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Major Phenolic Compounds in Olive Oil Modulate Bone Loss in an Ovariectomy/Inflammation Experimental Model

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This study was conducted to determine whether the daily consumption for 84 days of tyrosol and hydroxytyrosol, the main olive oil phenolic compounds, and olive oil mill wastewater (OMWW), a byproduct of olive oil production, rich in micronutrients, may improve bone loss in ovariectomized rats (an experimental model of postmenopausal osteoporosis) and in ovariectomized rats with granulomatosis inflammation (a model set up for senile osteoporosis). As expected, an induced chronic inflammation provoked further bone loss at total, metaphyseal, and diaphyseal sites in ovariectomized rats. Tyrosol and hydroxytyrosol prevented this osteopenia by increasing bone formation ($p < 0.05$), probably because of their antioxidant properties. The two doses of OMWW extracts had the same protective effect on bone ($p < 0.05$), whereas OMWW did not reverse established osteopenia. In conclusion, polyphenol consumption seems to be an interesting way to prevent bone loss.

KEYWORDS: Tyrosol; hydroxytyrosol; olive mill waste waters; bone-sparing effect; rat

INTRODUCTION

Osteoporosis is a major health problem in this millennium, occurring as a consequence of low bone mass and disturbed bone architecture. The main pathogenic factor is an estrogen deficiency, which elicits a chronic inflammatory status by increasing the local production of various cytokines, free radicals, and growth factors by cells located within the bone microenvironment. These processes are even further exacerbated with aging.

In fact, within Europe, conspicuous differences are encountered in the severity of osteoporosis, the lowest incidence being reported in the Mediterranean area. Indeed, epidemiological evidence suggests that the Mediterranean diet, among others, which is largely vegetarian in nature and includes the consumption of large quantities of olive oil, decreases the risk of developing chronic-age related conditions. In addition to being the predominant source of fat in the Mediterranean diet, olive oil is rich in phenolic compounds. Tyrosol and hydroxytyrosol, two characteristic olive oil phenolic compounds (*1*) present in olive oil as free or conjugated forms of secoiridoids or aglycones, have been thoroughly investigated for their antioxidant activities (*2*). In addition, during olive oil production a considerable amount of water is employed, and some of the olive biophenols end up in the water phase. Only about 1−2% of the total phenols of the olives are found in the oil, because the majority of them remain in the wastewaters and in the solid wastes (OSW). This water, in addition to that endogenously contained in the olives, makes up a black effluent, which is named "olive mill waste water" (OMWW). More than 30 biophenols and related compounds have been identified in OMWW (hydroxytyrosol, tyrosol, 3,4-dihydroxyphenyl acetic acid, *p*-hydroxyphenyl acetic acid, caffeic acid, *p*-coumaric acid, ferulic acid), and among them hydroxytyrosol, tyrosol, and (5-ethylidene-2-oxotetrahydropyran-4-yl) acetic acid (lactone) are the most abundant phenolic compounds (*3*). Even if polyphenols have been shown to prevent bone loss, there are few data to support the use of polyphenols from olive oil such as oleuropein to maintain bone health (*4, 5*). Thus, we investigated the possible influence of tyrosol, hydroxytyrosol, OMWW, and an OMWW extract on osteopenia in two experimental animal models: the ovariectomized (OVX) rat, with hormonal deficiency-induced bone loss, and the OVX rat in which inflammation had been induced by subcutaneous magnesium silicate, to mimic one of the multiple factors (inflammatory and oxidative status) occurring with aging.

