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Mechanisms of Olive Leaf Extract-Ameliorated Rat Arthritis Caused by Kaolin and Carrageenan

Dezheng Gong,[1] Chengyan Geng,[1] Liping Jiang,[1] Lihui Wang,[2] Hiroyuki Yoshimura[3] and Laifu Zhong[1]*

[1]China-Japanese Joint Institute for Medical and Pharmaceutical Science, Dalian Medical University, No. 9, West Segment of South Lvshun Road, Dalian 116044, China
[2]Department of Pathology Dalian Medical University, No. 9, West Segment of South Lvshun Road, Dalian 116044, China
[3]Eisai Food & Chemical Co., Ltd, Nihonbashi, Chuo-ku, Tokyo 103-0027, Japan

Olive leaf extract (OLE) has antioxidant and antiinflammatory actions. However, the role of OLE in mechanical inflammatory arthritis (osteoarthritis, OA) is unclear. This study investigated the effect of OLE on the development of kaolin and carrageenan-induced arthritis, a murine model of OA. Administration of OLE significantly ameliorated paw swelling, the paw Evans blue content and the histopathological scores. In the human monocyte cell line, THP-1, the OLE reduced the LPS-induced TNF-α production and was dose dependent. Croton oil-induced ear edema in mice also revealed that treatment with OLE suppressed ear edema, myeloperoxidase (MPO) production and was dose dependent. These results indicated that OLE is an effective antiarthritis agent through an antiinflammation mechanism. Also OLE may be beneficial for the treatment of OA in humans. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: olive leaf extracts; antiinflammatory; antiarthritis; cytokine.

INTRODUCTION

Olive tree leaves have been used widely in traditional remedies in European and Mediterranean countries such as Greece, Spain, Italy, France, Turkey, Israel, Morocco and Tunisia. They have been used in the human diet as an extract, an herbal tea and a powder, and they contain many potentially bioactive compounds that may have antioxidant, antihypertensive, antiatherogenic, antiinflammatory, hypoglycemic and hypocholesterolemic properties (El and Karakaya, 2009; Keys, 1995; McDonald *et al*., 2001). Hydroxytyrosol (HT) and tyrosol are some of the many phenol compounds in olives that contribute to the bitter taste, astringency and resistance to oxidation (Visioli *et al*., 1998, 2002).

It is well established that olive leaf extract (OLE) has antioxidant and antiinflammatory activities. The data reported showed that olive vegetation water (OVW) significantly reduced serum tumor necrosis factor-alpha (TNF-α) levels in lipopolysaccharide (LPS)-treated BALB/c mice, a model system of inflammation. It was also able to reduce LPS-induced TNF-α production in THP-1 cells, the human monocyte cell line. Thus OVW may be an effective therapy for a variety of inflammatory processes, including rheumatoid and osteoarthritis (OA). The major active ingredient of OVW was hydroxytyrosol (Bitler *et al*., 2005). Our previous studies demonstrated the antiinflammatory and antinociceptive effects of OLE in a rat model of acute inflammation and pain induced by carrageenan (Gong *et al*., 2009). Also shown was the effect of OLE on the inhibition of atherosclerosis, which is related to the suppressed inflammatory response (Wang *et al*., 2008).

Osteoarthritis, a disease characterized by joint pain and the loss of joint form and function due to articular degeneration, is not an inevitable consequence of aging, but a strong association exists between age and increasing evidence of OA (Buckwater and Mankin, 1997; Lawrence *et al*., 1966). Over the years, the presence of synovial inflammation has become obvious. Particularly at the clinical stage of the disease, it is among the significant structural changes that take place during the development of OA. The synthesis and release of a number of mediators by the inflamed tissue is believed to be an important factor in the development and progression of OA (Pelletier *et al*., 2001). Osteoarthritis is the most common form of arthritis, affecting tens of millions of people in the world. Kaolin and carrageenan-induced experimental arthritis are very similar to OA. However, the effect of OLE on this experimental arthritis in rats is not known.

The goal of this study was to investigate the effect of OLE on kaolin and carrageenan-induced mechanical inflammatory arthritis in rats. Moreover, the study aimed to explore the effects of such treatment on the major pathways involved in blocking the inflammatory state, including the inhibited production of TNF-α and MPO.

