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Vitamin E suppresses ex vivo osteoclastogenesis in ovariectomized rats

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Postmenopausal osteoporosis may be caused, in part, by oxidative stress and inflammation. Vitamin E is a strong antioxidant which has been shown to have anti-inflammatory and bone-protective effects. The objective of this study was to investigate the effects of various doses of supplemental vitamin E on osteoclastogenesis in ovariectomized rats. Sixty 12-month-old female Sprague-Dawley rats were sham-operated (Sham) or ovariectomized (Ovx; 4 groups) and fed a diet containing basal levels of vitamin E (75 mg d-alpha tocopherol acetate/kg diet) for 220 days. Rats in three of the Ovx groups were given supplemental doses of vitamin E (300, 525, and 750 mg d-alpha tocopherol acetate/kg diet) for the last 100 days. Femoral bone marrow cells were isolated, cultured, and osteoclasts were counted and normalized to 1000 total bone marrow cells. Blood monocyte and lymphocyte counts were also determined. Osteoclast number was significantly higher in the Ovx control group and was suppressed by all three doses of vitamin E, although more effectively in the Ovx group that received 300 mg/kg diet vitamin E. Additionally, vitamin E suppressed the Ovx-induced increase in monocyte and lymphocyte production. The results of this study suggest that vitamin E supplementation suppresses osteoclastogenesis, possibly by inhibiting monocyte and lymphocyte production.

Introduction

It is estimated that more than 54 million people living in the United States (U.S.) either have or are at risk of osteoporosis. Due to the growing population of individuals who are ≥ 50 years of age in the U.S., the incidence and prevalence of osteoporosis is likely to continue to increase with afflicted individuals being at a greater risk of falls and fractures, therefore increasing morbidity and mortality. The economic burden associated with osteoporosis is estimated to exceed $25 billion over the next few decades. The morbidity and mortality associated with osteoporosis as well as the growing costs of its associated medical care illustrate the importance of identifying safe, efficacious, and cost-effective ways to prevent its occurrence.

The underlying cause for age-related bone loss and its pathogenesis have been linked to a gradual increase in the production of pro-inflammatory cytokines and prostaglandins, as well as an imbalance in the formation of oxygen-derived free radicals both in the bone microenvironment and in osteoclast precursor cells and antioxidant defences. Higher levels of certain cytokines, oxidative stress, and prostaglandin E2 activate several pro-inflammatory pathways, including the nuclear factor kappa-B (NF-κB) pathway. NF-κB may be partially responsible for the age-related loss of bone due to its ability to increase osteoclastogenesis resulting in increased bone resorption. While osteoporosis can occur in both men and women, the abrupt cessation of ovarian hormone production that occurs in menopause leads to adverse increases in the production of pro-inflammatory cytokines and products of oxidative stress, placing women at a greater risk than men. It has been consistently observed in animal and clinical studies that ovarian hormone deficiency increases osteoclastogenesis from bone marrow-derived progenitor cells, such as monocytes and macrophages, which is inhibited by the administration of 17β-estradiol (E2). Additionally, both cell culture and animal studies have demonstrated that oxygen-derived free radicals generated in the bone microenvironment promote osteoclastogenesis and subsequently bone resorption. Estrogen replacement therapy is commonly avoided due to its association with adverse effects such as an increased risk of breast cancer. Hence, alternative therapies (e.g. functional foods and dietary supplements) to suppress osteoclastogenesis and therefore bone resorption are warranted.