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Table 1. Composition of the Soy Protein-Free and Fiber-Free Powdered Semipurified Diet Consumed by Female Wistar Rats[a]

ingredient	g/kg of diet
protein	
casein	200
carbohydrate	
cornstarch	660
fat	
peanut oil	25
rapeseed oil	25
vitamin mixture (vit E−D deficient)[b]	10
mineral mixture (Ca−P−Mg deficient)[c]	25
DL-methionine	3
choline bitartrate	2

[a] From INRA (Jouy en Josas, France); casein (Union des caséinerie, Surgères, France), cornstarch (Cerestar, Saint-Maur, France), cellulose (Durieux, Marne la Vallée, France), oil (Bailly, Aulnay sous Bois, France), vitamin mixture (Roche, Neuilly sur Seine, France), mineral mixture (Prolabo, Fontenay sous bois, France), DL-methionine and choline bitartrate (Jerafrance, Jeufosse, France). [b] With DL-α-tocopherol acetate, 1.1 mg/kg; cholecalciferol, 32.25 μg/kg. [c] With calcium, 4.2 g/kg; phosphorus, 4.01 g/kg; magnesium, 1.25 g/kg.

Table 2. Hydroxytyrosol, Tyrosol, and Lactone Content of Different Diets[a]

	tyrosol	hydroxytyrosol	OMWW	EM1	EM2
tyrosol	10		3.4	10	5
hydroxytyrosol			10	30	15
lactone			3.4	10	5

[a] Data expressed as mg/kg of BW/day.

MATERIALS AND METHODS

Animals and Treatments. The study was conducted in accordance with current legislation on animal experiments in France. Female Wistar rats ($n = 140$; 6 months old) were purchased from a laboratory colony (INRA: National Institute of Agricultural Research, Clermont-Ferrand, France) and housed individually at 21 °C, under 12/12 h light/dark cycles. The rats were either sham-operated (SH, controls; $n = 20$) or surgically ovariectomized (OVX; $n = 120$) under anesthesia using Imalgen 1000 (Merial, Lyon, France; 0.75 mL/kg of body weight, intraperitoneally) and Vetranquil 1% (Ceva santé animale, Libourne, France; 0.25 mL/kg of body weight, intraperitoneally). The animals were fed a semipurified diet (**Table 1**) (INRA, Jouy en Josas, France), the daily intake being 21 g/rat. They were randomly assigned to groups (20 per group) as follows: (1) SH controls; (2) ovariectomized controls (OVX). The others were given the same standard diet supplemented with either (3) 0.017% tyrosol (Tyr), (4) 0.017% hydroxytyrosol (OHT), (5) 0.17% olive mill wastewater (OM), or (6) 0.08% OMWW water extract (EM1) and (7) 0.0425% OMWW water extract (EM2), respectively (**Table 2**). During the whole experimental period (84 days), each compound was mixed with the semipurified diet and given orally. Food was humidified (1 mL/g). The rats had free access to water. Three weeks before the end of the investigation (day 63), an inflammation was provoked by subcutaneous injections of a sterile magnesium silicate suspension (3.2 g in 4.5 mL of saline) in half of the animals, in each experimental group (*6*). On day 83, the 24 h urine production was harvested to measure excretion of deoxypyridinoline (*7*) and isoprostane levels (*8*). Blood samples were collected on days 70, 77, and 84 into EDTA tubes to determine the number of granulocytes, as a marker of the inflammatory response. At necropsy (day 84), blood heparinized samples were collected from the abdominal aorta as well, to assess osteocalcin, fibrinogen, and ferric reducing ability of plasma (FRAP). The spleen and uterine horns were removed and weighed. Left and right femurs were cleaned from adjacent tissues and collected for mechanical testing and bone mineral density measurements, respectively.

Polyphenol Preparation. *Olive Mill Waste Water Extract (EM).* OMWW, EM1, and EM2 were prepared in the Pharmacognosy Laboratory (Athens, Greece) as previously published by Agalias et al. (*9*). A 25 L amount of an OMWW solution was filtered through 25 μm filters and then concentrated to dryness in a rotary evaporator. The residue was diluted in 5 L of MeOH and extracted with 5 L of *c*-hexane to remove the remaining olive oil. The MeOH extract (first extract) was evaporated and subjected to reverse phase medium-pressure liquid chromatography with decreasing polarity mixtures of H_2O/MeOH. The obtained water fraction (second extract, EM) was then evaporated, giving 15 g of an oily residue, which was identified by NMR spectroscopy as a mixture of mainly hydroxytyrosol, tyrosol, and (5-ethylidene-2-oxotetrahydropyran-4-yl) acetic acid (lactone) (3:1:1). The concentration of total polyphenol content assayed by the Folin−Ciocalteu method for EM was 42.5 mg of caffeic acid equiv (CAE)/g.

