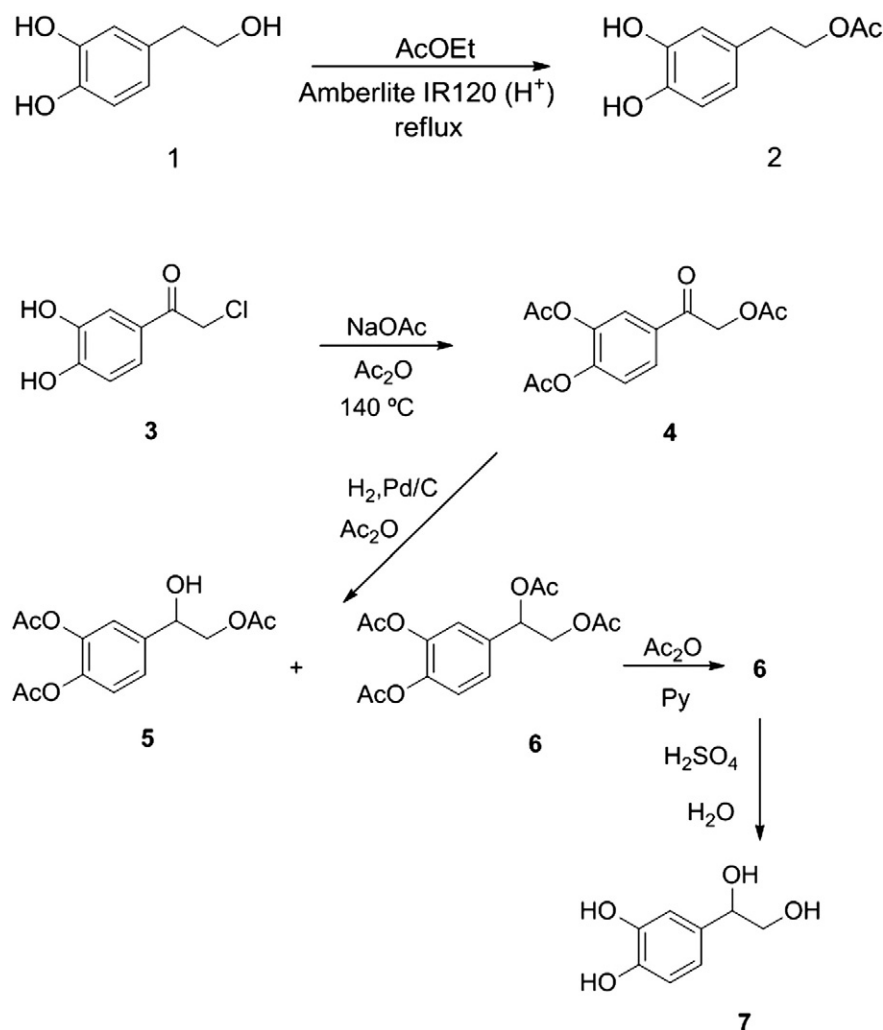


Fig. 1. Synthesis of HTy-Ac (2) from hydroxytyrosol and synthesis of DHPG (7) from 3,4-dihydroxyphenacyl chloride (3).

obtaining in the phosphorylation of JNK ($P < 0.05$ vs. DSS-control group) (Fig. 5).

3.7. Effects of dietary HTy-Ac supplementation on NF- κ B in DSS-induced acute colitis

NF- κ B is a crucial transcription factor which mediates transcriptional activation of many inflammatory genes. According to the results obtained, nuclear protein extracts of colon tissues showed that the nuclear translocation levels of p65 protein were increased in animals treated with DSS ($P < 0.05$ vs. sham group) (Fig. 6). However, animals fed with dietary HTy-Ac prevented the DSS-induced nuclear translocation level of p65 in colonic mucosa ($P < 0.01$ vs. DSS control group) (Fig. 6). In addition, the DSS treatment induced a significant cytosolic I κ B α degradation ($P < 0.01$ vs. sham group), which was consistent with NF- κ B-binding activity up-regulation; on the contrary, HTy-Ac supplementation was able to block the activation of NF- κ B pathway ($P < 0.05$ vs. DSS control group) (Fig. 6).