Vitamin E is a lipid-soluble vitamin found in a variety of foods including nuts, seeds, green leafy vegetables, and vegetable oils. Vitamin E has strong biological antioxidant properties and has been shown to suppress the production of pro-inflammatory cytokines including IL-1 and IL-6, both of which have been linked to increased bone loss, and enzymes such as cyclooxygenase-2 (COX-2).
Additionally, vitamin E has been reported to protect cartilage against cellular lipid peroxidation, maintain normal bone growth and modeling, and increase bone mass by lowering the concentrations of free radicals in young animals. \(^\text{19}\) Ahmad \textit{et al.}\(^\text{20}\) previously demonstrated that vitamin E reduced the oxygen-derived free radical stimulation of osteoclastic bone resorption. Our laboratory has also shown that vitamin E supplementation is protective against bone loss in four different animal models of osteopenia. First, a high dose of vitamin E supplementation (500 mg/kg diet) in aged mice, but not young adult mice, improved osteopenia. \(^\text{21}\) Second, in examining the effects of vitamin E on bone in a male rat model of orchidectomy-induced osteopenia, we demonstrated that vitamin E improved bone quality, although bone mineral density (BMD) and biochemical markers of bone turnover were unaffected. \(^\text{22}\) Third, we have shown that a high dose of vitamin E (500 mg/kg diet) was protective against bone loss in hindlimb unloaded male rats as it improved trabecular bone microarchitecture, and suppressed the hindlimb unloading-induced increase in COX-2 levels. \(^\text{23}\) Fourth, in a rat model of ovariectomy-induced osteopenia, vitamin E improved bone quality which may have been, in part, due to a reduction in bone resorption and increased bone formation; however, vitamin E was unable to prevent the loss of bone density and trabecular bone structure. \(^\text{24}\) Although there are a number of studies examining the effects of vitamin E on bone metabolism, there is a paucity of such studies with respect to the ability of vitamin E to suppress osteoclastogenesis. Therefore, as an extension of the latter parent study\(^\text{25}\), we aimed to investigate the degree to which vitamin E dose-dependently suppresses \textit{ex vivo} osteoclastogenesis in ovariectomized rats.

1. Materials and Methods

1.1. Animals and Diets

Sixty 12-month-old female Sprague-Dawley rats were housed in an environmentally-controlled animal laboratory upon arrival. All procedures were approved by the Florida State University Animal Care and Use Committee and were performed in accordance with National Institutes of Health Guidelines for the Care and Use of Animals. After a 5-day acclimation period, rats were either sham-operated (Sham; 1 group; \(n = 12\)) or ovariectomized (Ovx; 4 groups; \(n = 12\)) and maintained on a semi-purified casein-based diet (AIN 93M) that included a basal level of vitamin E (75 mg vitamin E/kg diet) for 120 days to induce osteopenia. \(^\text{26}\) Thereafter, the Ovx rats were randomly divided into three groups (\(n = 12/g\)roup): Ovx + 300 mg vitamin E/kg diet, Ovx + 525 mg vitamin E/kg diet, or Ovx + 750 mg vitamin E/kg diet for an additional 100 days. The Sham group and one of Ovx groups were continuously fed a diet with basal level of vitamin E (75 mg vitamin E/kg diet) for the entire 220 days and served as the control groups. Vitamin E was provided in the form of d-alpha-tocopherol acetate with 92% purity (Archer Daniels Midland Company, Chicago, IL, USA). The rats were pair-fed to the average food intake of Sham group and had free access to deionized water. Rats were fed their respective diets for a total of 100 days until terminated.

2.2. Necropsy and Blood Processing

One-hundred days after initiating treatment, rats were anesthetized with a mixture of ketamine/xylazine (60 and 3 mg/kg body weight, respectively). Rats were then bled via the abdominal aorta. Blood samples were collected in EDTA-treated containers and serum was separated by centrifugation at 1500 \(x\) g for 20 min at 4°C. Aliquots of serum were frozen and kept at -20°C for later analyses. The uterus of each rat was collected, blotted, and weighed to confirm the success of ovariectomy. Monocyte and lymphocyte counts were assessed using an automated combined impedance-light focusing hematology counter (Pentra 120 Retic Hematology Instrument, ABX Diagnostics, Inc., Irvine, CA, USA).

2.3. Assessment of Multinucleated Osteoclasts

Following sacrifice, femurs were cleaned, sawed in half, and the marrow was flushed out. Single cell suspensions of marrow were prepared and \(0.5 \times 10^6\) cells in 0.5 ml of culture medium were plated per well in 24-well plastic plates. The cells were cultured for 14 days at 37°C in a humidified atmosphere of 5% CO\(_2\) in air. The culture medium consisted of bicarbonate buffered αMEM containing 10% fetal bovine serum and \(10^{-8}\) M 1,25(OH)\(_2\) vitamin D\(_3\). Cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP; Sigma, St. Louis, MO, USA) and counter-stained with hematoxylin for 2 minutes. TRAP-positive cells with three or more nuclei were counted at a magnification of x100. TRAP-positive multinucleated cell numbers were adjusted by 1000 total bone marrow cell counts.

