Ann Ist Super Sanità 2015 | Vol. 51, No. 4: 382-386
DOI: 10.4415/ANN_15_04_21

TNF-α and IL-10 modulation induced by polyphenols extracted by olive pomace in a mouse model of paw inflammation

Valentina Carito[1], Stefania Ciafrè[2], Luigi Tarani[3], Mauro Ceccanti[4], Fausta Natella[5], Angela Iannitelli[6], Paola Tirassa[1], George N. Chaldakov[7], Marco Ceccanti[4], Chiara Boccardo[8] and Marco Fiore[1, 9]

[1]*Istituto di Biologia Cellulare e Neurobiologia, Consiglio Nazionale delle Ricerche, Rome, Italy*
[2]*Istituto di Farmacologia Traslazionale, Consiglio Nazionale delle Ricerche, Rome Italy*
[3]*Dipartimento di Pediatria e Neuropsichiatria Infantile, Sapienza Università di Roma, Rome, Italy*
[4]*Centro Riferimento Alcologico Regione Lazio, Sapienza Università di Roma, Rome, Italy*
[5]*Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Rome, Italy*
[6]*Dipartimento di Scienze e Biotecnologie Medico-Chirurgiche, Facoltà di Farmacia e Medicina, Sapienza Università di Roma, Rome, Italy*
[7]*Laboratory of Cell Biology, Department of Anatomy and Histology, Medical University, Varna, Bulgaria*
[8]*Scuola di Specializzazione Medicina di Emergenza-Urgenza, Sapienza Università di Roma, Rome, Italy*
[9]*Istituto di Ricovero e Cura a Carattere Scientifico, Fondazione Santa Lucia, Rome, Italy*

Abstract

Background. Polyphenols from olive are known to possess antioxidant and anti-inflammatory properties.

Aim. The aim of this study was to study whether or not 10 consecutive days i.p. administration of a blend of olive *(Olea europaea L.)* polyphenols (10 mg/kg) containing mostly hydroxytyrosol could have an effect on cytokines playing important roles in inflammatory processes as TNF-α and IL-10.

Materials and methods. Inflammation was induced in the mouse paw by 2 carrageenan injections (50 µl vol, 5 mg/kg each). TNF-α and IL-10 were measured by enzyme-linked immunosorbent assay.

Results. Carrageenan decreased IL-10 in the paws, however, this reduction appeared to be less evident in mice treated with carrageenan but administered with polyphenols. As for TNF-α, our findings did not reveal differences between groups but an increase in the polyphenol and carrageenan group if compared to the carrageenan only group. As for antioxidant polyphenols' properties, no differences between groups in the serum glutathione were found.

Conclusions. Altogether, this investigation shows that olive polyphenols in the mouse may modulate the levels of cytokines having a role in the process of inflammation as TNF-α and IL-10.

Key words
- polyphenol
- olive
- inflammation

INTRODUCTION

Polyphenols are a structural class of natural, synthetic, and semisynthetic organic compounds characterized by the presence of multiples of phenol units. The most abundant polyphenols are the condensed tannins, found in all families of plants, and comprising up to 50% of the dry weight of leaves. Polyphenols from plants, including those extracted from the olive tree, leaves and oil, possess antioxidant and anti-inflammatory properties [1].

In general, the process of inflammation involves yin-yang pattern of regulation expressed by a disbalance in the content of pro-inflammatory mediators (*e.g.* IL-1β, TNF-α, leptin) [2] and anti-inflammatory mediators (*e.g.* IL-10, adiponectin) [3]. Carrageenan-induced local inflammation in the paws is a useful model to

Address for correspondence: Marco Fiore, Consiglio Nazionale delle Ricerche, Via del Fosso di Fiorano 64, 00143 Rome, Italy. E-mail: marco.fiore@cnr.it.

assess the contribution of mediators. It appears that the onset of the carrageenan-induced acute inflammation has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals, such as hydrogen peroxide, superoxide and hydroxyl radical, as well as the release of other neutrophil-derived mediators [4].

