Olive oil exhibits osteoprotection in ovariectomized rats without estrogenic effects

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Abstract. The present study was designed to evaluate the effect of olive oil on bone and uterus in ovariectomized rats. A total of 34 surgically ovariectomized or sham-operated virgin Sprague-Dawley rats were divided into four groups: i) Sham-operated control rats (sham group); ii) Ovariectomized rats (OVX group); iii) Olive oil-supplemented ovariectomized rats (olive group); and iv) Diethylstilbestrol-supplemented ovariectomized rats (E2 group). At 12 weeks following left ventricular blood sacrificed to detect plasma estradiol (E2), interleukin-1β (IL-1β) and IL-6 levels. Bone mineral density (BMD) of the lumbar spine was evaluated using dual-energy X-ray absorptiometry, and the left femur proximal 1/3 slices were observed using transmission electron microscopy. Uterine wet weight and the uterus index (ratio of uterine wet weight and body weight) were compared, and the uterine endometrium was observed using a light microscope. In the OVX group, serum E2 was significantly lower and IL-1β levels increased and IL-6 levels decreased in the olive group, but showed no significant difference compared with the sham group. The lumbar spine BMD in the olive group was increased compared with OVX group. Electron microscopy revealed sparse collagen fibers in the OVX group, with decreased density and multi-cavity, showing pathological features of osteoporosis. By contrast, the situation was improved in the E2 and olive groups, in which organelles such as the rough endoplasmic reticulum, mitochondria and Golgi apparatus were visible and active. Compared with the sham group rats, the uterine wet weight and uterine index decreased in the OVX and olive groups; however, no statistically significant difference was observed in the E2 group. Furthermore, endometrial hyperplasia was not observed in the olive group, which were apparently different from E2 group. The present results suggest that olive oil can effectively reduce bone loss in ovariectomized rats, and with no or only mild effects on the uterine endothelium.

Introduction

Hormone replacement therapy (HRT) may exert beneficial effects in the bone of patients with postmenopausal osteoporosis; however, the chronic use of HRT has been associated with several risk factors (1). This is particularly apparent for hormone-dependent gynecological malignancies, and the appropriate usage of HRT has become a highly controversial issue (1,2). Therefore, there is currently an urgent requirement for non-estrogenic, bone protective compounds. Phytoestrogens, which have similar although milder effects to those of estradiol-17β benzoate, are involved in the maintenance of bone mass in the post-menopausal period for their putative function as osteoprotective agents (3). Numerous traditional Chinese medicine (TCM) compounds, particularly kidney-beneficial herbal medicines such as Zuogui Pill, Rehmannia glutinosa, Ferula, in addition to various extracts from food or TCM herbs such as isoflavones (e.g. daidzein), have been suggested to prevent ovariectomy-induced bone loss and other antiosteoporotic effects without influencing hormones such as estrogen (3-6). Phytoestrogenic isoflavones and their derivatives extracted from soy have been shown to increase bone density in postmenopausal women following high dietary intake (3-7).

The human diet may contain the phytochemicals possessing antioxidant and anti-inflammatory properties, and antioxidant nutrients may enhance bone formation and reduce the production of free radicals that contribute to bone resorption (8,9). Furthermore, the bone-protective effects of estrogen may involve the suppression of inflammatory cytokines such as interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α) (10). The lowest incidence of osteoporosis has been reported in the Mediterranean area within Europe (11,12), due to the so-called Mediterranean diet, which reportedly exerts a beneficial effect on gynecological malignancies, such as breast and endometrial cancer (13,14). Olive oil is one of the three most important and characteristic components of the Mediterranean diet, which contains a series of phenolic
minor compounds such as hydroxytyrosol and oleuropein, and is able to scavenge superoxide radicals and inhibit neutrophil respiratory burst (15). The other two primary components of the Mediterranean diet are fruits and vegetables, and nuts and cereals.

