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Vitamin E improves bone quality in the aged but not in young adult male mice

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Abstract

It is generally viewed that with advancing age, humans and other animals including mice experience a gradual decline in the rate of bone formation. This, in part, may be due to the rise in oxygen-derived free radical formation. Vitamin E, a strong antioxidant, functions as a free radical scavenger that potentially can suppress bone resorption while stimulating bone formation. Although the effects of vitamin E on immune functions are well documented, there is a paucity of information on its effect on skeletal health in vivo. The purpose of this study was to explore the influence of vitamin E supplementation on bone in young adult and old mice. Six and twenty-four month-old male C57BL/6NIA mice each were divided into two groups and fed a diet containing either adequate (30 mg/kg diet) or high (500 mg/kg diet) levels of vitamin E. Thirty days later, mice were killed and bones were removed for analyses including biomechanical testing using three-point bending and mRNA expressions of insulin-like growth factor-I (IGF-I), osteocalcin, and type 1α-collagen using Northern blot. In old but not the young adult mice, high-dose vitamin E enhanced bone quality as evident by improved material and structural bone properties in comparison with adequate. This improved quality was accompanied by increases in bone dry weight, protein, and mRNA transcripts for osteocalcin, type Iα−collagen, and IGF-I. These data demonstrate that high-dose vitamin E has pronounced effects on bone quality as well as matrix protein in old mice by augmenting bone matrix protein without reducing bone mineralization as evidenced by unaltered bone density.

Keywords: Aging; Alpha-tocopherol; Bone strength; Insulin-like growth factor-I; Mice

1. Introduction

Humans and certain animals, including mice, [1,2] experience bone loss as a result of aging. Underlying causes for age-related bone loss and its pathogenesis are uncertain beyond the consensus that it is related to senescence. The influence of confounding factors, such as hyperparathyroidism and changes in calcium-regulating hormones cannot explain the loss of bone due to aging [3]. Additionally, the inability of sex steroids in preventing the age-associated bone loss in both sexes [4,5] indicates the need for other effective preventative and therapeutic strategies. With advancing age in humans and other vertebrate animals, it is believed that the rate of bone formation gradually diminishes, while the rate of bone resorption either is unaltered or accelerated, resulting in net bone loss [6–8]. These events, in part, have been linked to the gradual age-associated modulation in immune cell mediators (cytokines and prostaglandins) and oxygen-derived free radical (ODFR) formation either in the bone microenvironment or in the cells that serve as osteoclastic precursors such as monocyte-macrophage lineage [9].

Bone contains a plethora of local cytokines and lipid mediators such as interleukin (IL)-1, -6, tumor necrosis factor, lymphotoxin, leukotrienes, and prostaglandins of the E series (PGE) [10]. The age-associated increase in certain immune cell mediators, such as IL-1, IL-6 [11], and in particular PGE_2 [12–14] may be partially responsible for the

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stimulated-osteoclastic bone resorption in senescence with unclear mechanism of action.

Oxygen-derived free radicals are formed by a number of phagocytes including monocytes, macrophages, and neutrophils [15] and have been reported to be increased in chronic inflammatory diseases [16], aging [17], and osteoporosis [18]. The *in vivo* and *in vitro* findings [19] indicate that free radicals generated in the bone environment enhance osteoclast formation and bone resorption.

Vitamin E is a strong biological antioxidant [20] and has been shown to suppress the production of certain cytokines such as IL-1 and IL-6 [21–24] that have been linked to increased bone loss. [1,25]. Furthermore, vitamin E has been reported to protect bone cells from damage as a result of lipid peroxidation [26]. More interestingly, recently the results of a population study by Melhus and colleagues [27] reported the important role of adequate intake of dietary vitamin E in reducing the risk of hip fracture in current smokers. These observations suggest that vitamin E influences skeletal health. The purpose of this study was to determine whether short-term supplementation of high-dose vitamin E modulates bone differently in young adult versus old mice.