2.4. Statistical Analysis

Statistical analysis of data involved estimation of means and standard error of the mean (SEM) for each of the groups. Values were compared using analysis of variance (ANOVA). When ANOVA indicated statistical significance, Tukey’s post hoc multiple comparisons test was used to determine which means were significantly different from each other. (SAS, version 8.2, Cary, NC). Significance was set at \(P < 0.05\).

3. Results

As presented in Table 1, Ovx rats had significantly higher final body weights despite no differences in food intake. Vitamin E had no effect on body weight. As expected, the number of TRAP-positive multinucleated cells was significantly increased in the Ovx-control group compared to the Sham group. The formation of TRAP-positive multinucleated cells was also inhibited in all vitamin E treated groups although most effectively in the Ovx group that received 300 mg/kg diet vitamin E (Figure 1). Similarly, both monocyte and lymphocyte counts were found to be significantly
increased in the Ovx-control group compared to Sham; yet, these increases were prevented by all three supplemental doses of vitamin E (Figures 2A and 2B).

Figure 1. Effects of ovariectomy and supplemental doses of vitamin E on osteoclastogenesis. Bars are mean ± SEM, n = 12. Bars with different letters are significantly different from each other (P < 0.05). Sham, sham-operated rats; Ovx, ovariectomized rats; LD, low dose (300 mg/kg diet), MD, medium dose (525 mg/kg diet); HD, high dose (750 mg/kg diet).

Figure 2. Effects of ovariectomy and supplemental doses of vitamin E on monocyte (A) and lymphocyte (B) counts. Bars are mean ± SEM, n = 12. Bars with different letters are significantly different from each other (P < 0.05). Sham, sham-operated rats; Ovx, ovariectomized rats; LD, low dose (300 mg/kg diet), MD, medium dose (525 mg/kg diet); HD, high dose (750 mg/kg diet).

Discussion
As a result of aging, as well as menopause, the production of hematopoietic-originated cells such as monocytes and lymphocytes increases.18,24 If sustained, these increases can lead to chronic inflammation and subsequently numerous inflammatory-related diseases including cardiovascular disease, cancer, and osteoporosis.18,25 There is ample evidence that vitamin E has antioxidative and anti-inflammatory properties, both of which have important implications for overall health including that of bone.26 Nonetheless, the role of vitamin E in bone health remains unclear as there is evidence suggesting that vitamin E may have both protective and detrimental effects on bone.27 The findings by Fujita et al.28 indicated that α-tocopherol increases bone resorption by increasing the production of osteoclasts. In contrast to their findings, the results of the present study suggest that vitamin E at doses of 300 mg/kg diet or higher not only suppresses osteoclastogenesis, but also its progenitor cells, e.g. monocytes. All doses of vitamin E moderately suppressed the production of lymphocytes, which are cells that support osteoclast differentiation. These observations, along with the findings of several other studies by our laboratory,3,5,21,22 suggest that vitamin E has bone-protective properties. This statement can be further supported by a mouse study conducted in our laboratory3 which indicated that vitamin E at a higher dose plays a role in protecting bone during aging. In that study, mice that received vitamin E at a dose of 500 mg/kg diet had higher levels of several bone matrix proteins when compared with an adequate dose of vitamin E (30 mg/kg diet).

In contrast to the findings of the present study, Ha et al.29 reported that vitamin E in the form of α-tocopherol did not inhibit osteoclast differentiation and activation in precursor cells while α-tocotrienol did. Similar findings were documented by Brooks and colleagues30 who indicated that both α- and γ-tocotrienol isomers, but not α-tocopherol, dose-dependently inhibited the formation of osteoclasts from CD14+ monocytes and its activity, although only γ-tocotrienol did so without toxicity. This may be partly explained by...
In our parent study, bone histomorphometry findings indicated that ovariectomy increased bone resorption parameters including osteoclast/bone surface (Oc.S/BS) and eroded surface/bone surface (ES/BS), and all doses of vitamin E prevented these increases. Our current finding that all doses of vitamin E prevented ovariectomy-induced osteoclastogenesis supports these findings, suggesting that supplemental doses of vitamin E inhibit bone resorption by suppressing osteoclastogenesis. Furthermore, bone formation parameters including mineralizing surface/bone surface (MS/BS) and bone formation rate/bone volume (BFR/BV) were significantly increased in Ovx animals. MS/BS was significantly increased by 42 and 50% in the groups receiving 300 and 525 mg/kg diet vitamin E, respectively, when compared to the Ovx control group. BFR/BV was significantly increased by 65% in the group receiving 525 mg/kg diet vitamin E when compared to the Ovx control group. Overall, these findings suggest that vitamin E may have upregulated osteoblastogenesis thereby increasing their bone-forming activities and suppressed osteoclastogenesis thus reducing bone erosion. However, since osteoblastogenesis was not assessed in the parent or current studies, this remains speculative and should be considered a limitation.