To date, the oil extracts of numerous plant species have been demonstrated to possess similar efficacy to 17β-estradiol in suppressing bone loss due to ovariectomy and to have a marked effect in anti-osteoporosis, for example of Korean safflower seed oil, virgin coconut oil and garlic oil (16,17). Furthermore, olive oil and its major phenolic compounds may exert a marked effect in osteoporosis. A prior study by Puel et al demonstrated that oleuropein and extra virgin olive oil were able to elicit protective effects on bone loss in a model of ovariectomy associated with inflammation, probably by modulating variables of inflammation, such as α-1-acid glycoprotein (18). In another study, OVX rats were administered oleuropein (a compound extracted from olive leaf) at 2.5, 5, 10 or 15 mg/kg body weight per day for 100 days, and plasma fibrinogen concentration (g/l) was found to be significantly higher in the OVX group compared with the SH group (19). A further increase was observed after inflammation in estrogen-deficient animals, while this marker for inflammation remained unchanged in intact rats (12,20,21). This pattern was partially improved by polyphenol supplementation, as fibrinogen was restored to values measured in OVX animals without talc administration (22). In this case, talc was used to stimulate and induce inflammation (22). In addition, an extra virgin olive oil total polyphenolic fraction (TPF) was isolated and its effect on the bone loss attenuation was investigated, the result showed that the TPF only slightly induced the uterine wet weight (P=0.058) (23). Garia-Villalba et al results suggest that postmenopausal women could be a target population for olive oil supplementation in order to prevent age-related and oxidative stress-related processes such as osteoporosis (23).

Consequently, the aim of the present study was to investigate the effects of olive oil supplementation in OVX rats modeling post-menopausal osteoporosis. Furthermore, olive oil was compared with E2 to determine whether olive oil has functions at the uterine tissue level in a manner similar to 17β-estradiol.

Materials and methods

Animals and treatments. A total of 34 female Sprague-Dawley rats (weight, 190±20 g) were provided by the Division of Comparative Medicine of Fuzhou General Hospital of Nanjing Command PLA (permission no. SCXK (2012-0001); Fuzhou, China) were used for the present experiments. All rats were maintained in individual cages in a temperature-controlled room (21°C) with a 12-h light/dark cycle with free access to water. The rats were fed a diet constructed in the laboratory according to the normal nutritional dietary requirement (59% carbohydrates, 7% fat, 21% protein and 13% minerals and ash). The protocol was approved by the Ethics committee of Fuzhou General Hospital of Nanjing Command PLA and was conducted in accordance with current legislation on animal experiments.

All rats were sham-operated or surgically ovariectomized under anesthesia using chloral hydrate (4 ml/kg; Fluka Chemie AG, Buchs, Switzerland) in 80 g/l saline solution (9 g NaCl/l), and were randomly allocated into four groups: i) Sham-operated control rats (sham group, n=8); ii) Ovariectomized rats (OVX group, n=9); iii) Diethylstilbestrol-supplemented ovariectomized rats (E2 group, n=8); and iv) olive oil-supplemented ovariectomized rats (olive group, n=9). All rats were orally administered with their allocated diet (1 ml/100 g) by oral tubal feeding for 12 weeks. Sham and OVX groups were administered daily with saline. E2 group mice received a solution of diethylstilbestrol (0.0033 mg; Zhunzi H34021250; batch number: 20100920; Hefei Lifeon Pharmaceutical Co., Ltd., Hefei, China). Olive group mice received Terra Creta Kolymvari Chania Cretei Extra Virgin Olive Oil (Terra Creta SA, Chania, Crete, Greece), with the following component values: Acidic, <0.5%; fatty acid composition, 79% monounsaturates and 7% polyunsaturates; and trans fatty acid C18 composition, 1T≤0.05 (C18:2T + C18:3T; integrated value ≤0.05). All administered substances were gavaged at 1 ml/100 g once daily for 12 consecutive weeks. Body mass was weighed once a week to adjust the dosage. To prevent hyperphagia associated with ovariectomy, the OVX rats were pair-fed to the mean intake of those in the sham group. At the end of the experiments, animals were sacrificed using ether (Shanghai Lianshi Chemical Reagent Co., Ltd., Shanghai, China) inhalation and exsanguination.

Serum analyses. Blood samples were centrifuged (2,500 x g for 20 min) and the was serum stored at -20°C for further analysis. Serum E2 levels were determined by radioimmunoassay kit (Fu Sheng Industrial Co., Ltd., Shanghai, China), while serum IL-1β and IL-6 levels was measured using an ELISA (Fu Sheng Industrial Co., Ltd.) double antibody sandwich method. The clinical laboratory of Fuzhou General Hospital was entrusted to complete all of the above test indexes according to the specific measurement of their indicators by double-blind testing.