Olive oil is an integral ingredient of the traditional Mediterranean diet and several studies attribute many of the healthy advantages of this diet to olive oil's unique characteristics. It is well-known for its minor components exerting either anti-inflammatory or antioxidant effects. Among the several minor constituents of virgin olive oil, there are vitamins such as α- and γ-tocopherols and β-carotene, phytosterols, pigments, terpenic acids, flavonoids such as luteolin and quercetin, squalene, and phenolic compounds, usually termed polyphenols. Many of the biological activities attributed to natural phenolic derivatives have anti-inflammatory components so various health benefits seem to overlap with those attributed to non-steroidal anti-inflammatory drugs. Indeed, in addition to their antioxidant properties, polyphenolic compounds have been shown to exhibit a range of indirect actions that may be beneficial to health, including the inhibition of enzymes involved in the inflammatory process [1]. Therefore, the phenolic fraction is responsible for the stability and flavor of olive oil and is endowed with "pharmacological" and "nutraceutical" properties as an additional, valuable marker of olive oil quality [5]. In light of the above considerations and of the increasing interest in the Mediterranean diet, in the present studies we decided to evaluate the effects of polyphenols extracted by olive oil in a mice model of acute inflammation, the carrageenan-induced inflammation in the paws [2]. We investigated the presence of both a pro-inflammatory cytokine and an anti-inflammatory cytokine, tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) respectively in CD-1 adult male laboratory mice following treatment with or without a blend of polyphenols containing mostly hydroxytyrosol extracted from the olive pomace [6].

MATERIALS AND METHODS

Animals and polyphenols' administration

CD-1 outbred male mice were housed singularly in plexiglas cages (33 x 13 x 14 cm) under standardized conditions with pellet food (enriched standard diet not containing olive products purchased from Mucedola, Settimo Milanese, Italy). A 12L:12D lighting regime was used. Animals were divided in 4 groups, polyphenol group (Poly, n = 6), carrageenan group (Carr, n = 6), polyphenol and carrageenan group (Poly + Carr n = 6) and Control group (n = 6). The Poly group received daily for 10 consecutive days 10 mg/kg i.p. of a blend of polyphenols containing mostly hydroxytyrosol extracted from the olive pomace and dissolved in saline. Such residues were obtained during the production of extra virgin olive oil and dissolved in saline. Polyphenols (Phenolea Active Complex, www.phenofarm.it) were purchased from Leadergy Light, Italy. The Carr group received 2 injections of carrageenan (Sigma-Aldrich, USA) (50 µl vol, 5 mg/kg each) in both rear paws on day 1 and day 5 of the experimental schedule. The Poly + Carr group received polyphenols and carrageenan (same time and dose schedule of the previous groups) while controls received daily for 10 consecutive days saline solution i.p. and 2 saline injections in both rear paws on day 1 and day 5 of the experimental schedule. Animals' body weight was measured on day 0, 5 and 10 of the experimental schedule. Food and water consumption were measured daily. All efforts were made to minimize and reduce animal suffering and for limiting the number of animals used. All animal experiments were carried out following the procedure described by the guidelines of the European Community Council Directive of 24 November 1986 (86/609/EEC) and all experiments were authorized by the local ethical committees (Ministero della Salute, Regione Lazio, following the Decreto Legislativo 116/92; no permit number or approval ID are required since we only inform the committees of our experimentations without receiving any reply in case of positive answer).

Polyphenols' description

The polyphenols used in the present study [6], as indicated by the manufacturer, derive from a natural standardized olive pulp (*Olea europaea* L.) extract obtained by an eco-sustainable patented mechanical process. It is a by-product of the olive residues (pomace) obtained following olive pressing during the production of extra virgin olive oil. Specifications of the mixture, as indicated by the manufacturer, are shown in *Table 1*. We used a blend of phenolic compounds since it has been proposed that most of the health benefits associated with virgin olive oil are due to its minor components [5]. Indeed, mixtures of biophenols are supposed to possess a stronger action for counteracting different stages of oxidative damage instead of single compounds due to their possible synergism in scavenging hydroxyl radicals than the well known and studied hydroxytyrosol [7].

Animals sacrifice

Animals were sacrificed by a guillotine the day after the last i.p. polyphenols' injection between 10.00 h and 12.30 h. The blood for GSH/Hydroxytyrosol assessments was collected in heparin vials and quickly centrifuged at 10 000 *g* for 15 min. The rear paws were quickly removed and tissues dissected out and stored at -70 °C until used. Paw tissues were then homogenized and centrifuged at 8500 *g* and the supernatant used for TNF-α and IL-10 analyses.