3.8. Effects of dietary HTy-Ac supplementation on FOXP3 in DSS-induced acute colitis

Next, we postulated that HTy-Ac supplementation could modulate the Tregs, which are characterized by the expression of FOXP3.

The results showed that it was reduced in DSS group and administration of dietary HTy-Ac resulted in the induction of its FOXP3 expression, although no significant differences were observed (Fig. 7).

4. Discussion

Previous data from our research group demonstrated, for the first time, that dietary extra virgin olive oil (EVOO) polyphenol extract (PE) supplementation possessed marked protective effects on experimental colitis [11]. Moreover, these improved effects observed could be due to a possible synergistic effect among EVOO constituents, since it is not clear whether all the possible beneficial mechanisms act independently of each other or whether they have a synergistic or competitive action. Based on this background, our research group previously demonstrated that a diet made with EVOO enriched with HTy could improve inflammatory response [18]. However, no reports are available regarding the evaluation of other bioavailable olive oil polyphenols such as HTy-Ac and DHPG in inflammation and particularly in acute colitis induced in mice.

Our results revealed for the first time that dietary administration of HTy-Ac but not DHPG was able to improve the clinical signs of the disease ameliorating the first stage in a model of ulcerative colitis in C57BL/6 mice. These data suggest that the presence of the ester group may contribute significantly to the above-reported effects.