2. Materials and Methods

2.1. Animals and diet

The short-term influence of adequate- and high-dose vitamin E on age-related changes in bone was investigated using six- and twenty-four-month old (young adult and old, respectively) C57BL/6NIA male mice. Twenty-two young adult and thirty old mice were each divided equally into two treatment groups and fed *ad libitum* a semi-purified casein-based diet containing either adequate (30 mg/kg diet) or high (500 mg/kg diet) levels of vitamin E (DL-α-tocopherol acetate) as reported elsewhere [21]. All conditions and handling of animals were approved by the Animal Care and Use Committee of Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University and followed the NIH guidelines for the care and use of animals. The animals were euthanized by CO_2 asphyxiation 30 days after the initiation of the treatment; left tibiae and femurs were removed, cleaned of soft tissues and stored at -20°C for later measurements of physical, biochemical, and biomechanical parameters. Right femurs were also removed, cleaned of soft tissues, snap frozen in liquid nitrogen, and kept at -80°C for total RNA extraction.

2.2. Bone density, dry weight, and total protein

Bone volume and density of left tibiae and femurs were measured by Archimedes' principle as described elsewhere [28]. For tibial bone total protein determination, a modified method of Farley et al. [29] was used. Tibiae were cut, rinsed, and transferred to tubes containing PBS buffer overnight to remove any contaminants. Each tibia was then transferred to a solution of 0.1 g/L triton X-100 for 72 h. Bone extracts were vortexed, centrifuged and stored at 4°C prior to measurements of bone total protein using Bio-Rad assay reagents and following the manufacturer's instructions.

2.3. Biomechanical testing of bone

The biomechanical properties of femoral bones were assessed using a three-point bending test [30] utilizing an Instron Universal Testing Machine (Model 1125; Instron Corp; Canton, MA). Each left femur was placed on two lower supports that were 5 mm apart. Force was applied at mid diaphysis on the anterior surface such that the anterior surface was in compression and the posterior surface in tension. Ultimate load, yield load, and stiffness were determined from load-displacement curves. Ultimate stress, yield stress, and flexural modulus of elasticity were calculated from the beam bending equations [31]. All biomechanical tests were conducted at room temperature and at a rate of 1 mm/min. Cross sectional area and moment of area/inertia of compact (cortical) bone were calculated using equations for an ellipse [30,31].

2.4. RNA isolation and Northern blot analyses

Four bone specimens from each treatment group were randomly selected for Northern blot analyses, Total cellular RNA from right femur was extracted by the acid-guanidium-chloroform method of Chomczynski and Sacchi [32] and quantified by UV absorbance. The abundance of mRNAs was analyzed with appropriate cDNA probes for insulin-like growth factor-I (IGF-I), type Iα-collagen, and osteocalcin. Probes for IGF-I and 28S were provided by Roberts et al. [33] and type Iα-collagen, and osteocalcin by Klein and Wiren [34]. Adjustments in the amounts of total RNA were made with a cDNA probe for 28S RNA. For Northern blotting, 30 μg of total cellular RNA was fractionated electrophoretically on 0.12 g/L agarose gel containing 2.2 mol/L formaldehyde. All RNA samples were denatured prior to loading by incubation for 20 min at 65°C in a solution of 10x MOPS (3-[n-morpholino]-propanesulfonic acid), 37% formaldehyde, and deionized formamide. Electrophoresis was performed in 1$\times$ MOPS (pH 7.0) at 80V for approximately 2 h. Ribosomal 18S and 28S RNA were visualized under ultraviolet light after staining with ethidium bromide to confirm the integrity of RNA and ensure equal loading.

The RNA was transferred overnight from the gel to Qiabrane Nylon Plus membrane (Qiagen, Santa Clarita, CA) by capillary action in 10x standard saline citrate (3 mol/L NaCl, 0.3 mol/L trisodium citrate). Membrane was then pre-hybridized for 6 h at 65°C in hybridization buffer containing 1.0 mol/L Na_2HPO_4 (pH 7.0), 0.5 mol/L EDTA,

Table 1
Effects of vitamin E-supplementation on bone physical parameters and tibial total protein of young adult and old C57BL/6NIA mice