Bone mineral density (BMD) measurements. The BMD of the lumbar spine was measured using a Discovery dual-energy X-ray absorptiometer (DXA) (S/N 806409) bone densitometer (Hologic, Inc., Bedford, MA, USA), equipped with appropriate software for bone density assessment in small laboratory animals. Unit: (g/cm²). The intra-assay and inter-assay coefficients of variability for lumbar spine assays were 0.22 and 0.24% respectively.

Femoral head visualization. Two left femoral heads of the proximal femur were selected from each group rat, cut into 1x1x1 mm pieces and washed in phosphate-buffered saline (PBS) (Fuzhou Maixin Bio, Co., Ltd., Fuzhou, China). The samples were then placed in 2.5% glutaric aldehyde solution (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for 3 h at 4°C, decalcified in 5% ethylenediaminetetraacetic acid (EDTA) (Fuzhou Maixin Bio, Co., Ltd.) for three weeks by changing EDTA solution every week. Subsequently, the samples were sent to the Department of Pathology to be fixed in 1% osmium tetroxide, embedded and cut into thin sections with Epon812 (both purchased from SPI Supplies, West Chester, PA, USA), double-stained using uranyl acetate (China National Nuclear Corporation, Beijing, China) and
lead citrate (Sinopharm Chemical Reagent Co., Ltd.), finally observed using transmission electron microscope (EM208S; Philips, Amsterdam, Netherlands).

**Uterine endothelium visualization.** Samples of uterine tissues were fixed, embedded in paraffin and cut into serial cross sections. The skin was cut vertically in the median of the lower abdomen and the peritoneum was opened into the abdominal cavity. The ‘Y’ type uterus was extracted and adipose tissue removed. Wet weight was recorded and the uterus index calculated (uterine wet weight / body weight). Then, two embedded rat uteri were selected from each group, and made into paraffin sections. The extent of endometrial hyperplasy was observed under a light microscope [2506; Motic (Xiamen) Electric Group Co., Ltd., Xiamen, China] and photographed using a microscope with a digital camera (BX51; Olympus Corporation, Tokyo, Japan).

**Statistical analysis.** Data are expressed as the mean ± standard deviation. Statistical significance for data was determined using one-way analysis of variance with post-hoc test, significance calculated using a Least Significant Difference multiple range-test to find inter-group significance. P<0.05 was considered to indicate a statistically significant difference.

### Results

**Comparison of serum E₂ levels among groups.** By analysis of variance indicated statistically significant differences in E₂ expression among groups (F=9.934; overall significance, P=0.000). Serum E₂ values in the olive group were not significantly different compared with the sham group (P>0.05); however, they were significantly increased compared with the OVX group (P=0.001), as shown in Table I.

**Comparison of serum IL-1β levels among groups.** Serum IL-1β concentration was similar in the sham, E₂ and olive groups (P>0.05). This marker was increased in the OVX animals, but was mitigated in the olive and E₂ group rats (Table II).

**Comparison of serum IL-6 levels among groups.** In OVX group, IL-6 levels were significantly higher than Sham group and E₂ group, the difference was statistically significant (P<0.01), but no statistical significance with Olive group.
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There was not statistically significant in E$_2$ group compared with Sham group (P>0.05), as shown in Table III.

**Comparison of absolutes values (g/cm$^2$) of BMD of the lumbar spine among groups.** Lumbar spine BMD in the olive group was significantly higher compared with the OVX group, but showed no significant difference compared with the sham group. However, lumbar spine BMD was not significantly different between the E$_2$ and OVX groups (P>0.05), as shown in Table IV.

**Femoral head under transmission electron microscopy.** Collagen fibers in the sham group showed light and dark stripes, closely and neatly arranged. In addition, osteoblasts and numerous Golgi apparatus, endoplasmic reticula and other organelles could be seen, as shown in Fig. 1.

Collagen fibers in the OVX group were sparse, exhibited bending and breaking, and numerous voids are visible, also osteoclast were clearly visible, presenting pathological features of osteoporosis, as shown in Fig. 1.