Hydroxytyrosol. The previous water fraction was further subjected to chromatographic fractionation with a CH_2Cl_2/MeOH solvent system of increasing polarity, to give 50 fractions. Fractions 21−23 were eluted with 90% CH_2Cl_2/10% MeOH and gave 2.4 g of pure hydroxytyrosol. Purity was determined by HPLC and NMR spectral analysis.

Tyrosol. This was purchased from Sigma (St. Louis, MO).

Bone Mineral Density (BMD). BMD was assessed by dual-energy X-ray absorptiometry (DEXA), using a Hologic QDR-4500 A X-ray bone densitometer (Hologic, Massy, France). The total femur BMD (T-BMD) and the BMD of two subregions, one corresponding to the distal femur metaphyseal zone (M-BMD), which is mainly cancelous bone, and the other to the diaphysis (D-BMD), rich in cortical bone, were determined (*4, 5*).

Femoral Mechanical Testing. Femoral length and mean diaphyseal zone were measured with a precision caliper (Mitutoyo, Shropshire, U.K.). The femoral failure load was determined using a three-point bending test (*10*), with a Universal Testing Machine (Instron 4501, Instron, Canton, MA). The two lower supports were separated by a 20 mm distance to guarantee that 85−90% of the bone flexure was due to bending. The cross-head speed was 0.5 mm/min.

Marker of Osteoblastic Activity. Plasma osteocalcin (OC) was measured by RIA, using rat ^{125}I-labeled OC, goat anti-rat OC antibody and donkey anti-goat second antibody (Biochemical Technologies, Stoughton, MA). The sensitivity was 0.01 ng/mL. The intra- and interassay precisions were 6.8 and 8.9%, respectively.

Marker of Bone Resorption. Urinary deoxypyridinoline excretion (DPD) was determined by competitive RIA, using rat monoclonal anti-DPD antibody coating on the inner surface of a polystyrene tube and ^{125}I-labeled DPD (Pyrilinks-D RIA kit, Metra Biosystems, Mountain View, CA). The sensitivity was 2 nmol/L. The intra- and interassay precisions were 4 and 6%, respectively. Results were expressed as nanomoles of DPD per millimole of creatinine (*7*). The urinary creatinine assay (Bio Merieux SA, Marcy-l'Etoile, France), based on a modified Jaffe's method in which picric acid forms a colored solution in the presence of creatinine (*11*), was used to adjust DPD values for variation in urine volume.

Markers of Inflammation. Plasma fibrinogen was measured by a chronometric assay (Biodirect, Les Ulis, France), which evaluates the precipitation of fibrinogen in the presence of ammonium sulfate (*12*). Colorimetric measurement was performed at 340 nm, and the reaction was monitored for up to 190 s on 1.23 mL samples. Results were calculated from a standard scale of fibrinogen.

Blood granulocyte counts were determined with an automatic hematology counter ABC vet (Scil Animal Care Company France, Holtzheim, France) on fresh blood.

Markers of Oxidative Stress. The ferric reducing ability of plasma (FRAP) was determined in plasma using the method of Benzie and Strain (*13*), which measures the reduction of ferric iron to the ferrous form in the presence of antioxidant components. The colorimetric measurement was performed at 593 nm, and the reaction was monitored for up to 8 min on 25 μL samples. Results were calculated from a standard scale of $FeSO_4$.

Urinary isoprostane (*8*) was assessed by ELISA, using 15-isoprostane F2t conjugated to horseradish peroxidase and a polyclonal antibody specific for 15-isoprostane F2t (Oxford Biomedical Research, Oxford, MI).