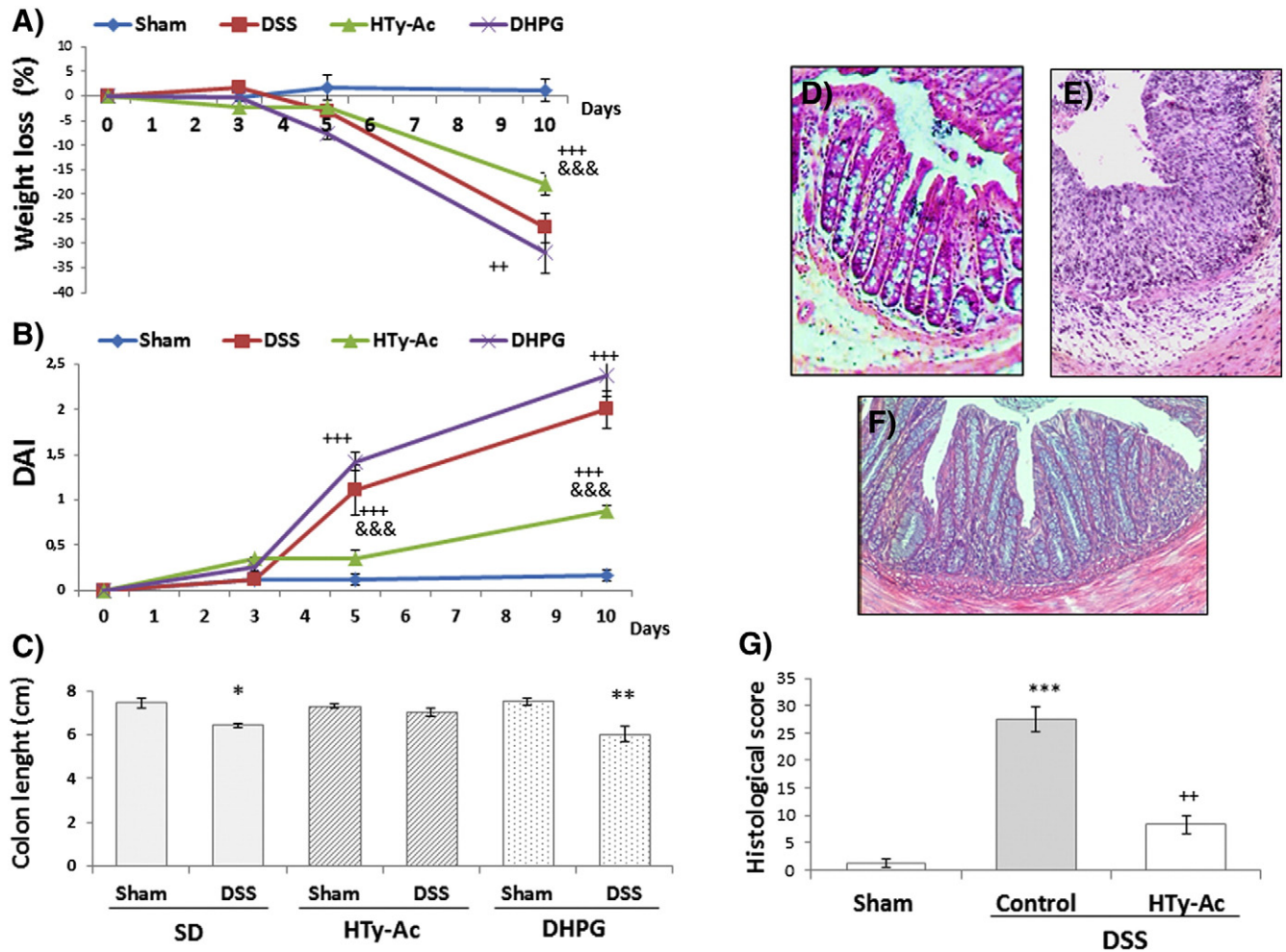


Fig. 2. Effect of dietary HTy-Ac and DHPG supplementation, polyphenols from olive oil, on DSS-induced acute colitis. Mice received 3% DSS for 5 days and drinking water for the next 5 days. Animals (sham and DSS) were fed with SD and 0.1% HTy-Ac and DHPG enrichment diet, respectively. (A) Body weight loss; (B) DAI evaluated as average of score of clinical parameters as body weight changes, rectal bleeding and stool consistency; (C) length of the colon of animals; (D,E,F) representative histological sections of colon from sham, control DSS and HTy-Ac dietary groups, respectively were examined microscopically after H&E staining with original magnification $\times 100$; (G) histological analysis using a colitis score as previously described by Dieleman *et al.* (1998). Data are reported as means \pm S.E.M. (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$ versus sham group; (++) $P < 0.01$ and (++++) $P < 0.001$ versus DSS control group; (&&&) $P < 0.001$ versus DHPG group.

HTy-Ac was described for the first time in olive oil by Brenes *et al.* [36] and is found in most Spanish virgin olive oils. Moreover, recently, it was reported by Mateos *et al.* [37] that HTy-Ac is more soluble in the lipophilic phases than HTy, due to the presence of the ester group, which was demonstrated in a Caco-2 cell model. Thus, this increased

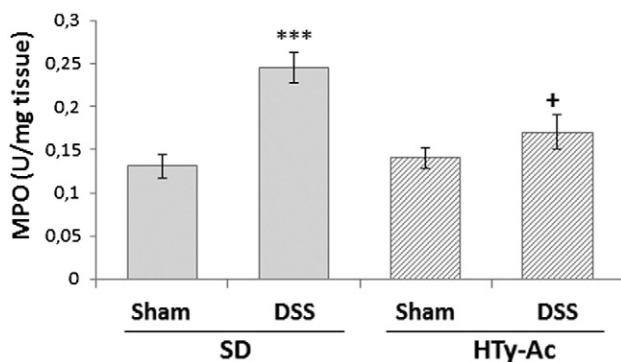


Fig. 3. Effect of dietary HTy-Ac supplementation on MPO activity in mice DSS-induced acute colitis. Data are expressed as mean \pm S.E.M. (***) $P < 0.001$ versus control sham group; (+) $P < 0.05$ versus DSS control group.

lipophilicity means that HTy-Ac is better absorbed across intestinal epithelial cell monolayers than free HTy [38]. Moreover, high digestive stability, evaluated by a simulated digestion procedure, has been shown with HTy-Ac [16].

Our results demonstrated that dietary HTy-Ac supplementation improved histological signs of inflammation and reduced MPO values as index of neutrophil infiltration, down-regulated inflammatory COX-2 and iNOS proteins, as well as inhibited the JNK activation in mice colonic tissue when compared with DSS control group. Moreover, HTy-Ac blocked I κ B degradation and induced an inhibition of NF- κ B activation, and although no significant changes in FOXP3 were observed, there was a tendency to maintain the levels of its expression next to healthy control.