The arrangement of collagen fibers in the E$_2$ and olive groups improved compared with OVX group, with organelles such as the rough endoplasmic reticulum, mitochondria and Golgi apparatus being visible and active, as shown in Fig. 1.

**Body weight pre-treatment and post-treatment.** None of the groups exhibited a significant difference in mean initial body weight (P>0.05). At 90 days after operation, weights of OVX group rats were increased by 57.16%, which was increased compared with the sham group (30.95%). The two groups showed a significant difference in body weight after 90 days (sham, 269.63±6.70 g vs. OVX, 316.33±14.20 g, P=0.007). Weights of E$_2$ group rats showed no significant difference compared with the sham group rats, but were reduced compared with the OVX group rats. Weights of the olive group rats did not significantly differ from those of the OVX and sham groups (P>0.05) (Fig. 2).

**Uterine weight.** Uterine weights and uterine index values in the OVX and olive group rats were significantly decreased compared with the sham group rats (P<0.001). Treatment of E$_2$ group significantly increased uterine weights, significantly different with OVX group, but had no significant difference with sham group (P>0.05). The E$_2$ group exhibited no significant difference compared with the OVX group (P>0.05) (Table V).

**Comparison of histomorphometric variables of uterine among groups.** HE staining and visualization using light microscopy at low magnification (magnification, x40) revealed that the olive and OVX groups showed thin endometria, with no

### Table IV. BMD of the lumbar spine among groups (mean ± standard deviation).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BMD (g/cm$^2$)</th>
<th>P$^a$</th>
<th>P$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>8</td>
<td>0.2476±0.0049</td>
<td>-</td>
<td>0.004</td>
</tr>
<tr>
<td>OVX</td>
<td>9</td>
<td>0.2262±0.0049</td>
<td>0.004</td>
<td>-</td>
</tr>
<tr>
<td>E$_2$</td>
<td>8</td>
<td>0.2291±0.0053</td>
<td>0.015</td>
<td>0.679</td>
</tr>
<tr>
<td>Olive</td>
<td>9</td>
<td>0.2431±0.0046</td>
<td>0.521</td>
<td>0.018</td>
</tr>
</tbody>
</table>

$^a$P-value vs. sham group; $^b$P-value vs. OVX group. BMD, bone mineral density; OVX, ovariectomized group; E$_2$, diethylstilbestrol group.
proliferation. However, in the E$_2$ group the columnar epithelial thickened, the number of glands increased, with evident hyperplasia (Fig. 3).

Discussion

Ovarian, cervical and endometrial cancers are common gynecological malignancies, whose incidence are increasing (24-26). This is particularly evident in the case of ovarian cancer, which poses a serious threat to female health (25). With the continuous development of medical diagnosis and treatment technology, the cure rate and disease-free survival of patients has improved significantly (27). However, radical surgery and adjuvant chemotherapy-induced menopause syndrome are becoming serious factors affecting the quality of life of patients (28), particularly osteoporosis, whose risk is significantly higher than natural menopause, and the problems caused by its fracture in postmenopausal women has been described as a ‘silent killer’ (29). Hormone replacement therapy (HRT) has been publicly recognized as the first choice prevention and treatment for postmenopausal osteoporosis, and the majority of obstetrician-gynecologists are aware of the osteoporosis problem. However, non-HRT users are often screened, and in a recent survey 69.4% of gynecologists speculated that HRT could increase the risk of breast cancer, and 52.9% speculated that HRT may increase the risk of endometrial cancer (30). The appropriate use of HRT for the treatment of osteoporosis and postoperative gynecological malignancies has become a highly controversial issue, particularly in the case of hormone-dependent gynecological malignancies (31). Therefore, there is a requirement for treatments for osteoporosis that have fewer undesirable side effects on the female reproductive system as estradiol-17β benzoate.

The present results show that the administration of olive oil to ovariectomized rats resulted in increased serum levels of E$_2$ compared with the OVX group, but with no statistically significant difference compared with the sham group. The serum IL-1β level in the olive group rats showed no statistically significant difference compared with the OVX group. The serum IL-6 level in the olive group rats did not differ significantly compared with the OVX group. These results indicate that olive oil supplementation can result in

Table V. Uterine weight and uterine index among groups (mean ± standard deviation).