Serum hydroxytyrosol measurement

Hydroxytyrosol was measured following methods previously described with minor modifications [6, 8]. Hydroxytyrosol, tyrosol, hydroxytyrosol-sulphates and salicylic acid were detected with MRM transition of 153/123, 137/106, 233/153 and 137/93 [M-H]-, respectively. Data acquisition and processing were performed using Analyst software 1.5.1. The quantification of phenolic compounds were performed by using a calibration curve; due to lack of a standard, the quantification of hydroxytyrosol sulfates was tentatively quantified using the calibration curve of hydroxytyrosol.

Table 1
Specifications of Phenolea Active Complex as indicated by the manufacturer and the chemical and phenolic composition

Specification of Phenolea Active Complex as indicated by the manufacturer		
Appearance		red rubin molasse
Solubility in water	%	> 99
Microbiological		
Salmonellae Spp.		absent in 25 g
Escherichia coli		absent in 1 g
Yeast and moulds	CFU/g	≤ 5 × 102
Enterobacteria	CFU/g	≤ 1 × 102
Total plate count	CFU/g	≤ 5 × 105
Pesticides		absent
Aflatoxins		absent
Ochratoxin		absent
PAHs (polycyclic aromatic hydrocarbons)	µg/kg	< 1
Chemical composition of Phenolea Active Complex (%)		
Moisture		28
Carbohydrates		61
Ashes		6.5
Proteins		2.5
Fats		0
Crude fibre		2
Phenolic composition of Phenolea Active Complex (%)		
Total polyphenols		5
Phenolic families (% on total polyphenols)		
Hydroxytyrosol		30
Other hydroxyyrosol derivatives		20
Ligstroside and derivatives		6
Total secoiridoid acids		14
Total phenolic acids		10
Oleocanthal		2
Other polyphenols		18

Glutathione assay

Glutathione (GSH), a nonprotein thiol, is an antioxidant found in eukaryotic cells, a change in GSH levels is important for assessing toxicological responses and oxidative stress in cells. GSH level was measured using GSH- GloTM Glutathione Assay (Promega, USA) on whole blood samples. Blood was collected in heparinized vials, and the whole blood lysate with the anticoagulant was diluted 1:2 in GSH-Glo Reaction Buffer and incubated on ice for 15 minutes. Blood was collected on day 5 of the experimental protocol from the mouse tail and during animal sacrifice the day after the last i.p. injection. Then blood lysate was centrifuged at 10 000 *g* for 20 minutes at 4 °C and the supernatant (lysed whole blood sample) was collected and stored at -20 °C. Prior the measurements the blood required additional dilution (1:15 in deionized water). In the assay procedure 10µl of diluted blood lysate was transferred to multiple wells of a 96-well plate. Then 100 µl of 1X GSH-Glo Reagent was added in the sample wells and the plate was mixed briefly and incubated at room temperature for 30 minutes. For the luminescence reaction 100 µl of Luciferin Detection Reagent was added to each well, plate mixed briefly and incubated for 15 minutes. Finally the plate was read in a luminometer to obtain the Glutathione concentration in the sample (µM). To measure total GSH (reduced GSH plus oxidized GSSH), a reducing agent β-mercapto-ethanol has been added to test wells at a concentration of 500 µM-1 mM that does not interfere significantly with the GSH-Glo Glutathione Assay and will reduce any oxidized glutathione present in the samples.

IL-10 and TNF-α determination

IL-10 and TNF-α (n = 6) were analyzed in the in the rear paws of adult male mice with ELISA kits "IL-10 Emaxtm ImmunoAssay System" and "TNF-α Emaxtm ImmunoAssay System" by Promega (Madison, WI, USA) following the indication provided by the manufacturer by a researcher who was unaware of the tissue group assignment. Data are represented as pg/mg total proteins and all assays were performed in duplicate which were averaged for statistical comparisons [9, 10].

Statistical analysis

Data were analyzed by ANOVA with the Carrageenan' and Polyphenols' administration as main factors. Post-hoc comparisons were performed using the Tukey's HSD test.