MATERIALS AND METHODS

Reagents. The RPMI and fetal bovine serum (FBS) were purchased from Gibco. A TNF-α enzyme-linked immunosorbent assay (ELISA) kit was purchased

* Correspondence to: Laifu Zhong, China-Japanese Joint Institute for Medical and Pharmaceutical Science, Dalian Medical University, No. 9, West Segment of South Lvshun Road, Dalian 116044, China.
E-mail: zhong@dlmedu.edu.cn

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from Genzyme Techne (MA, USA). The LPS (*E. coli* 055: B5) and other chemicals used in this study were of analytical grade and purchased from Sigma (St Louis, MO, USA).

Preparation of OLE. The commercially produced OLE (lot 604281) was supplied by Eisai Food & Chemical Co., Ltd, Japan. The aqueous extract of olive leaves was prepared from dried material (100 g) in boiling water (250 mL). The mixture was boiled for 45 min, filtered and then lyophilized (HT yield: 22%). The composition of the OLE was HT (22%), polyphenol (4%), saccharide (67%), lipid (2%), ignition residue (4%) and moisture (1%). The HT content, determined using the liquid chromatography method, was 22%. The polyphenol content, determined using the colorimetric analysis method, was 4%. The major active ingredient of OLE is HT. The OLE powder was dissolved in distilled water before use.

Animals. Male BALB/c mice weighing 20–25 g and male Sprague-Dawley rats (148–163 g) from Laboratory Animal Center, Dalian Medical University (Dalian, China) were acclimatized to laboratory conditions for 1 week and observed for any sign of illness. Rats were housed in plastic cages (5 rats/cage) on chip bedding. The animal room was maintained at 23–25°C and 50–60% relative humidity with a 12 h light/dark cycle and the room air was changed about 12 times/h. All animals were supplied with a sterile commercial diet and tap water *ad libitum* throughout the acclimation and testing periods. The experimental protocol was approved by the Animal Ethics Committee of Dalian Medical University, in accordance with 'Principles of Laboratory Animal Care and Use in Research' (Ministry of Health, Beijing, China).

Group composition and treatment of rat arthritis. The animals were assigned randomly to five groups of 20 rats each. The rats in the experimental groups received daily gavage of OLE at 25, 50 or 100 mg/kg b.w. for 5 consecutive days. Rats in the control group and in the model control group received distilled water or indomethacin 2 mg/kg b.w. by daily gavage for 5 consecutive days, respectively.

After the rats were anesthetized with pentobarbital sodium (30 mg/kg b.w.) by intraperitoneal injection, arthritis was induced by an injection of 0.2 mL of 10% suspension of kaolin in sterile saline with a 27 gauge needle into the tibio-tarsal articulation after sterilization of the right hind paws with 75% ethanol solution. Then the articulation was slowly flexed and extended for 5 min. Fifteen minutes after the kaolin injection, 0.07 mL of a 2% carrageenan solution was given intradermally into the subplantar region of the right hind paw, and then the region was massaged for 5 min. The arthritis induction occurred 1 h after administration of OLE.

Degree of right hind paw swelling. The volumes (mL) of the left and right hind paws for ten rats in each group were estimated by a volumetric measuring tube 1, 3 and 5 days after induction of arthritis (day 0). The degree of right hind paw swelling was calculated by:

$$\frac{\text{Volume (mL) of right hind paw} - \text{Volume (mL) of left hind paw}}{\text{Volume (mL) of right hind paw}} \times 100$$

Evans blue content in right hind paw. Five days after the induction of arthritis, 1 mL/kg b.w. of a 1% solution of Evans blue was injected intravenously into ten rats (under pentobarbital sodium anesthesia) in each group. After 2 h the rats were killed, and the right hind paws were removed and digested in 3 mL concentrated HCl at 37°C for 18 h. At the end of the digestion, 4 mL of a 12.8% benzalkonium chloride solution was added to the samples and the mixture was shaken for 30 min. Benzalkonium forms a complex with Evans blue that is selectively soluble in chloroform. Therefore 7 mL of chloroform was added to each sample, which was then shaken vigorously. After a few minutes, the chloroform layer containing the Evans blue complex was collected, centrifuged (3000 rpm) for 5 min and brought to a final volume of 10 mL. The concentration of Evans blue was photometrically measured at 620 nm against the chloroform blank. The results are expressed as μg of Evans blue per mg of weight of the paw.