<table>
<thead>
<tr>
<th>Group</th>
<th>Uterine weight (mg)</th>
<th>Uterine index (mg/g)</th>
<th>P$^a$</th>
<th>P$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>674.05±68.40</td>
<td>2.485±0.227</td>
<td>-</td>
<td>0.000</td>
</tr>
<tr>
<td>OVX</td>
<td>116.71±15.54</td>
<td>0.358±0.035</td>
<td>0.000</td>
<td>-</td>
</tr>
<tr>
<td>E$_2$</td>
<td>539.61±58.15</td>
<td>2.061±0.299</td>
<td>0.118</td>
<td>0.000</td>
</tr>
<tr>
<td>Olive</td>
<td>233.69±22.17</td>
<td>0.785±0.073</td>
<td>0.000</td>
<td>0.097</td>
</tr>
</tbody>
</table>

$^a$P-value vs. sham group; $^b$P-value vs. OVX group. OVX, ovariectomized group; E$_2$, diethylstilbestrol group.

Figure 3. Morphology of the endometrium among the four groups. OVX, ovariectomized group; E$_2$, diethylstilbestrol group.
increased serum E₂ and decreased IL-1β levels. By contrast, diethylstilbestrol supplementation of the ovariectomized rats increased serum E₂ and decrease IL-1β and IL-6 levels.

Ovariectomy induced a significant reduction in total BMD (OVX, 0.2262±0.0049 g/cm²; sham, 0.2476±0.0049 g/cm²; P=0.004). However, olive oil reduced this bone loss and showed no significant difference compared with the E₂ group (olive, 0.2431±0.0046 vs. E₂, 0.2291±0.0053 g/cm²; P=0.053). The arrangement of collagen fibers in the olive group were more tightly packed and regular compared with the OVX group, while organelles such as the rough endoplasmic reticulum, mitochondria and Golgi apparatus were visible and active, indicating micro proliferation of bone.

Estrogen deficiency induces a mild increase in the production of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, systemically and locally, which promotes osteoclastogenesis in the bone marrow (32,33). IL-1β and IL-6 are proinflammatory cytokines involved in bone turnover. IL-1β is a potent stimulant of bone resorption, acting directly on osteoclasts and osteoclast precursors (34). Numerous studies have investigated the associations between IL-1β gene polymorphisms and susceptibility to osteoporosis and BMD in various populations (35,36). A prior study enrolled 226 postmenopausal women with a diagnosed BMD T-score <−2.5 standard deviation (SD) and 224 postmenopausal women with a BMD T-score >−2.5 SD to investigate the associations between cytokine gene polymorphisms (IL-1β, IL-2 and IL-6) and BMD values (37). The result suggested that among the women with T-scores <−2.5 SD, the BMD values were significantly lower in the carriers of the IL-6 GG genotype compared with those with the CC and GC genotypes. Furthermore, there was a statistically significant association between the IL-6 −174 G/C polymorphism and osteoporosis in postmenopausal women (37). As the immune system plays such a critical role in the pathophysiology of postmenopausal osteoporosis, cytokines have the ability to influence each other; just as a mild increase in the production of proinflammatory cytokines would be found in a patient with osteoporosis, an increase in proinflammatory cytokines may exacerbate osteoporosis (37). Terauchi has suggested that counteracting these interactions could be a novel strategy for osteoporosis treatment (38). Puel et al showed that the daily consumption of the major phenolic compounds in olive oil for 84 days modulated bone loss in an ovariectomy/ inflammation experimental model, concluding that polyphenol consumption of olive oil may be associated with the mitigation of bone loss (22).

The aforementioned results suggest that olive oil is able to increase E₂ levels, and have positive effects on proinflammatory cytokines IL-1β and bone loss, as indicated by the collagen fibers of femoral, indicating that olive oil supplementation in OVX-rats attenuated ovariectomy-induced osteoporosis. A possible reason for this improvement in bone loss could be attributed to the elevated levels of E₂ and the high content of monounsaturated fatty acid in olive oil, which has been reported to affect BMD (39). However, as supplementation with diethylstilbestrol was also able to increase serum E₂ and decrease IL-1β and IL-6 levels in the ovariectomized rats, the estrogenic effects were more marked compared with olive oil. It is possible that the total quantity of olive oil administered in this experiment were insufficient to equal the effects of diethylstilbestrol.