RESULTS

As predicted [2, 3] carrageenan injections in the paws elicited swelling. However, at the time of animal sacrifice, 5 days after the last carrageenan injection swelling was not evident in animals exposed to paw carrageenan. Furthermore there were not gross differences in swelling dimensions between groups on day 2 and day 7 of the experimental schedule. At the time of animal sacrifice no differences in animal body weight between groups, or in food and water consumption were also found. Similarly, no statistically significant differences in body weight between groups, or in food and water consumption were found during the other days of the testing. No differences between groups were found in GSH or GSSG (or their ratio) in the serum the day after the last i.p. injection of the experimental schedule. Hydroxytyrosol and hydroxytyrosol sulphates were present in the serum of polyphenol-administered animals, both groups (hydroxytyrosol 1.6 ng/mL; hydroxytyrosol sulphate 1, 7.1 ng/mL; hydroxytyrosol sulphate 2, 8.3 ng/mL) [6]. However, quite interestingly, hydroxytyrosol (0.9 ng/mL) was found in the serum of Controls and Carr animals but not hydroxytyrosol sulphate 1 and hydroxytyrosol sulphate 2. *Figure 1* shows the data on IL-10 and TNF-α in the paws of mice exposed to carrageenan with or without treatment with polyphenols extracted by olive pomace. Statistical analysis revealed that carrageenan decreased IL-10 in the paws, however,

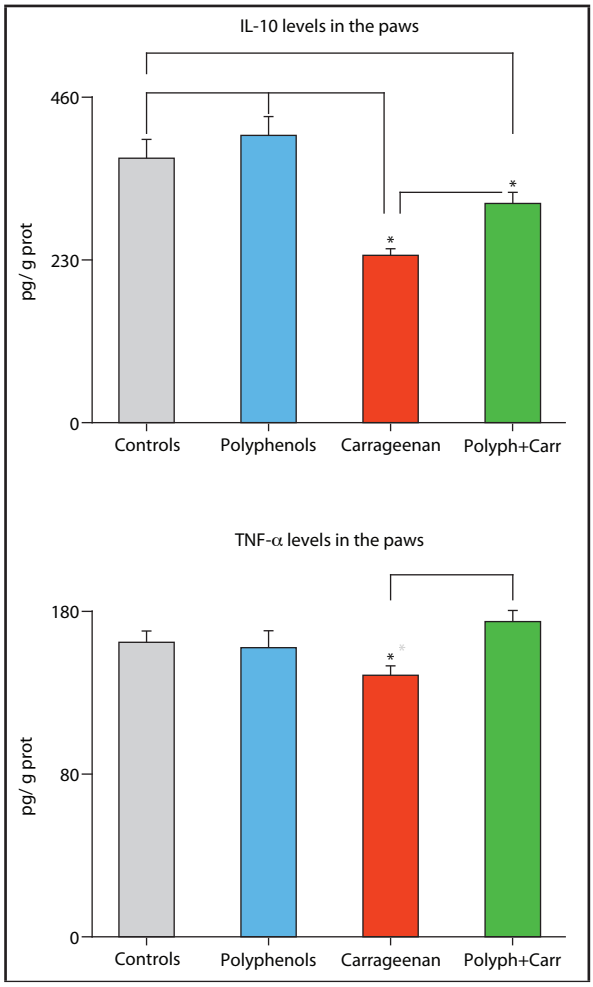

Figure 1
IL-10 and TNF-α levels in the paws of adult male mice administered daily for 10 days with 10 mg/kg i.p. of polyphenols extracted from olive oil and their respective controls or with carrageenan (5 mg/kg) on day 1 and 5 of the experimental schedule. Asterisks indicate significant differences between groups (* $p < 0.05$). The vertical lines in the figure indicate pooled SEM derived from appropriate error mean square in the ANOVA.

this reduction appeared to be less evident in mice treated with carrageenan but administered with polyphenols ($p < 0.01$ in the ANOVA; $p < 0.05$ in post-hoc comparisons, see *Figure 1*). As for TNF-α, our findings did not reveal differences between groups but an increase in polyphenol and carrageenan group if compared to the carrageenan only group ($p = 0.05$ in the ANOVA; $p < 0.05$ in post-hoc comparisons).