Histopathological examination and scoring. Five days after OLE administration, the right hind paws were isolated from another ten rats in each group under ether anesthesia, fixed in 10% neutral buffered formalin, decalcified with 5% formic acid–formalin, embedded in paraffin and sectioned. The sections were stained with hematoxylin–eosin (HE) and observed under a light microscope.

All histopathological evaluations were carried out blind by the same pathologist. The histopathological severity of arthritis was evaluated and scored based on edema and inflammatory cell infiltration into the soft tissue around the synovial membrane and articulation, proliferation of synovial cells, fibrosis and the destruction of articular cartilage and bone.

Histopathological scoring for the arthritis of each rat was as follows: **1**, minor inflammation: slight edema, a little inflammatory cell infiltration, and no proliferation of synovial cells and fibrosis; **2**, moderate inflammation: moderate edema and inflammatory cell infiltration, and no or slight proliferation of synovial cells and fibrosis; **3**, severe inflammation: severe edema, a large inflammatory cell infiltration, proliferation of synovial cells and fibrosis; **4**, very severe inflammation: severe edema, a large inflammatory cell infiltration, proliferation of synovial cells, fibrosis and destruction of articular cartilage and bone.

TNF-α measurement in THP cells. The THP-1 cells [American Type Culture Collection (ATCC) TIB-202] were obtained from Peking Union Medical College (Peking, China). The OLE was added to the THP-1 cells in 96-well microtiter plates for 1 h before stimulation with LPS (10 μg/L) for 3 h at 37°C. After LPS stimulation, the supernatants were collected by centrifugation at 200 × **g** for 20 min and evaluated for levels of TNF-α by ELISA. Dexamethasone (DEX, 100 nmol/L), an inhibitor of TNF-α release, was used as a positive control.

Mouse ear edema and MPO activity measurement. The animals were randomly assigned to five groups of ten mice. Edema was induced in the right ear of each mouse by topical application of 100 μg/ear of croton oil as a phlogistic agent dissolved in 10 μL of acetone. This was applied by an automatic pipette in 10 μL volumes to both inner and outer surfaces of the right ear. Mice in

experimental groups, the positive control group and the model control group received OLE which was dissolved in distilled water before use, indomethacin which was dissolved in acetone, or acetone, respectively. The OLE, indomethacin or acetone was applied to the right ear, before the application of croton oil. The left ear (blank) received the same volume of vehicle acetone. The dose levels of OLE were 0.5, 1.0 and 2.0 mg/ear and the dose of indomethacin was 0.5 mg/ear. A section (8 mm diameter) of the central portion of both ears was weighed, 6 h after the inflammatory challenge. The degree of swelling induced by croton oil was assessed in terms of the increase in the weight of the right ear punch biopsy over that of the left ear. Inhibition percentages were calculated by comparison with the model control group that received the croton oil but no OLE.

The MPO activity was determined in the supernatants from the homogenates of the ear biopsies prepared. Briefly, the complete section of punched ear was placed in 1.5 mL of 50 mM sodium phosphate buffer, pH 6.0, containing 0.5% hexadecyltrimethy-ammonium bromide (HTAB) was homogenized for 45 s at 0°C in a motor-driven homogenizer. The assay of MPO was performed in a microplate reader. The following reagents were added in the following order to wells of a 96-well microtiter plate: 50 μL of supernatant, 50 μL pH 6.0 phosphate buffer containing 0.5% HTAB, 50 μL o-dianisidin 0.68 mg/mL in distilled water, and 50 μL freshly prepared 0.005% hydrogen peroxide to start the reaction. The optical density at 450 nm was read immediately and thereafter at 5 min intervals. The amount of enzyme in the samples was obtained by comparison of the rate of reaction with that in wells containing supernatants from the model control group treated with croton oil only.

Statistical analysis. The values are presented as mean ± standard deviation (SD). The data were statistically analysed by one-way analysis of variance (ANOVA) followed by Student's *t*-test or Mann-Whitney *U*-test for nonparametric analysis using SPSS software vision 13.0. A value of $p < 0.05$ was considered significant.

RESULTS

Effect of OLE on degree of right hind paw swelling

The results are shown in Table 1. Rats in the indomethacin group and OLE 50 and 100 mg/kg b.w. groups displayed a significant reduction in the degrees of right hind paw swelling 1, 3 and 5 days after dosing ($p < 0.05$ or $p < 0.01$), compared with the model control rats.