Statistical Methods. Results were expressed as means ± standard errors of means (SEM). A parametric one-way ANOVA was performed to test for any difference among groups. If the result was found to be significant ($p < 0.05$), the Student−Newman−Keuls multiple-

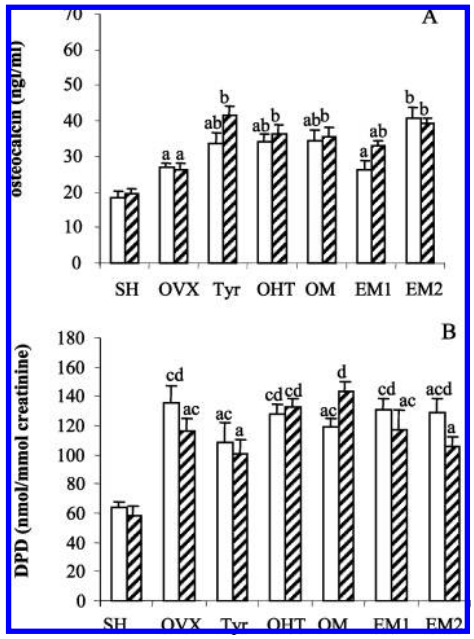

Figure 1. Bone turnover measured in sham-operated (SH) and ovariectomized rats (OVX), ovariectomized + tyrosol (Tyr), ovariectomized + OHtyrosol (OHT), ovariectomized + OMWW (OM), ovariectomized + OMWW extract 1 (EM1), and ovariectomized + OMWW extract 2 (EM2) rats with (hatched bars) or without inflammation (white bars): (**A**) plasma osteocalcin concentrations; (**B**) urinary deoxypyridinoline excretion. Values are means ± SEM, $n = 10$. Means on day 84 not sharing a letter differ significantly, $p < 0.05$.

comparison test was then used to determine specific differences between means. If a parametric ANOVA was not feasible (when there were significant differences between the SD groups, tested by Kolmogorov−Smirnov test), a Kruskall−Wallis test followed by the Mann−Whitney Wilcoxon *U* test was performed to compare differences between groups.

RESULTS

Body Weight and Uterine Weight. In each group, body weight (g) increased with aging [day 0 (SH, 290 ± 7) and day 84 (SH, 349 ± 13) ($p < 0.01$)]. Subcutaneous talc injection did not elicit any change in this parameter (SHinf, 353 ± 13), whereas excess weight was demonstrated in all OVX rats (OVX, 393 ± 7), as early as 3 weeks. This body weight gain did not differ between treated groups (mean = 398 ± 2).

Uterine weight (g), as expected, decreased after castration (0.121 ± 0.007 vs 0.929 ± 0.100 in SH, $p < 0.0001$). Neither inflammation (OVXinf, 0.126 ± 0.005) nor the intake of any tested compounds had any further significant effect (mean = 0.130 ± 0.003).

Bone Turnover (Figure 1). Plasma OC concentrations (ng/mL), measured on day 84, were lower in the SH than in the other experimental groups (SH, 18.43 ± 1.93, vs OVX, 27.08 ± 1.04; $p < 0.05$). Inflammation by itself did not modify this pattern (OVXinf, 26.30 ± 1.72). Nevertheless, in the castrated animals with chronic inflammation, a further increase was elicited by each experimental diet, except for the highest dose of the OMWW extract (EM1). This trend was also demonstrated in the OVX rats after consumption of an EM2-enriched diet.

Urinary DPD excretion (nmol/mmol of creatinine) was significantly higher in the ovariectomized rats (135.74 ± 11.04) than in the SH (63.40 ± 4.24), regardless of the talc injections or not. None of the treatments had any significant effect. Except