DISCUSSION

In this study, we show that a blend of polyphenols administered i.p., containing mostly hydroxytyrosol and extracted by olive pomace, may affect IL-10 and TNF-α in an animal model of paw inflammation induced by injecting carrageenan. In particular our findings have shown that: *i)* no differences in GSH or GSSG (or their ratio) have been observed after 10 days of polyphenols administration; *ii)* the presence of the anti-inflammatory cytokine IL-10 normally depleted following carrageenan exposure is partially restored by polyphenols; *iii)* no TNF-α differences between controls and animals exposed to carrageenan or polyphenols were found but increased TNF-α in animals administered with both carrageenan and polyphenols compared to carrageenan only.

IL-10 is a well known anti-inflammatory cytokine [11]. This cytokine is produced by monocytes and to a lesser extent by lymphocytes. This cytokine has pleiotropic effects in immunoregulation and inflammation. It has been also shown that IL-10 is also produced by mast cells, counteracting the inflammatory effect that these cells have at the site of an allergic reaction. It is capable of inhibiting the synthesis of pro-inflammatory cytokines like IFN-γ, IL-2, IL-3, and TNF-α made by cells such as macrophages and regulatory T-cells. Quite interestingly, a previous paper has shown that hydroxytyrosol extracted from olive leaves has no effect on IL-10 levels following carrageenan paw injections in the rat [12].

Tumor necrosis factor (TNF-α, cachexin or cachectin formerly known as tumor necrosis factor-alpha) is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction [13]. The primary role of TNF-α is in the regulation of immune cells. TNF-α is able to induce apoptotic cell death, to induce inflammation, and to inhibit tumorigenesis and viral replication. TNF-α was thought to be produced primarily by macrophages, but it is produced also by a broad variety of cell types including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts, and neuronal tissue. In a previous study TNF-α resulted to be elevated following paw carrageenan in the presence of swelling but slightly decreased following oral hydroxytyrosol administration [12]. As far as we know there is not a reliable explanation for the low TNF-α levels of the carrageenan group of the present study analyzed in absence of swelling when compared to the polyphenol and carrageenan group and further studies are needed. However, it may be possible to hypothesize that polyphenols could prolong the TNF-α recovery processes following carrageenan induced inflammation [4].

According to the present experimental conditions, there were no differences in GSH levels. Although polyphenols are known to possess antioxidant properties protecting by inflammatory processes by scavenging free radicals [5], they could also act on cell physiology and signaling by different pathways affecting the presence of TNF-α and/or IL-10 [14] or neurotrophins as recently observed in alcoholics administered with pillows containing blends of olive polyphenols during withdrawal [15].

Another implication of the present study is that administering i.p. in the mouse a blend of polyphenols extracted by olive pomace and containing mostly hydroxytyrosol, it is possible to potentiate the presence in the serum of hydroxytyrosol per se and the presence of metabolites of the hydroxytyrosol as the hydroxytyrosol sulphate 1 and hydroxytyrosol sulphate 2. Quite interestingly, control mice fed with pellet food containing enriched standard diet without olive products showed only

low levels of hydroxytyrosol but not hydroxytyrosol sulphate 1 and hydroxytyrosol sulphate 2 indicating that: i) hydroxytyrosol also known as DOPET (3,4-dihydroxyphenylethanol), a well-known metabolite of dopamine, is known to be endogenously produced and present in body fluids at low concentrations [16]; ii) a limitation of the present investigation is that under certain circumstances different routes of administration or higher concentrations could be needed to obtain visible effects.

In conclusion, since natural polyphenols' consumption represents a key factor in human health, the present study may be considered a further step in the attempt to disclose some biomolecular aspects of the action of olive polyphenols.

Author's contribution statement

Valentina Carito, Luigi Tarani, Mauro Ceccanti and Marco Fiore were responsible for the study concept and design. Valentina Carito and Paola Tirassa contributed to the acquisition of animal data. Valentina Carito and Fausta Natella performed the biomolecular analyses. Mauro Ceccanti, Angela Iannitelli, Valentina Carito and Marco Fiore contributed to data analysis and interpretation of findings. Valentina Carito and Marco Fiore drafted the manuscript. George Chaldakov provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version for publication.