With respect to the role of vitamin E in estrogen deficiency, vitamin E may exert bone-protective properties by reducing the production of monocytes and therefore macrophages, precursors of osteoclastic cells, and/or their production of pro-inflammatory cytokines by lymphocytes. In support of this view, there are several studies that have shown ovarian hormone deficiency unfavorably alters hematological parameters. For instance, Ben-Hur and associates reported that postmenopausal women 50 to 60 years of age had higher monocyte counts than young women 18 to 24 years of age. Using peripheral blood mononuclear cells taken from premenopausal and postmenopausal women, D’Amelio et al. demonstrated that estrogen deficiency increases the production of circulating osteoclastic precursors, osteoclast formation, as well as monocyte and T cell production of pro-osteoclastogenic cytokines, i.e. tumor necrosis factor-α (TNF-α) and receptor activator of nuclear factor kappa-B ligand (RANKL). The postmenopausal period is also a time in which women tend to lose the largest amount of bone mass leading to osteoporosis. Our findings clearly indicate that vitamin E at a dose of 300 mg/kg diet in Ovx rats had a pronounced effect in reducing the Ovx-induced increases in monocytes and lymphocytes. However, doses higher than 300 mg/kg diet had no further effects on these parameters implying that vitamin E absorption had reached its plateau. In fact, in our parent study, we found that providing doses of vitamin E greater than 300 mg/kg diet did not lead to further increases in circulating vitamin E levels thereby supporting this notion. In accordance with this, a study by Traber et al. showed that vitamin E absorption reaches a plateau at its dose increases. However, despite our previous observation that circulating vitamin E levels did not increase as the dose was increased, we found that different doses of vitamin E led to differential effects on bone quality. We speculate that circulating vitamin E levels may not reflect that of the tissues, such as bone.

Although the mechanisms by which vitamin E suppresses ex vivo osteoclastogenesis in ovariectomized rats cannot be determined from the results presented here, one possible mechanism is through inhibiting the production of bone-marrow derived progenitor cells such as monocytes. Considering the anti-inflammatory properties of vitamin E, other possible mechanisms include targeting the RANK/RANKL/osteoprotegerin (OPG) system including stimulating the production of OPG, a decoy receptor for RANKL, by osteoblasts and decreasing the expression of RANKL by osteoblasts. The possible underlying mechanisms by which vitamin E and vitamin E-rich foods inhibit osteoclastogenesis are illustrated in Figure 3. However, the RANK/RANKL/OPG system was not examined in this study and these mechanisms are therefore speculative. Future studies are warranted to examine the mechanisms by which vitamin E suppresses osteoclastogenesis.

Figure 3. Putative underlying mechanisms by which vitamin E and vitamin E-rich foods inhibit osteoclastogenesis.

4. Conclusions
Although there are numerous factors leading to enhanced osteoclastogenesis in the postmenopausal period, oxidative stress and inflammation are known to play a major role in this process. As such, it is reasonable to suggest that vitamin E may play a role in reducing osteoclastogenesis by inhibiting the production of bone-marrow derived progenitor cells such as monocytes. The observation that vitamin E at higher doses not only beneficially affects the cardiovascular system, but also favorably affects bone by suppressing its resorption is novel and warrants further investigation. Nonetheless, the findings of this study cannot be directly extrapolated to humans and long-term randomized, controlled clinical trials are needed to confirm the bone-protective role of vitamin E, particularly in high risk populations such as postmenopausal women.
Acknowledgements

This work was funded by United States Department of Agriculture NRI grant No. 99-35200-7606. The vitamin E used was generously provided by Archer Daniels Midland Company (Decatur, IL). The authors wish to thank Mr. Antonio M. Johnson for his assistance with graphics.

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This study presents the effects of various doses of supplemental vitamin E on *ex vivo* osteoclastogenesis in ovariectomized rats.