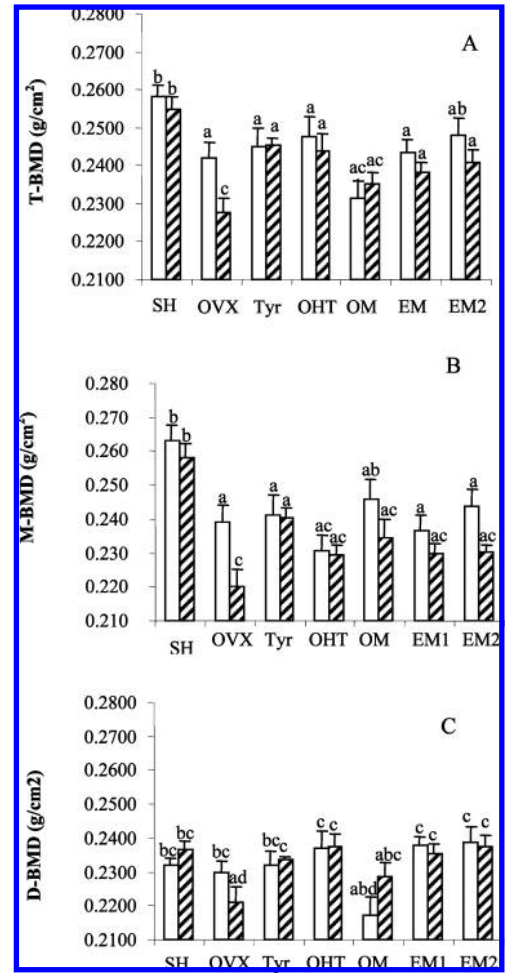

Figure 2. Bone mineral density (BMD) measured in sham-operated (SH) and ovariectomized rats (OVX), ovariectomized + tyrosol (Tyr), ovariectomized + OHtyrosol (OHT), ovariectomized + OMWW (OM), ovariectomized + OMWW extract 1 (EM1), and ovariectomized + OMWW extract 2 (EM2) rats with (hatched bars) or without inflammation (white bars): (**A**) total femur (T-BMD); (**B**) femur metaphysis (M-BMD); (**C**) femoral diaphysis (D-BMD). Values are means ± SEM, $n = 10$. Means on day 84 not sharing a letter differ significantly, $p < 0.05$.

for the OMWW (which actually worsened this increase: 143.62 ± 6.80; $p < 0.05$ vs OVXinf), every treatment was devoid of any significant effect.

Bone Mineral Density (Figure 2). As previously reported, ovariectomy induced a significant decrease in T-BMD (g/cm^2) (OVX, 0.2420 ± 0.0041, vs SH, 0.2580 ± 0.0031; $p < 0.01$). Inflammation exacerbated this osteopenia, but was devoid of any effect in SH rats [OVXinf, 0.2275 ± 0.0039 ($p < 0.01$, compared to OVX); SHinf, 0.2549 ± 0.0034 (NS)]. This decrease in T-BMD was partially prevented by all of the enriched diets (Tyrinf, 0.2453 ± 0.0020; OHTinf, 0.2440 ± 0.0044; EM1inf, 0.2381 ± 0.0029; EM2inf, 0.2410 ± 0.0031; $p < 0.05$, compared to OVXinf). Nevertheless, OMWW consumption was devoid of any protective effect (OMinf, 0.2351 ± 0.0030). As far as metaphyseal-BMD was concerned, castration was associated with osteopenia, this bone loss being even greater after talc injection. Nevertheless, only tyrosol consumption appeared to be efficient in preventing ovariectomy/inflammation-induced demineralization. Regarding diaphyseal-BMD, a decrease was observed only when administration of magnesium silicate was associated with the surgery. The

Table 3. Inflammatory Status and Oxidative Status in Sham-Operated (SH) and Ovariectomized Rats (OVX), Ovariectomized + Tyrosol (Tyr), Ovariectomized + OHtyrosol (OHT), Ovariectomized + OMWW (OM), Ovariectomized + OMWW Extract 1 (EM1), and Ovariectomized + OMWW Extract 2 (EM2) Rats with or without Inflammation[a]