Measures	Young		Old	
	Adequate	High	Adequate	High
Femur				
Length (cm)	1.54 ± 0.01	1.57 ± 0.01	1.67 ± 0.01	1.65 ± 0.01
Dry Weight (mg)	35 ± 1.0	36 ± 1.1	37.0 ± 1.1	41.0 ± 1.2*
Density (g/cm^1)	1.319 ± 0.01	1.329 ± 0.02	1.307 ± 0.01	1.313 ± 0.01
Tibia				
Dry weight (mg)	27.8 ± 1.3	29.2 ± 0.8	30.8 ± 0.6	31.3 ± 0.6
Density (g/cm^1	1.337 ± 0.01	1.337 ± 0.01	1.287 ± 0.01	1.292 ± 0.01
Total Protein (μg)	204 ± 24	232 ± 43	173.0 ± 38.5	263.3 ± 35.1
Protein/day weight (μg/mg)	7.18 ± 0.69	8.03 ± 1.59	5.72 ± 1.30	8.48 ± 1.17

Data shown are mean ± SE; n = 11-15. * Significantly different from mice of the same age group fed the adequate diet at $p < 0.05$ by Student's t-test. Adequate and high represent 30 and 500 mg vitamin E/kg diet, respectively.

0.05 g/L bovine serum albumin, 2.0 g/L sodium dodecyl sulfate, and probed overnight at 65°C with 4×10^6 cpm/L ^{32}P-labeled cDNA. The cDNA was labeled with [^{32}P]dCTP (specific activity of approximately 3000 Ci/ml; Amersham) by random priming using Primer-it-II Random Primer labeling system (Stratagene Cloning Systems, La Jolla, CA) and purified by push column (Stratagene). The blots were washed under the conditions of increased stringency, up to 0.1× SSC and 0.01 g/L SDS at 65°C and then exposed using Kodak X-Omat AR-5 film (Eastman Kodak, Rochester, NY, U.S.A.) at −80°C [35,36]. The Northern blots were quantitated using a phosphorimager (Model 445-S1, Molecular Dynamics, Sunnyvale, CA).

2.5. Statistical analysis

In each age group data were analyzed using unpaired Student t-test (GraphPad Instat Software version 3.00, 1998–99) and are presented as mean ± standard error (SE). Significant differences were determined at $p < 0.05$, unless otherwise stated.

3. Results

Within each age category, neither the food intakes nor the body weights differed significantly between the treatments. Although there were no significant differences in femoral bone densities, high-dose vitamin E increased ($p < 0.01$) femoral dry weights of old but not the young adult mice (Table 1).

Vitamin E status had no effect on tibial density and dry weight in either age group. However, in old mice the mean total protein content of tibiae tended to be higher ($p < 0.09$) in high-dose vitamin E-supplemented animals versus the adequate (Table 1). The same trends existed when tibial protein levels were normalized to bone dry weight (Table 1).

Biomechanical properties of bone are reported in Table 2. Although high-dose vitamin E supplementation had no effects on parameters of bone quality in young adult mice, it significantly ($p < 0.05$) enhanced femoral strength as evident by higher ultimate load, yield load, and stiffness (structural parameters) as well as improving material properties of bone, i.e. significantly higher yield stress and tendency ($p < 0.08$) of elevating flexural modulus of elasticity in older mice.

Northern blot analyses of total cellular RNA isolated from femurs detected 0.6 kilobase (kb) osteocalcin, 0.8 kb IGF-I, and 4.7 and 5.7 kb type Iα collagen mRNA transcripts. High-dose vitamin E in both young adult and old mice significantly ($p < 0.05$) increased osteocalcin (Figs. 1a and 1b) and IGF-I (Figs. 2a and 2b) mRNA transcripts versus adequate. Although, in old mice mRNA transcript for type Iα collagen tended ($p < 0.07$) to be higher in high-dose vitamin E, it did not differ in the young adult mice (data are not shown). Percent increases in mRNA transcripts for osteocalcin, IGF-I, as well as type 1α-collagen are represented in Fig. 3 and Fig. 4 for old and young mice, respectively. In old mice, high-dose vitamin E increased mRNA transcripts by 70, 28, and 32% for osteocalcin, IGF-I, and type 1α-collagen, respectively in comparison with adequate vitamin E dose. Whereas, in young adult mice, high-dose vitamin E increased percent mRNA transcripts for osteocalcin (78%) and IGF-I (44%) but not type 1α-collagen in comparison to the adequate.