It is already established that inducible enzymes COX-2 and iNOS are inflammatory proteins predominantly expressed at sites of inflammation and their activation may affect to colon mucosa integrity and contribute to the development of intestinal damage [8,39]. In this regard, COX-2 activation produces excessive PGE₂ and TXB₂, which are important inflammatory mediators that contribute to the intestinal hiperemia edema and even dysfunction, and iNOS activation leads to excessive production of NO which may be detrimental to the integrity of the colon based on the generation of reactive nitrogen species

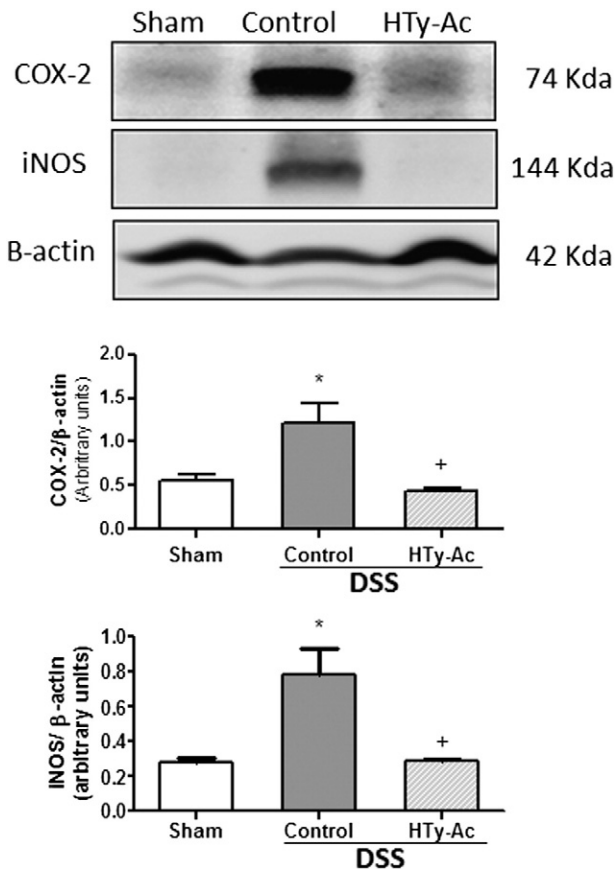


Fig. 4. Effect of dietary HTy-Ac supplementation on the COX-2 and iNOS expression in the colon tissue after 3% DSS for 5 days followed by 5 days of water. Densitometry was performed following normalization to the control (β -actin housekeeping gene). Data are expressed as the means \pm S.E.M. (*) $P < 0.05$ versus control sham group; (+) $P < 0.05$ versus DSS control group.

causing cellular degeneration in various tissues and contributing to the development of intestinal damage [40]. In addition, iNOS acts in synergy with COX-2 to promote the inflammatory reaction [41]. Furthermore, both COX-2 and iNOS expression are up-regulated by MAPKs and NF- κ B and AP-1 nuclear transcription factors in intestinal epithelial cells [42]. Our data are in agreement with previous reports where other olive oil polyphenols such as HTy [18] and oleuropein [43] could reduce inflammatory protein expression in ulcerative colitis. Thus, our results suggest that both proinflammatory proteins represent a potential molecular target susceptible to HTy-Ac modulation, which has not been demonstrated previously.

An important potential target for antiinflammatory therapeutic is the regulation of the inflammation mediators' synthesis at the transcription level and translation. Among them, MAPKs are kinases serine/threonine protein families that are implicated in regulate cytokine production [9,18,44] and COX-2 and iNOS up-regulation in intestinal epithelial IBD patients cells [45]. In addition to controlling the activity of leukocytes, MAPKs play a crucial role in the control of the activity of nonimmune cells suggesting that their blockade could offer a molecular target for blockade of leukocyte recruitment to the intestine [46]. Our study showed that phosphorylation of p38 and JNK MAPKs were increased in acute phase of colitis and HTy-Ac-supplemented diet reduced significantly JNK activation, although no changes in p38 phosphorylation were observed.

