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Protective effects of hydroxytyrosol-supplemented refined olive oil in animal models of acute inflammation and rheumatoid arthritis

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Abstract

Virgin olive oil is the primary source of fat in the Mediterranean diet, and its beneficial health effects have been related with oleic acid and phenolic compounds content. Hydroxytyrosol, a typical virgin olive oil phenolic compound, has beneficial antioxidant and anti-inflammatory properties as previously reported. The aim of this study was to evaluate the effect of hydroxytyrosol-supplemented refined olive oil at 0.5 and 5 mg/kg in a rodent model of rheumatoid arthritis. Rheumatoid arthritis was induced by intradermic administration, in male Wistar rats, of Freund's adjuvant with collagen type II on days 1 and 21. Hydroxytyrosol-supplemented refined olive oils were administrated by gavage from day 23 until day 35. The treatment at 5-mg/kg dose significantly decreased paw edema ($P<.01$), histological damage, cyclooxygenase-2 and inducible nitric oxide synthase expression, and markedly reduced the degree of bone resorption, soft tissue swelling and osteophyte formation, improving articular function in treated animals. Acute inflammation, induced by carrageenan, was also evaluated for hydroxytyrosol-supplemented refined olive oils at 0.5 and 5 mg/kg. Both doses significantly reduced paw edema ($P<.001$). Our results suggest that the supplementation of refined olive oil with hydroxytyrosol may be advantageous in rheumatoid arthritis with significant impact not only on chronic inflammation but also on acute inflammatory processes.

Keywords: Hydroxytyrosol; Olive oil; Rheumatoid arthritis; Inflammation; Phenolic compounds

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic joint inflammation [1,2]. The chronic inflammation, localized and systemic [3], causes swelling, pain and stiffness in joints besides cartilage and bone destruction [1,4]. This disease is associated with progressive disability and systemic complications, has impact on mobility and causes early death and socioeconomic costs [4]. RA has a prevalence of about 2% worldwide, and the associated complications of osteoporosis and cardiovascular disease make RA important in public health terms [3]. Nonsteroidal anti-inflammatory drugs (NSAIDs) [1,3,5], slow-acting antirheumatic drugs (e.g., methotrexate) [6], corticosteroids [3] or biological agents [6] can be used to relieve symptoms and to reduce disease progression. However, the side effects of these pharmacological therapies frequently turn patients to complementary or supportive therapies, such as dietary modification or supplementation [1,3]. There is good evidence today to suggest that fish oil supplements, containing eicosapentaenoic acid and docohexaenoic acid — long-chain n-3 polyunsaturated fatty acids — may be helpful in relieving some RA symptoms [3,5] at 3–6 g of daily consumption for a minimum of 12 weeks [5], and evidence for the use of vitamins (e.g.: vitamin E) and mineral supplements (e.g.: iron and zinc) as adjuvant treatment of RA was also reported [1,3]. It is also possible that monounsaturated fatty acids have potential effects in the immune response [5]. Moreover, antioxidants may provide an important defense against the increased oxidant stress in patients with RA [3]. Dietary changes have been proposed to have beneficial impact on RA through a number of mechanisms, such as a general decrease in the severity or impact of the inflammatory process, an increase in antioxidant levels, altered lipid profiles and/or possible changes in gut microbiota composition [7].

Abbreviations: BSA, bovine serum albumin; CII, type II collagen; CFA, complete Freund's adjuvant; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; NSAIDs, nonsteroidal anti-inflammatory drugs; PBS, phosphate buffer saline; PFA, paraformaldehyde; PMA, phorbol myristate acetate; RA, rheumatoid arthritis; ROO, refined olive oil; ROS, reactive oxygen species; RT, room temperature; SEM, standard error of the mean

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The Mediterranean-style diet, in which olive oil is the primary source of fat [8], was awarded an Immaterial Human Heritage title by The United Nations Education, Scientific & Cultural Organization [9]. Diet and lifestyle of Mediterranean populations are related with health benefits such as decreased rates of cancer, diabetes and heart disease [10].