4. Discussion

Studies in humans and animals suggest that aging impairs the ability to form bone [6–8]. Principally, an important contributing factor to the development of osteoporosis appears to be a decrease in the number and activity of osteoblasts responsible for synthesizing new bone matrix [7]. These events, in part, have been linked to increased

Table 2
Effects of vitamin E-supplementation on femoral structural and material properties of young adult and old C57BL/6NIA mice

Treatment group	Structural properties			Material properties		
	Ultimate load (N)	Yield load (N)	Stiffness (N/mm)	Ultimate stress (N/mm^2)	Yield stress (N/mm^2)	Modulus (N/mm^2)
Young						
Adequate	15.5 ± 0.5	13.4 ± 0.6	548 ± 31	50.6 ± 2.3	43.7 ± 2.3	5430 ± 472
High	15.6 ± 0.4	14.2 ± 0.4	549 ± 20	52.9 ± 2.8	48.2 ± 2.8	5906 ± 361
Old						
Adequate	14.1 ± 0.6	7.3 ± 0.5	491 ± 32	36.3 ± 1.6	14.3 ± 0.8	3540 ± 300
High	16.5 ± 0.5*	9.3 ± 0.5*	589 ± 32*	36.9 ± 2.1	18.6 ± 1.4*	5900 ± 1320

Data shown are mean ± SE. * Significantly different from mice of the same age group fed the adequate diet at $p < 0.05$ by Student's t-test. Adequate and high represent 30 and 500 mg vitamin E/kg diet, respectively.

production of oxygen radicals and decreased levels of antioxidants [26]. Because vitamin E is an effective antioxidant and free radical scavenger, it can potentially protect against age-associated bone loss.

The findings of the present study strongly suggest that high-dose of vitamin E enhances the synthesis of bone matrix proteins in both young adult and old mice as evidenced by higher mRNA levels for osteocalcin, a specific marker of osteoblastic bone formation [37] and IGF-I, an important local regulator of bone metabolism [37]. Furthermore, high-dose vitamin E supplementation had a more pronounced effect on bone of old but not young adult mice by increasing mRNA level of type 1α-collagen, a predominant bone matrix protein [38] and total tibial protein content.

Osteoblasts have appreciable amounts of polyunsaturated fatty acids (PUFA) and hence are highly susceptible to oxidative stress [9]. Therefore, vitamin E can reduce the

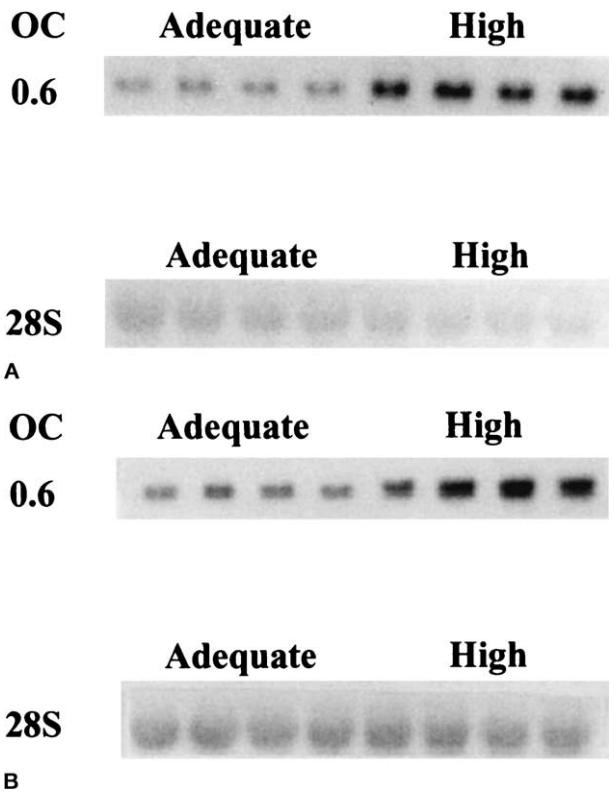

Fig. 1. (a and b) Northern blots of femur total cellular RNA probed with ^{32}P-labeled cDNA for osteocalcin (OC; top panel) and 28S (bottom panel) RNA. Each lane represents RNA extracted from a femur of one animal. The femurs were from young adult (Fig. 1a) and old (Fig. 1b) mice on adequate (adequate) or high-dose (high) vitamin E diets.