	inflammatory state parameters				oxidative status parameters			
	fibrinogen (g/L)		spleen wt (g)		isoprostane (ng/mg of creatinine)		FRAP (μM Fe^{II}/L)	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
				Without Inflammation				
SH	1.67	0.11	0.81	0.04	1.90	0.26 a	191	10 b
OVX	1.88	0.06	0.75	0.02	1.38	0.30 a	167	6 bc
Tyr	1.86	0.09	0.82	0.03	1.17	0.19 a	192	10 b
OHT	1.94	0.10	0.84	0.04	1.94	0.21 a	149	7 ac
OM	2.18	0.07 a	0.88	0.05	2.76	0.33 bc	149	7 ac
EM1	1.86	0.08	0.81	0.05	1.24	0.20 a	200	15 b
EM2	1.97	0.05	0.84	0.03	2.13	0.28 ab	153	8 ac
				With Inflammation				
SH	1.68	0.08	0.96	0.04 a	2.15	0.45 ab	126	11 a
OVX	2.20	0.13 a	0.95	0.06 a	2.77	0.49 bc	130	6 a
Tyr	2.46	0.16 a	1.09	0.06 ab	1.66	0.13 ab	118	10 a
OHT	2.42	0.06 a	0.98	0.05 a	1.98	0.22 a	134	7 a
OM	2.58	0.19 a	1.15	0.08 b	3.34	0.23 c	116	8 a
EM1	2.72	0.14 a	1.06	0.05 ab	1.76	0.38 a	148	4 a
EM2	2.95	0.15 a	1.11	0.02 ab	1.53	0.06 a	136	5 a

[a] Values are means ± SEM. Mean values (10 rats per group) within a row with unlike letters differ ($p < 0.05$).

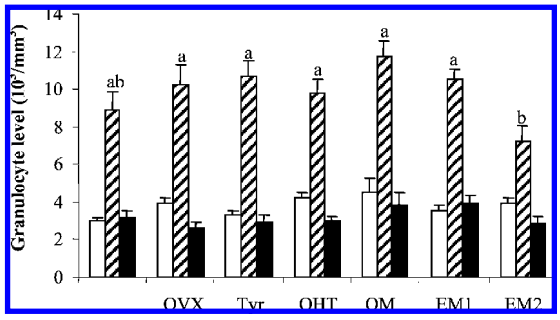

Figure 3. Granulocyte levels in sham-operated (SH), ovariectomized (OVX), ovariectomized + OW (OM), ovariectomized + tyrosol (Tyr), ovariectomized + OHtyrosol (OHT), ovariectomized + EM1 (EM1), ovariectomized + EM2 (EM2) rats at three times: before (white bars), during (hatched bars), and 3 weeks after inflammation (black bars). Values are means ± SEM, $n = 10$; means on day 84 not sharing a letter differ significantly: $p < 0.001$ (a); $p < 0.05$ (b).

different treatments were able to restore this parameter, up to values measured in both OVX and SH animals, except for OMWW intake.

Femoral Mechanical Testing. No significant difference in the femoral failure load (N) (mean value = 123 ± 1) was demonstrated among groups (data not shown).

Markers of Inflammation (Table 3 and Figure 3). Plasma fibrinogen concentrations (g/L) were significantly higher in all OVX groups with granulomatous inflammation ($p < 0.05$), but not in the SH. The different diets did not show any effect on this acute phase protein, although higher values were measured in the OVX animals that had been given the OMWW (**Table 3**).

Spleen weight (g) was significantly higher in animals receiving subcutaneous talc injections, compared to controls ($p < 0.05$). This splenomegaly was exacerbated by OMWW ($p < 0.05$). The other treatments did not improve this parameter (**Table 3**).

Two weeks after injection of magnesium silicate, the number of blood granulocytes (10^3/mm^3) increased ($p < 0.001$) in all groups [SH, 8.91 ± 0.98; OVX, 10.25 ± 1.02, compared to basal values (SH, 3.01 ± 0.16; OVX, 3.95 ± 0.24)]. This inflammation was partially prevented by the lowest dose of OMWW extract (EM2, 7.21 ± 0.83, vs OVXinf; $p < 0.05$). At the end of the experimental period, this parameter returned to initial levels (SH, 3.13 ± 0.44; OVX, 2.67 ± 0.28) (**Figure 3**).

Marker of Oxidative Stress (Table 3). Urine isoprostane levels (ng/mg of creatinine), a marker for lipid peroxidation, increased after the injection of talc ($p < 0.05$). Each compound added to the diet prevented this rise, except for OMWW ($p < 0.05$).