Previously published data demonstrated that RA patients did obtain a reduction in inflammatory activity [11] and that risk of RA was inversely associated with olive oil consumption [12]. Berbert et al. [13] reported a human intervention trial in patients with RA where supplementation of fish oil and olive oil was evaluated. In this study, an accentuated improvement was observed when fish oils were used in combination with olive oil, favoring the hypothesis that adding oleic acid, a major olive oil monounsaturated fatty acid, to the diet decreases inflammation in RA patients who already consume fish oil supplements. Studies focusing specifically on olive oil intake in RA are limited, and further elaboration is required for a translational approach aiming at an adjunctive pharmacological action.

The major components of olive oil are glycerols, which represent more than 98% of the total oil weight, including triacylglycerol esters of oleic acid that account for 55% to 83% of olive oil composition [14]. Minor components of virgin olive oils with about 2% of the total oil weight include sterols (e.g., β-sitosterol), tocopherols, phenolic compounds such as hydroxytyrosol and tyrosol [14–16], and the secoiridoids that account for more than 50% of the phenolic content of the oil [17]. It is worth noting that phenolic compounds are lost during oil-refining process, and therefore, refined olive oils (ROOs) have a low phenolic content.

As one of the major phenolic compounds present in virgin olive oils, hydroxytyrosol presents a variety of pharmacological activities, such as antioxidant properties and anticancer, anti-inflammatory and neuroprotective activities, and beneficial effects on the cardiovascular system, which enhance its potentiality for the development of dietary supplements [18]. Hydroxytyrosol has good stability in the free form, and its main source in diet is virgin olive oils, where it mainly exists in combined (with secoiridoids) and free forms [18]. Toxicological evaluation of pure hydroxytyrosol was recently reported by Aunon-Calles et al. [19] and, based on the results, a 'No Observed Adverse Effects Level' of 500 mg/kg/day was proposed, ensuring a safety profile for this compound. Assays focused on acute inflammation and hyperalgesia in rats were performed using HT-20 (22% hydroxytyrosol) [20]. Results demonstrated that HT-20 inhibits acute inflammation and hyperalgesia induced by carrageenan in rats. Studies on acute and chronic inflammation, held in rats, showed protective effects of a polyphenol-supplemented virgin olive oil diet [21]. In those studies, acute inflammation was induced by carrageenan, and chronic inflammation was evaluated in an induced arthritis model. The authors concluded that virgin olive oil with a higher content of polyphenolic compounds had protective effects in both models of inflammation [21]. In addition, beneficial effects diets enriched with extra virgin olive oil were observed in a chronic colitis model induced in mice, and increased benefits were obtained through extra virgin olive oil supplemented with hydroxytyrosol [22].

Until now, no studies have investigated the impact of hydroxytyrosol intake on RA. Our hypothesis is that the supplementation of ROO with hydroxytyrosol can reduce acute and chronic inflammation induced in rats. Acute and chronic inflammation was evaluated using carrageenan and an RA model, respectively. Two hydroxytyrosol doses were evaluated. A mechanistic approach was also attempted for hydroxytyrosol evaluating neutrophil's oxidative burst, and concerning RA model histological assessment, radiographic analysis and immunohistochemistry for inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were studied.

2. Materials and methods

2.1. Materials

The following reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA): acetic acid *p.a.*, bovine type II collagen (CII), λ-carrageenan, complete Freund's adjuvant (CFA), D-(+)-glucose, dimethylsulfoxide, Dulbecco's phosphate buffer saline (PBS) without calcium chloride and magnesium chloride, H_2O_2, 4-hidroxy-tempol, Histopaque 1077, Histopaque 1119, indomethacin, paraformaldehyde (PFA), phorbol myristate acetate (PMA), Tris/EDTA, Trizma, Trolox and trypan blue solution 0.4%. Luminol was obtained from Fluka Chemie GmbH (Steinheim, Germany). Calcium chloride dihydrate and magnesium sulfate were obtained from Merck (Darmstadt, Germany). Potassium chloride was obtained from Pronalab (Abrunheira, Portugal), sodium chloride from Honeywell Riedel-de Haën (Hanover, Germany) and sodium pentobarbital from Eutasil (Sanofi Veterinária, Algés, Portugal). An olive oil producer supplied ROO, used as vehicle for hydroxytyrosol supplementation, and hydroxytyrosol was obtained from Extrasynthese (Lyon, France). Analysis of fatty acids, free acidity and peroxide value in the ROO was carried out according to European Union regulation [23] (Supplementary Table 1).