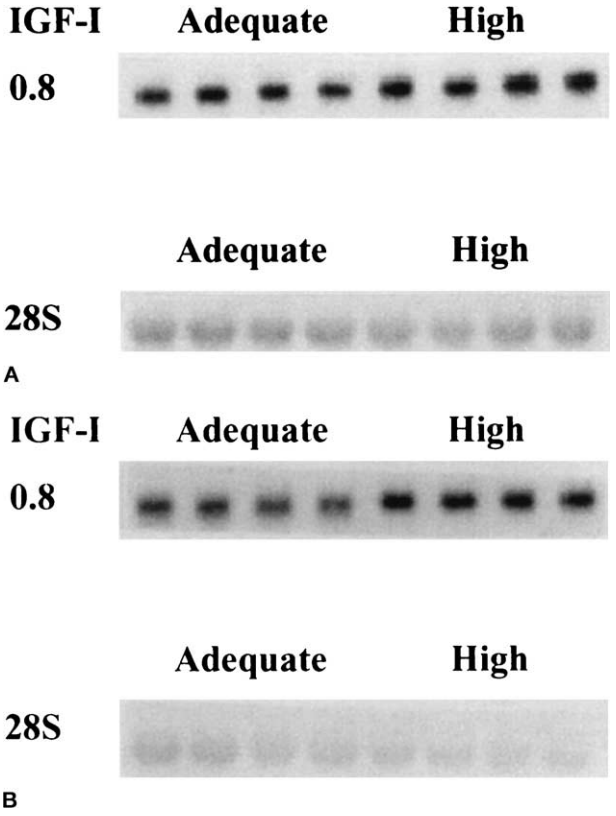

Fig. 2. (a and b) Northern blots of femur total cellular RNA probed with ^{32}P-labeled cDNA for insulin-like growth factor-I (IGF-I; top panel), and 28S RNA (bottom panel). Each lane represents RNA extracted from a femur of one animal. The femurs were from young adult (Fig. 2a) and old (Fig. 2b) mice on adequate (adequate) or high-dose (high) vitamin E diets.

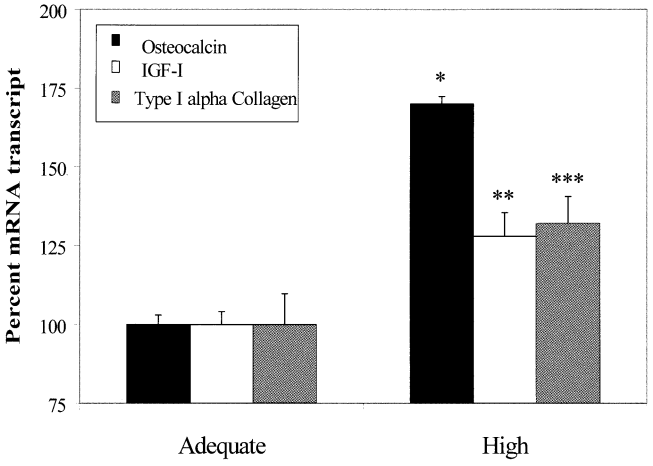

Fig. 3. Percent mRNA transcript for osteocalcin, insulin-like growth factor-I (IGF-I), and Type Iα-collagen gene expression in femurs of old mice on either adequate (adequate) or high-dose (high) vitamin E diet. Bars represent mean + SE, n = 4. *, **, ***Higher than mice fed the adequate diet at $p < 0.0001$, 0.03, and 0.07, respectively by Student's t-test.

predisposition of osteoblasts to oxidation and stimulate their activity resulting in higher rates of protein synthesis. Hence, as observed in this study, high-dose vitamin E supplementation may exert a greater beneficial effect on bone during aging, where the oxidative stress is higher.