One of the well-studied transcription factors downstream of MAPKs signaling is the nuclear factor NF- κ B, which has been established that is activated in mucosal cells on DSS-induced ulcerative colitis mice

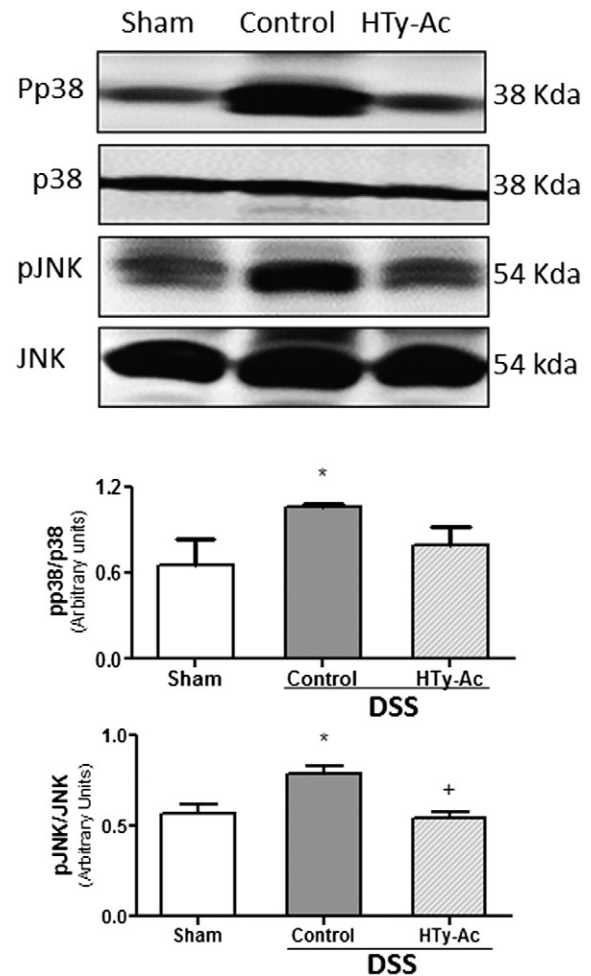


Fig. 5. Effect of dietary HTy-Ac supplementation on p-p38 and p-JNK activation in the colon tissue after 3% DSS for 5 days followed by 4 days of water. Densitometry was performed following normalization to the control (p38, JNK housekeeping genes, respectively). Data are expressed as the means \pm S.E.M. (*) $P < 0.05$ versus control sham group; (+) $P < 0.05$ versus DSS control group.

[9,11,47]. Phosphorylation of I κ B α generally leads to the degradation of I κ B α , with the concomitant release of NF- κ B and its nuclear translocation, which binds to the promoter of many genes that are important for the activation of immune responses as iNOS and COX-2 among others [42,48]. Dietary HTy-Ac supplement prevented the I κ B α degradation, which inhibited the NF- κ B activation and the subsequent COX-2 and iNOS proteins expression reduction, contributing to a minimization on the development and maintenance of intestinal inflammation. These data provide further evidence in the role of NF- κ B pathway in the beneficial effects of HTy-Ac in colitis. This is in accordance with other polyphenols, for example dietary treatment with ellagic acid has shown an NF- κ B-p65 reduction, contributing significantly in the beneficial effect on colitis [47].

Dysfunction of the Treg suppressive mechanism is presumed to be causative of autoimmune and immunopathological diseases [49]. Clinical and experimental studies have shown that the down-regulation of the established inflammatory response and the induction and maintenance of Tregs functions could ameliorate IBD [50–52]. Our results show that DSS treatment reduced the FOXP3 expression, a marker for Tregs, in the colon tissue. Although there is no significant changes, HTy-Ac improved its expression indicating that up-regulation of Tregs response could be another mechanism for the beneficial effect of HTy-Ac on colitis.