Effect of OLE on content of Evans blue in right hind paw

Rats in the indomethacin group and OLE 50 and 100 mg/kg b.w. groups had a statistically significant decrease in Evans blue content in the right hind paw 5 days after dosing, compared with the model control rats ($p < 0.05$ Table 2). Thus OLE, as well as indomethacin, can reduce the tissue edema associated with OA damage.

Histopathological findings

The results are shown in Figs 1 and 2. The histopathological features of experimental rat arthritis in the model control group were characterized as follows: severe edema of the synovial membrane and soft tissue around the articulation, inflammatory cell infiltration including a large number macrophages and lymphocytes and few neutrophilic granulocytes, a slight exudation of fibrin, proliferation of synovial cells, fibrosis, destruction of articular cartilage and bone, and an abundance of inflamed exudates in the articular space (Figs 1 and 2). Compared with the model control rats, histopathological examination of the indomethacin group and of the OLE 50 and 100 mg/kg b.w. groups showed significant attenuation of the synovial membrane and periarticular soft tissue edema, decreased inflammatory infiltration including macrophages and lymphocytes. The indomethacin group and in OLE 50 and 100 mg/kg groups, also scored significantly lower for right hind paw histopathology, compared with the model control group ($p < 0.05$ or $p < 0.01$ Fig. 2).

Effect of OLE on TNF-α level in THP-1 cells

To examine the effects of OLE on cytokine expression associated with inflammation, the effects on TNF-α production were evaluated in the human monocyte cell line, THP-1. The OLE significantly reduced the content of TNF-α relative to the control (only LPS-treated; $p < 0.05$ or $p < 0.01$, Fig. 3).

Table 1. Effects of OLE given by gavage on degree of right hind paw swelling in kaolin and carrageenan-induced arthritis model in rats

Group	Dose (mg/kg)	Body weight (g)	Degree of swelling (%)		
			1 day after dosing	3 days after dosing	5 days after dosing
Model control		155.5±3.74	52.5±4.05	45.0±2.91	40.4±4.01
Indomethacin	2	155.3±5.47	31.9±2.22[a]	27.5±3.39[a]	23.0±4.19[a]
OLE	25	154.3±3.77	49.7±2.90	43.2±3.39	39.1±5.48
OLE	50	155.9±3.91	46.7±4.15[b]	40.4±4.79[b]	34.5±4.85[b]
OLE	100	156.1±4.39	44.8±6.78[b]	37.4±5.71[b]	31.7±5.26[b]

Values are mean ± SD, $n = 10$.

[a] Significantly different from model control at $p < 0.01$.

[b] Significantly different from model control at $p < 0.05$.

Table 2. Effects of OLE given by gavage on Evans blue content in right hind paw in kaolin and carrageenan-induced arthritis model

Group	Dose (mg/kg b.w.)	Evans blue content (g/g)
Model control	0	16.25±2.58
Indomethacin	2	13.61±0.89[a]
OLE	25	15.64±3.25
OLE	50	13.52±1.91[a]
OLE	100	13.32±2.02[a]

Values are mean ± SD, $n = 10$.

[a] Significantly different from model control at $p < 0.05$.

Effect of OLE on croton oil-induced cutaneous inflammation

The results are shown in Fig. 4. The degree of ear swelling in the indomethacin and OLE groups at doses of 2.0 mg/ear and 1.0 mg/ear was significantly lower than that of the model control group ($p < 0.05$ or $p < 0.01$). The anti-swelling effect of OLE was dose dependent.

Effect of OLE on MPO activity

The results on MPO activity are shown in Fig. 5. The MPO activity of indomethacin and OLE groups at doses of 2.0 mg/ear and 1.0 mg/ear was significantly lower than that of the model control group ($p < 0.05$ or $p < 0.01$). The inhibition of MPO activity by OLE was dose dependent.

DISCUSSION

The present study clearly demonstrated the effect of OLE in a model of mechanical arthritis in rats. The OLE treatment resulted in a significant reduction in the degree of right hind paw swelling 1, 3 and 5 days after dosing, compared with the model control rats, the OLE-treated groups also displayed a statistically significant decrease in Evans blue extravasation in the right hind paw 5 days after dosing, compared with the model control rats. Histopathological examination of rat arthritis in the OLE groups revealed significant attenuation of edema of the synovial membrane and soft tissue surrounding the articulation, decreased inflammatory cell infiltration including macrophages and lymphocytes, compared with the model control rats. In this study OLE was found to be effective in a model of mechanical-induced inflammatory arthritis, the rat model of kaolin and carrageenan-induced arthritis. The efficacy of OLE in this model confirms the antireactive activity of OLE in sub-acute inflammation and arthritis and the OLE was reported to be free of adverse effects even at a repeated dose of 2000 mg/kg/day for rats (Christian *et al*., 2004), therefore the rationale for the human therapeutic use of OLE in OA may be considered.