2.2. Isolation of human neutrophils

Venous blood was collected from healthy adult volunteers, and neutrophils were isolated by gradient density, as previously described Freitas et al. [24]. The obtained cell suspensions contained more than 99% of neutrophils, and the control of their viability showed more than 95% of the cells excluding trypan blue solution 0.4%. Isolated neutrophils were kept in ice until use. Tris glucose (25 mM Tris, 1.26 mM $CaCl_2.2H_2O$, 5.37 mM KCl, 0.81 mM $MgSO_4$, 140 mM NaCl and 5.55 mM D-glucose) was the incubation medium used, as previously recommended by Freitas et al. [25].

2.3. Evaluation of neutrophils' oxidative burst

The measurement of neutrophils' oxidative burst was undertaken by chemiluminescence, by monitoring reactive oxygen species (ROS)-induced oxidation of luminol, according to a previously described procedure [26]. The reaction mixtures contained neutrophils (1×10^6 cells/ml) and the following reagents at the indicated final concentrations (final volume of 250 μl): tested compounds at various concentrations, luminol (500 μM) and PMA (160 nM). Cells were preincubated with luminol and the tested compounds for 5 min before the addition of PMA, and the measurements were carried out at 37°C, under continuous soft shaking. Kinetic readings were initiated immediately after cell stimulation. Measurements were taken at the peak of the curve (around 10 min). Effects are expressed as the percent inhibition of luminol oxidation. Each study corresponds to, at least, four individual experiments, performed in triplicate in each experiment.

2.4. Animal models

Experiments were conducted according to the Home Office Guidance in the Operation of Animals (Scientific Procedures) Act 1986, published by Her Majesty's Stationary Office, London, UK, and the Institutional Animal Research Committee Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), as well as to the currently adopted EC regulations. The studies are in compliance with the ARRIVE Guidelines for Reporting Animal Research summarized at www.nc3rs.org.uk. All studies were carried out using male Wistar rats weighing 100–150 g (Harlan Iberica, Barcelona, Spain). All animals received a standard diet and water *ad libitum*.

2.4.1. Carrageenan-induced edema

Paw edema was induced by subplantar injection of 0.1 ml of 1% w/v sterile λ-carrageenan in saline solution into the rat left hind paw. At 0 and 6 h after λ-carrageenan administration, the paw volume was measured with a plethysmometer (LE7500; Letica, Spain). The increase in paw volume was taken as edema volume (V) and was calculated as the difference between the final volumes of the λ-carrageenan-injected paw (V_f) and the initial volume of the same paw before injecting λ-carrageenan (V_0).

Animals were randomly divided and allocated into eight groups as described below:

(a) Negative control group: animals subjected to subplantar injection into the rat left hind paw of 0.1 ml sterile saline and administered with saline (1 ml/kg, gavage.) (n=6); (b) carrageenan group: animals subjected to paw edema induction and administered with saline (1 ml/kg, gavage) (n=6) 30 min before λ-carrageenan injection; (c) ROO group: animals subjected to paw edema induction and pretreated with ROO (1 ml, gavage) 30 min before λ-carrageenan injection (n=5); (d) hydroxytyrosol-supplemented ROO low-dose group: animals subjected to paw edema induction and pretreated with hydroxytyrosol-supplemented ROO (1 ml, 0.5 mg/kg hydroxytyrosol, gavage) 30 min before λ-carrageenan injection (n=5); (e) hydroxytyrosol-supplemented ROO high-dose group: animals subjected to paw edema induction and pretreated with hydroxytyrosol-supplemented ROO (1 ml, 5 mg/kg, hydroxytyrosol, gavage) 30 min before λ-carrageenan injection (n=5); (f) indomethacin group: animals subjected to paw edema induction and pretreated with indomethacin (10 mg/kg, gavage.) 30 min before λ-carrageenan injection (n=6); (g) Trolox group: animals subjected to paw edema induction and pretreated with trolox (30 mg/kg, gavage) 30 min before λ-carrageenan injection (n=6); (h) tempol group: animals subjected to paw edema induction and pretreated with tempol (30 mg/kg, gavage) 30 min before λ-carrageenan injection (n=6). For indomethacin, trolox and tempol, sterile saline was used as vehicle.