Conflict of interest statement

Authors declare that they have no conflict of interest.

Received on 19 May 2015.
Accepted on 15 October 2015.

REFERENCES

1. Biesalski HK. Polyphenols and inflammation: basic interactions. *Curr Opin Clin Nutr Metab Care* 2007;10(6):724-8.
2. Rocha AC, Fernandes ES, Quintão NL, Campos MM, Calixto JB. Relevance of tumour necrosis factor-alpha for the inflammatory and nociceptive responses evoked by carrageenan in the mouse paw. *Br J Pharmacol* 2006;148(5):688-95.
3. Yang J, Li S, Xie C, Ye H, Tang H, Chen L, Peng A. Anti-inflammatory activity of ethyl acetate fraction of the seeds of *Brucea javanica*. *J Ethnopharmacol* 2013;147(2):442-6.
4. Morris CJ. Carrageenan-induced paw edema in the rat and mouse. *Met Mol Biol Clifton NJ* 2003;225:115-21.
5. Visioli F, Bernardini E. Extra virgin olive oil's polyphenols: biological activities. *Curr Pharm Des* 2011;17:786-804.
6. De Nicoló S, Tarani L, Ceccanti M, Maldini M, Natella F, Vania A, *et al.* Effects of olive polyphenols administration on nerve growth factor and brain-derived neurotrophic factor in the mouse brain. *Nutrition* 2013;29(4):681-7.
7. Cardinali A, Cicco N, Linsalata V, Minervini F, Pati S, Pieralice M, *et al.* Biological activity of high molecular weight phenolics from olive mill wastewater. *J Agric Food Chem* 2010;58:8585-90.
8. Rodriguez-Gutierrez G, Wood S, Fernandez-Bolanos Guzman J, Duthie GG, de Roos B. Determination of 3, 4-dihydroxyphenylglycol, hydroxytyrosol and tyrosol purified from olive oil by-products with HPLC in animal plasma and tissues. *Food Chem* 2011;126:1948-52.
9. Ceccanti M, Mancinelli R, Tirassa P, Laviola G, Rossi S, Romeo M, *et al.* Early exposure to ethanol or red wine and long-lasting effects in aged mice. A study on nerve growth factor, brain-derived neurotrophic factor, hepatocyte growth factor, and vascular endothelial growth factor. *Neurobiol Aging* 2012;33:359-67.
10. Carito V, Venditti A, Bianco A, Ceccanti M, Serrilli AM, Chaldakov G, *et al.* Effects of olive leaf polyphenols on male mouse brain NGF, BDNF and their receptors TrkA, TrkB and p75. *Nat Prod Res* 2014;1-15.
11. Gabryšová L, Howes A, Saraiva M, O'Garra A. The regulation of IL-10 expression. *Curr Top Microbiol Immunol* 2014;380:157-90.
12. Gong D, Geng C, Jiang L, Cao J, Yoshimura H, Zhong L. Effects of hydroxytyrosol-20 on carrageenan-induced acute inflammation and hyperalgesia in rats. *Phytother Res* 2009;23:646-50.
13. Olmos G, Lladó J. Tumor necrosis factor alpha: a link between neuroinflammation and excitotoxicity. *Mediators Inflamm* 2014; ID 861231.
14. Cárdeno A, Sánchez-Hidalgo M, Alarcón-de-la-Lastra C. An up-date of olive oil phenols in inflammation and cancer: molecular mechanisms and clinical implications. *Curr Med Chem* 2013;20(37):4758-76.
15. Ceccanti M, Carito V, Iannuzzi S, Vitali M, Tarani L, De Nicolo S, Ceccanti M, Ciafre' S, Tirassa P, Capriglione I, Coriale G, Iannitelli A, Chaldakov GN, Fiore M. Serum BDNF and NGF modulation by olive polyphenols in alcoholics during withdrawal. *J Alcohol Drug Dep* 2015;3:214. doi:10.4172/23296488.1000214
16. De la Torre R. Bioavailability of olive oil phenolic compounds in humans. *Inflammopharmacology* 2008;16(5):245-7.