Olive leaf extract is marketed as a natural medicine with wide-ranging health benefits. Particular emphasis is placed on the antioxidant activity of the extract and the corresponding health benefits such as the cardioprotective effects (Fito *et al*., 2007). However, the health benefits such as antiarthritis effects of OLE are not emphasized. This study showed that OLE significantly decreased the production of TNF-α after LPS treatment in THP-1 cells, a model of joint inflammation. TNF-α is the primary cytokine induced in this system and the cytokine responsible for the perpetuation of the inflammatory response in monocytes. TNF-α is also a major contributor to inflammation associated with OA (Pelletier *et al*., 2001).

Hydroxytyrosol is a phenol in the OLE. It was shown to be effective *in vitro* and *in vivo* as an antioxidant agent. It was also shown to inhibit free radical

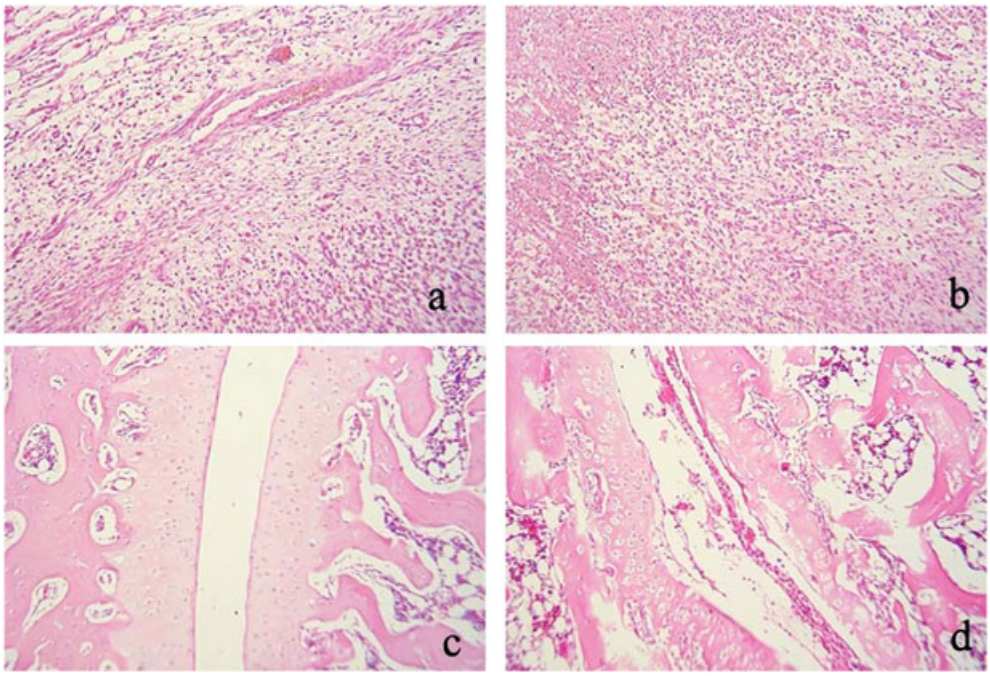

Figure 1. Photomicrographs of tibio-tarsal articulation sections and soft tissue around the articulation of kaolin and carrageenan-induced arthritis rats. (a) A mild inflammatory cell infiltration is seen in tissue of OLE group. (b) A large inflammatory cell infiltration is observed in tissue from the model control group. (c) No destruction of articular cartilage in OLE group. (d) Destruction of articular cartilage in the model control group. The dose of OLE was 100 mg/kg b.w. The OLE was given by gavage. HE × 100. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

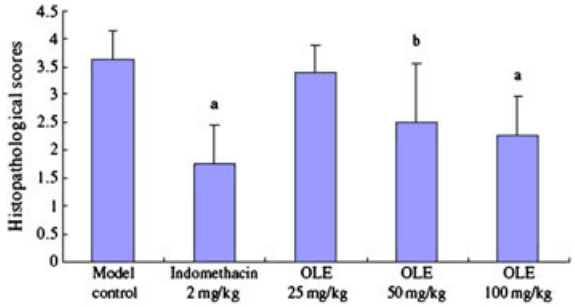

Figure 2. Right hind paw histopathological scores in kaolin and carrageenan-induced arthritis rats. Values are mean ± SD, $n = 10$. [a]Significantly different from model control at $p < 0.01$. [b]Significantly different from model control at $p < 0.05$. The OLE and indomethacin were given by gavage.