Ferric-reducing potential value (FRAP) (μM Fe^{II}/L), which reflects the antioxidant capacity of plasma, was significantly decreased in rats under inflammatory conditions ($p < 0.005$). None of the tested diets was able to restore the plasma antioxidant capacity to values measured in controls.

DISCUSSION

This study was carried out to assess the bone-sparing effect of tyrosol and hydroxytyrosol, bioactive metabolites of oleuropein with anti-inflammatory and antioxidant properties. In the experiment, we selected the dose of phenolic compounds according to a previous study on the sparing effect of oleuropein (10 mg/kg of BW/day; 0.017% in the diet) in the same experimental model (*4*). Moreover, Martinez-Dominguez et al. (*14*) had demonstrated that virgin olive oil with a higher content of polyphenolic compounds shows protective effects in experimental models of inflammation.

In our conditions, the olive oil polyphenols such as tyrosol and hydroxytyrosol and the OMWW extract appeared to be able to prevent bone loss caused by estrogen deficiency associated with inflammation in rats, whereas the OMWW was not.

Estrogen Deficiency State. As expected, ovariectomy (confirmed by uterine atrophy) greatly reduced BMD in the distal femur resulting from increased bone turnover, as indicated by the higher plasma OC concentrations and urinary DPD excretion rate in the OVX group compared to the SH group. These results are in agreement with those from Wronski et al. (*15*), which demonstrated that bone remodeling in rats is accelerated after the cessation of ovarian function. Osteopenia due to ovariectomy remained unchanged under any polyphenol-enriched diet effect, as shown by values of bone mineral density, which were not improved. Moreover, ovariectomy did not induce a modification

of the antioxidative capacity of plasma (ferric-reducing potential) in rats. However, Maggio et al. (*16*) found a marked decrease in plasma antioxidants in a group of elderly osteoporotic women. Under such conditions, increasing the levels of natural antioxidants was of little interest. In addition, with respect to the inflammatory parameters, it is well recognized that estrogens modulate the immune response and inflammatory processes. Nevertheless, in our experiment a discrepancy should be noted because we failed to demonstrate an increase of spleen weight and granulocyte levels after castration. In postmenopausal women, accelerated bone loss that occurs following lack of estrogens can be correlated to an increase in cytokine production by peripheral blood monocyte culture. In any case it is well-known that molecules with estrogenic activity are effective in the prevention of bone loss associated with early menopause. Probably only polyphenols with intrinsic estrogenic activity could directly target bone cells.

Estrogen Deficiency with an Inflammatory Condition. We demonstrated here that subcutaneous implantation of magnesium silicate inducing chronic granulomatous inflammation (IMO) (*6*) was associated with a decrease of both trabecular and cortical BMD in estrogen-deficient animals. This trabecular bone loss was the result of the suppression of the longitudinal bone growth and the inability of osteoblast to synthesize bone matrix and form normal secondary spongiosa (*6*). Talc administration also led to an increase of inflammatory parameters (*17*), as shown by a higher blood level of granulocytes or fibrinogen (an acute phase protein) 2 weeks after injection, and splenomegaly. The IMO model induces an inflammatory response mediated at least in part by arachidonic acid metabolism, nitric oxide, prostaglandins, and cytokines [IL1, IL6, and TNFα (*18*)]. TNF was the first cytokine released into the plasma, and direct administration of anti-TNFα antibody neutralizes the effect of inflammation on bone (*19*). Concomitant with inflammation there was generation of reactive oxygen species (ROS) or free radicals, which increase oxidation of proteins and lipids, resulting in signals that trigger more inflammation (*20*). In this study, oxidative stress was elicited as well, as indicated by a decrease of the antioxidative capacity of plasma (FRAP) and an enhanced urinary excretion of isoprostanes.