The body weights of the E₂ group rats were similar to the sham group rats, but reduced compared with the OVX group rats. Body weights of the olive group rats showed no significant difference compared with sham group rats (P>0.05), suggesting that olive oil may not share the positive effect of controlling body weight increase induced by ovariectomy as exhibited by estrogen supplement. A diet rich in unsaturated fatty acids from olive oil reduces waist girth, a significant indicator of metabolic disease, in addition to reducing body mass index (40). In addition, a study supports the hypothesis that extra virgin olive oil phenols protect low density lipoproteins in the plasma against oxidation (41).

Uterine wet weight and uterine index values of the olive group rats decreased compared with sham group (P<0.01), but showed no significant difference compared with the OVX group (P>0.05). By contrast, the uterine weight and uterine index of the E₂ group rats significantly increased compared with the OVX group (P<0.01), and the uterine index was not statistically significant compared with the sham group. Furthermore, the endometria in the olive group were atrophic, thinner and the number of glands was reduced, as imaged using a light microscope. The endometria exhibited hyperplasia in the E₂ group, and were significantly different compared with the olive and OVX groups. These results suggest that olive oil had no significant or only a mild effect on the uterus, indicating that it may be safe to use as a form of hormone replacement therapy in treating female genital mutilation. The results may be similar to Keiler AM and colleagues’ study, in which female Lewis rats ovariectomized and fed a diet enriched with a total phenolic extract of extra virgin olive oil in a concentration of 800 mg/kg diet for 12 weeks (42). The results showed that oleocanthal, a compound of the polyphenolic fraction, showed a higher relative estrogen receptor binding affinity to the estrogen receptor α (ERα) compared to the ERβ. While the total polyphenolic fraction (TFP) only mildly increased the uterine wet weight (490.7±53.7 vs. 432.7±23 mg/kg body weight; P=0.058), it regulated estrogen response genes in the uterus (progesterone receptor, antigen identified by monoclonal antibody Ki67, complement C3). These results suggested that the administration of extra virgin olive oil polyphenols regulated uterine estrogen response marker genes in an E₂-agonistic manner (42).

Olive oil may be rich in phytoestrogens, similar to other plants or their ingredient extracts. Traditional Chinese medicinal herbs such as Rehmannia glutinosa and Ferula, or oils extracted from various plant species, which have similar though milder effects to that of estradiol-17β benzoate, may be associated with the maintenance of bone mass in the post-menopausal period due to their putative function as osteoprotective agents (42).

Estrogen exerts certain effects by binding to different estrogen receptors, including ERα and ERβ. ERα is predominant in a number of tissues and is primarily involved in the reproductive system, whereas ERβ is expressed in numerous tissues including bone (34). ERα and ERβ may be present in rat and human osteoblasts in cortical and trabecular compartments of bone (36,37), and a number of phytoestrogens,
including genistein and coumestrol, show a higher affinity to ERβ (20).

A possible explanation for olive oil exhibiting bone protective effects but no estrogenic effects may be its phytoestrogen effect; olive oil is structurally related to 17β-estradiol, but has much stronger affinity for ERβ than 17β-estradiol. As Annekathrin Martina Keiler do has proved the polyphenols impact on estrogen response genes because of the aromatic ring structurally related to 17β-estradiol binding to estrogen receptors. Seidlova-Wuttke et al previously showed that β-ecdysone has an antiestoteric effect which does not involve the activation of ER in ovariectomized rats. Thus, olive oil may a exert a similar bone protective effect, with no associated estrogenic effects (21).

Furthermore, olive oil also has anti-cancer effect as there has been reported that olive oil contains a number of compounds which by their antioxidative action reduce the risk of cell damage and their consequent uncontrolled growth and division (43). Therefore, olive oil may be more suitable to the treatment of osteoporosis associated with postoperative gynecological malignancies, displaying desirable estrogenic effects but with fewer undesirable side effects.

In conclusion, the present study demonstrates the beneficial effects of olive oil on osteoporosis via serum proinflammatory cytokines and lumbar spine BMD. However, further research is required in order to determine the mechanism of its effects, possibly using an ER-ligand binding assay or phytoestrogen.

Acknowledgements

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