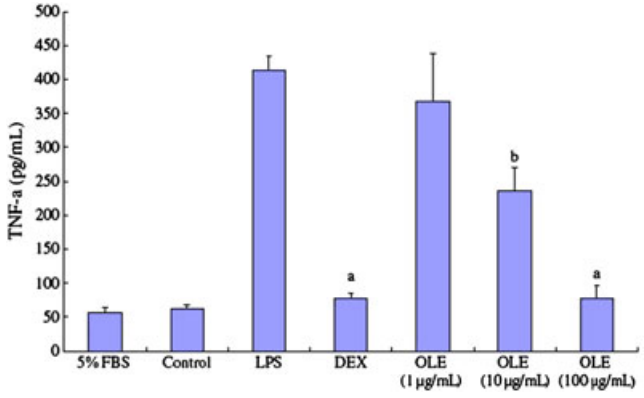

Figure 3. Effects of OLE on LPS-induced TNF-α production in human THP-1 cells. THP-1 cells were cultured in the absence of LPS with 5% FBS or with (control), in the presence of LPS alone, or with DEX (100 nmol/L, positive control), OLE. Values are mean ± SD, $n = 10$. [a]Significantly different from LPS alone at $p < 0.01$. [b]Significantly different from LPS alone at $p < 0.05$.

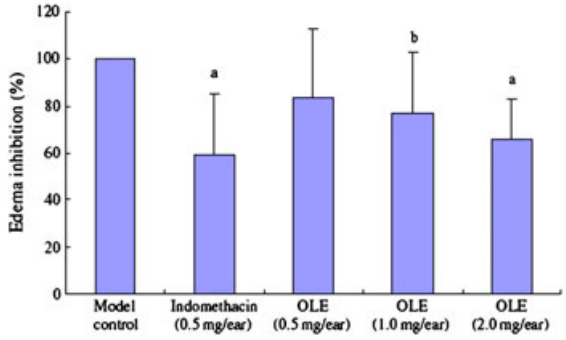

Figure 4. Effects of OLE in croton oil-induced mouse ear edema model. Values are mean ± SD, $n = 10$. [a]Significantly different from model control at $p < 0.01$. [b]Significantly different from model control at $p < 0.05$.

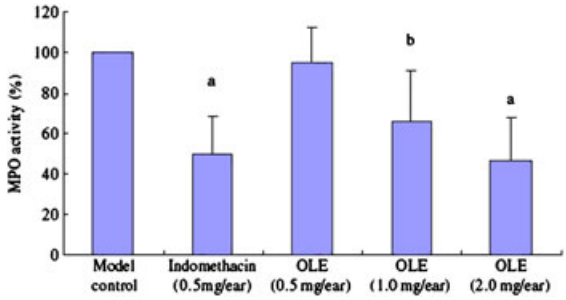

Figure 5. Effects of OLE on MPO activity in croton oil-induced mouse ear edema model, Values are mean ± SD, $n = 10$. [a]Significantly different from model control at $p < 0.01$. [b]Significantly different from model control at $p < 0.05$.