Another mechanism by which vitamin E can modulate bone metabolism is through its effects on prostaglandin E_2 (PGE_2) production locally in bone tissue [26,39] and other cells including macrophages [14,21,40,41,42]. Age-associated increase in PGE_2 production has been reported in both mice and humans [12,13,40,43]. Prostaglandins are multifunctional regulators of bone metabolism where higher concentrations stimulate bone resorption [44] and lower concentrations are shown to enhance bone formation in vivo [9]. In this context, high doses of vitamin E may suppress the age-associated rise in PGE_2 production and, therefore, exert positive effects on bone. Previously, it has been reported that high-dose vitamin E (500 mg/kg diet) in 24 month-old C57B4 NIA male mice, for the same duration as that used in this study, significantly reduced PGE_2 production by macrophages of old mice [14,40,45,46]. The vitamin E-induced decrease in PGE_2 production can lead to increased production of IGF-I or to increase the responsiveness of bone cells to IGF-I [47]. Accordingly, in this study, high-dose vitamin E increased IGF-I mRNA expression in bone, indicating that vitamin E not only elevates circulating levels of IGF-I but also stimulates local synthesis of IGF-I, an important observation which supports a direct role for vitamin E on bone. It is well recognized that IGF-I enhances osteoblastic activity in humans [48] and that higher serum IGF-I concentrations are reflective of an elevated rate of bone formation [49,50].

Our biomechanical data indicate that high-dose vitamin E increases bone strength in old mice (quality) perhaps through alterations in organic phase of the bone as evident by higher protein content of tibiae (52% higher vs. adequate group) without significant changes in bone mineral density. Bone strength is not only dependent on mineral but also on other factors such as bone architecture and matrix protein content [51–53]. In humans [54] and animals [55,56], collagen contents of bone have been consistently shown to be the strongest predictor of bone strength [57]. Collagen is the main organic component of bone that confers tensile strength. Therefore, our observation of improved bone quality and bending strength in old mice due to high-dose vitamin E can be attributed to the higher protein content of bone. A possible explanation for the ineffectiveness of vitamin E in younger animals may be because their bones were perhaps exposed to lesser degree of oxidative stress.

In conclusion, the findings of this study indicate that high-dose vitamin E supplementation can stimulate bone formation and improve bone quality in old mice more effectively than young adult mice. These results can have significant implications for quality of life in elderly. Clinical studies are needed to evaluate the efficacy of vitamin E in improving skeletal health in older humans.

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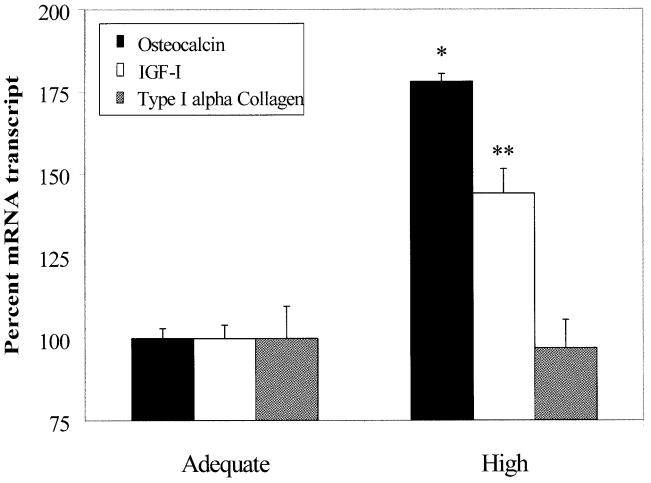

Fig. 4. Percent mRNA transcript for osteocalcin, insulin-like growth factor-I (IGF-I), and Type Iα-collagen gene expression in femurs of young adult mice on either adequate (adequate) or high-dose (high) vitamin E diet. Bars represent mean + SE, n = 4. *, **Significantly higher than mice fed the adequate diet at $p < 0.0009$ and 0.0009 respectively by Student's t-test.

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