In our experimental conditions, when tyrosol and hydroxytyrosol were given as simple phenols at the same concentration (10 mg/kg of BW/day), they were able to prevent inflammation-induced osteopenia in ovariectomized rats. This significant effect on T-BMD and D-BMD was probably explained by an improvement of osteoblastic activity, as shown by osteocalcinemia data. Nevertheless, tyrosol was the only efficient compound on M-BMD.

When they were given as the mixture of mainly hydroxytyrosol, tyrosol, and lactone in an extract of OMWW at two different doses, they prevented inflammation-induced bone loss in OVX rats. The highest dose (EM1, 10 and 30 mg/kg of BW/day of tyrosol and hydroxytyrosol, respectively) seemed to act only thanks to antioxidant properties, whereas the lowest dose (EM2, 5 and 15 mg/kg of BW/day of tyrosol and hydroxytyrosol, respectively) seemed to work through antioxidant and anti-inflammatory properties. Data published by Visioli et al. (*2*) show that a hydroxytyrosol-rich OMWW extract prevented oxidative stress in rats exposed to sidestream smoke for 20 min once a day. Moreover, Covas et al. (*21*) showed that the degree of oxidative stress was modulated, in an inverse relationship, by the phenolic content of olive oil.

When they were administered as the mixture such as OMWW that provided 3.4 and 10 mg/kg of BW/day of tyrosol and hydroxytyrosol, respectively, they did not prevent bone loss and induced a pro-inflammatory status, which could explain this lack of protective effect. This could be explained by the composition of OMWW, which is complex and may include toxic compounds (*22*).

Concerning the antioxidant properties of the two phenols, which have been thoroughly investigated, administration of hydroxytyrosol has been shown to reduce the consequences of passive smoking-induced oxidative stress in rats (*2*) and to decrease the amount of isoprostanes excreted in urine in humans (*23*).

Tyrosol is also effective in reverting H_2O_2 production induced by oxLDL and acid arachidonic release and PGE2 synthesis in RAW 264.7 macrophage (*24*). The mechanism by which hydroxytyrosol, like other simple phenols, scavenges free radicals probably involves proton donation to radical species (*25*) or binding to iron ions (*26*), via the decrease of reactive oxygen species and the increase of glutathione (*27*). Tyrosol exhibits a lower antioxidant activity than does OHtyrosol, in agreement with the assumption that an *ortho*-hydroxyl group confers a strong action. Miles et al. (*28*) suggested that phenolic compounds with a single structure, involving only a single phenol ring, may be unlikely to exert anti-inflammatory effects by inhibiting the production of inflammatory cytokines or PGE2 and that a more complex structure may be required to elicit these effects.

Phenolic compounds can exert anti-inflammatory action in the cell by interacting with a wide spectrum of molecular targets central to the cell signaling machinery. They could inhibit cellular enzymes, such as PLA2, COX, and LOX, to reduce arachidonic acid, prostaglandin, and leukotriene production (*29*). Tyrosol inhibited COX-2 and iNOS gene expression in RAW 264.7 macrophages stimulated by gliadinin association with interferon-γ (IFNγ), probably through the NFκB pathway (*30*). In fact, NFκB plays a pivotal role by coordinating the induction of a wide range of genes encoding pro-inflammatory cytokines (e.g., IL-1, IL-2, IL-6, and TNFα), chemokines (e.g., IL-8, MIP-1α, and MCP-1), adhesion molecules (e.g., ICAM, VCAM, and E-selectin), acute-phase proteins, COX-2, iNOS, etc.

Hydroxytyrosol can inhibit the production of LTB4 from endogenous arachidonic acid (*31*), and it was able to inhibit COX-2 and iNOS gene expression in LPS-stimulated J774 murine macrophages (*32*). This anti-inflammatory activity in LPS-induced murine macrophages was determined by preventing NFκB, STAT-1α, and IRF-1 activation, consequently inhibiting iNOS and COX-2 gene expression (*32*).

In conclusion, this study suggests the possible relevance of the dietary intake of olive oil, based on the capability of its major polyphenols, hydroxytyrosol and tyrosol, to lower the risk of inflammation-induced osteopenia in estrogen-deficient animals by their antioxidant activity.

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LITERATURE CITED

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