generation, scavenge reactive oxygen and nitrogen, inhibit smoking-induced oxidative stress in rats, and increase plasma antioxidant capacity (Visioli *et al.*, 2000). In human trials, the consumption of olive oil with increasing HT and tyrosol resulted in a dose-dependent decrease in oxidized LDL, a decrease in 8-oxo-7,8-dihydro-2′-deoxyguanosine in mitochondrial DNA, and a decrease in urine malondialdehyde levels (Weinbrenner *et al.*, 2004; Marrugat *et al.*, 2004). The evidence indicates that chondrocytes are potent sources of reactive oxygen species, which may damage cartilage collagen and synovial fluid hyaluronate. Recent reports have postulated that oxidative damage to cartilage may affect chondrocyte function, resulting in changes in cartilage homeostasis that are relevant to cartilage ageing and the development of OA (Yudoh *et al.*, 2005). Interleukin-1 beta (IL-1β) and TNF-α are the predominant pro-inflammatory cytokines synthesized during the arthritic process. They have been found in elevated levels in the synovial membrane, the synovial fluid, and the cartilage of OA patients and play an essential role in the cartilage destruction and inflammation process (Martel-Pelletier *et al.*, 1999). It was reported previously that OLE has antiinflammatory and antinociceptive effects and the mechanisms appear to involve a decrease in the content of the pro-inflammatory cytokine IL-1β and TNF-α in rats (Gong *et al.*, 2009). Nuclear factor kappa B (NF-κB) is a pivotal transcription factor involved in the activation of the TNF-α and IL-1β genes (Tak and Firestein, 2001). Activation of NF-κB in synovial cells is a feature seen in both OA and rheumatoid arthritis (RA) patients (Gilston *et al.*, 1997). NF-κB activation provides a potential link between inflammation and hyperplasia in the arthritic joint (Miagkov *et al.*, 1998). Therefore NF-κB represents a potential target for the treatment of a variety of disorders, such as OA and RA (Yamamoto and Gaynor, 2001). Our recent study found that HT has antioxidant activity to suppress intracellular oxidative stress and NF-κB activation in THP-1 cells (Zhang *et al.*, 2009). These reports by other groups and our own findings provide support for the hypothesis that the antiarthritic effect of OLE provides a defense mechanism against inflammatory state protective articular cartilaginous injuries.

Croton oil or 12-O-tetradecanoylphorbol acetate (TPA), which is an active component of croton oil, is a widely used agent that induces cutaneous inflammation in experimental animals. The topical application of TPA was reported to induce a longer-lasting inflammatory response with a transient increase in prostanoid production. The inflammation response induced by croton oil is associated with a marked influx of neutrophils, resulting in a gradual increase in MPO levels that persisted up to 24h. Therefore, the croton oil-induced inflammation was used to investigate the OLE effect on MPO. The MPO level is known as a marker for neutrophil content in inflamed tissue. A variety of anti-inflammatory drugs, such as diclofenac, indomethacin, naproxen, peroxicam and tenoxicam, suppresses MPO activity during inflammation (Paino *et al.*, 2005; Halici *et al.*, 2007). In the field of human medicine the activity and/or the content of MPO in synovial fluid as a marker

of joint disease has long been included in the special consideration of rheumatoid arthritis (Edwards *et al*., 1988). An increase of MPO activity in the synovial fluids of dogs with OA has been reported (Spellmeyer, 2003). Our results demonstrated that the obvious topical anti-inflammatory effect of OLE is likely associated with inhibiting the neutrophil influx and MPO activity. In a recent study, it was clearly shown that administration of OLE effectively prevents cartilaginous injuries and was promisingly beneficial for cartilage repair in rabbits (Gong *et al*., 2011). Therefore, a high dietary intake of OLE could be postulated to protect against OA.

The findings presented in this report are very encouraging and support the need for further studies. Extending the experimental duration to 30–90 days would investigate the long-term efficacy of OLE. Our results suggest that OLE treatment is beneficial in the treatment of age-related joint disease, most likely through its joint modifying and antiinflammatory properties.

This study aimed to evaluate the potential effect of OLE at lower dose levels in rat kaolin and carrageenan-induced arthritis model. Right hind paw swelling appeared following the injection of inducers to rats and histopathological features of arthritis, including edema of tissue, inflammatory cell infiltration, proliferation of synovial cells, fibrosis and destruction of articular cartilage and bone, were observed 5 days after the injection of inducers to rats, suggesting the successful establishment of a subacute unilateral mechanical arthritis model in rats. The destruction of articular cartilage is the primary pathological basis of arthritis due to trauma, infection and immune and so on, and the destruction of articular cartilage and bone was found in sections of rat arthritis in the present study. Therefore the rat arthritis model would be useful in evaluating the efficacy of the antiarthritis effect of OLE. The present findings suggest that OLE is effective in preventing the destruction of cartilage bone, as well as limiting peri-articular soft tissue inflammation in the rat model of kaolin and carrageenan-induced arthritis. Collectively, the data provide evidence supporting the dietary supplementation of OLE, which in turn may have a beneficial effect of slowing and reducing inflammation in the pathogenesis of degenerative joint diseases in man.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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