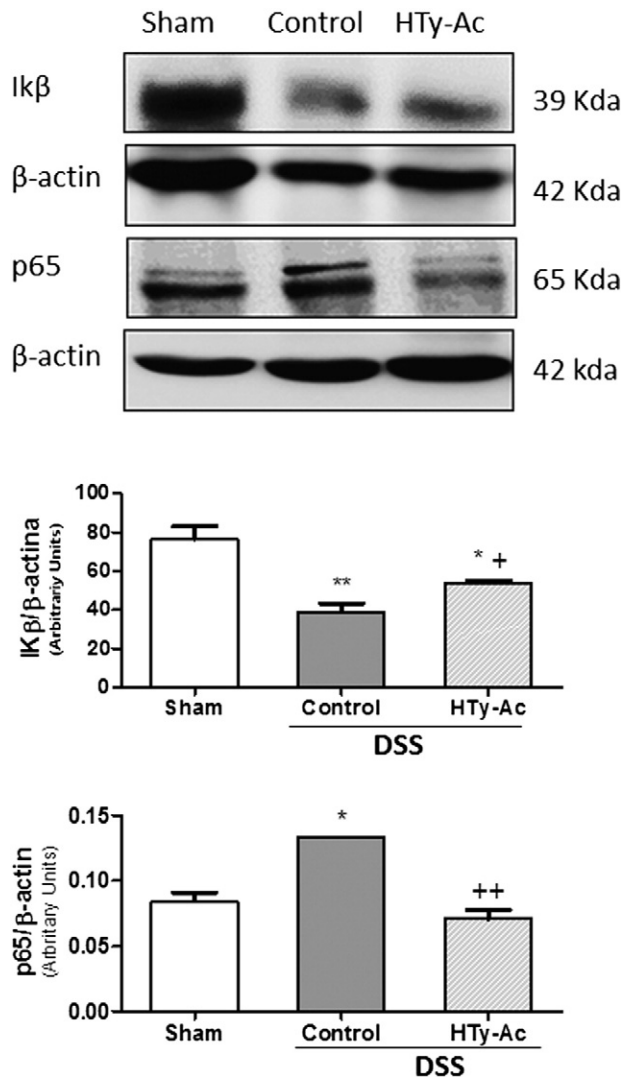


Fig. 6. Effect of dietary HTy-Ac supplementation on IkBα degradation and NF-κB p65 in colonic mucosa after 3% DSS for 5 days followed by 5 days of water. Densitometry was performed following normalization to the control (β-actin housekeeping gene). Data are expressed as the means±S.E.M. (*) $P<0.05$ and (**) $P<0.01$ versus control sham group; (+) $P<0.05$ and (++) $P<0.01$ versus DSS control group.

In summary, the present results show for the first time that HTy-Ac, but no DHPG, two polyphenols present in olive oil, improves DSS-induced acute ulcerative colitis. This beneficial effect involves a decrease in COX-2 and iNOS expression probably through JNK MAPK and NF-κB signaling pathways. The present findings, together with the reported antioxidant effect of HTy-Ac [53], suggest the need for further studies of the effects of HTy-Ac on the suite of mechanisms that regulate inflammation. We concluded that HTy-Ac supplement might provide a basis for developing a new dietary strategy for the prevention of ulcerative colitis.

Acknowledgments

The research was supported by grants from Ministerio de Ciencia y Tecnología (AGL2008-02475), Ministerio de Economía y Competitividad (AGL 2011-26949) and Junta de Andalucía (AGR-6609 and P08-AGR-03751), Spain. A.M. also thanks the Junta de Andalucía for the award of a fellowship. The authors gratefully acknowledge the assistance of Center for Technology and Innovation Research, University of Seville (CITIUS).

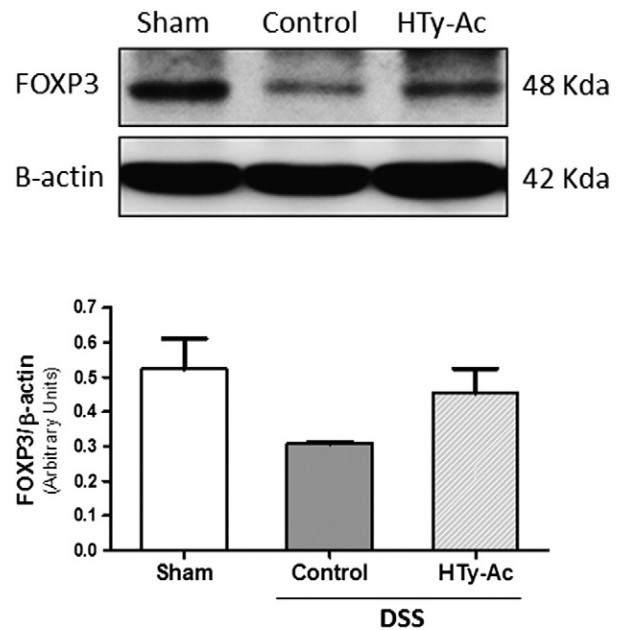


Fig. 7. Effect of dietary HTy-Ac supplementation on FOXP3 expression in colonic mucosa after 3% DSS for 5 days followed by 5 days of water. Densitometry was performed following normalization to the control (β-actin housekeeping gene). Data are expressed as the means±S.E.M.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jnutbio.2014.12.001>.

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