AN ABSTRACT OF THE THESIS OF

Katherine G. Kasper for the degree of Master of Science in Nutrition presented on November 24, 2015.

Title: Examining the Effects of High Vitamin E Diets on the Rodent Skeleton

Abstract approved:

________________________________________________________________________

Maret G. Traber

The primary goal of my study was to evaluate the effects of high dietary vitamin E on the rodent skeleton. The outcomes from my study assess the impact of high intakes of vitamin E and increased concentrations of its metabolite, carboxy ethyl hydroxy chromanol (CEHC), on bone turnover and skeletal pathology. These findings contribute to a better understanding of the potential skeletal consequences of high intakes of vitamin E. Our central hypothesis was that bone loss mediated by high dietary vitamin E is due to high extra-hepatic tissue concentrations of its metabolite, CEHC. Our study consisted of two specific aims:

In Aim 1, we evaluated the hypothesis that high dietary vitamin E induces bone loss in rodents. Sprague Dawley rats, 11-weeks-old, were fed for 18 weeks semi-purified diets containing adequate alpha-tocopherol (78 mg/kg diet), high alpha-tocopherol (518 mg/kg diet), or mixed-tocopherols and tocotrienols (234 mg/kg diet). Alterations in bone mineral density, bone mass, and microarchitecture were assessed using dual x-ray absorptiometry and micro-computed tomography, respectively. Serum osteocalcin, bone formation rates, and osteogenic gene expression were also examined using an osteocalcin immunoassay, histomorphometry and polymerase chain reaction (PCR) analyses, respectively. Cancellous bone volume fraction in proximal tibia and lumbar vertebra were unaffected by dietary treatments. Additionally, no significant differences between
groups were detected for serum osteocalcin, osteogenic gene expression, or bone formation rates. Given these findings, we conclude that vitamin E added to otherwise adequate diets has no adverse effects on bone mass and bone turnover in young adult male rats.

In Aim 2, we evaluated the hypothesis that increased concentrations of the anti-inflammatory metabolite, CEHC, mediate bone loss in rodents. Using the study design from Aim 1, we measured serum and tissue concentrations of CEHC in each treatment group. Rats consuming diets containing high alpha-tocopherol had increased plasma, bone marrow, and liver alpha-CEHC concentrations, whereas rats consuming diets of mixed-tocopherols and tocotrienols had increased plasma gamma-CEHC compared with other treatment groups. Only plasma gamma-CEHC was correlated with serum osteocalcin, a marker of bone turnover, with the highest plasma gamma-CEHC and lowest serum osteocalcin concentrations from the mixed-tocopherols and tocotrienols group. Given these findings, we conclude that plasma gamma-CEHC may play a role in bone turnover, as evidenced by a correlation with lower serum osteocalcin.

In summary, my study has shown that high intakes of dietary vitamin E and its metabolite, CEHC, have no adverse effects on the rodent skeleton. Interestingly, plasma gamma-CEHC is correlated with low serum osteocalcin; however, the physiological significance of this relationship is unclear.
Examining the Effects of High Vitamin E Diets on the Rodent Skeleton

by

Katherine G. Kasper

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APPROVED:

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Katherine G. Kasper, Author
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Urszula Iwaniec, Russ Turner, and Maret Traber were involved in the design, analysis, and writing of all experiments and Chapter 2. Additionally, Maret Traber was involved in the writing of Chapters 1 and 3. Scott Leonard contributed to the vitamin E and CEHC measurements. Carmen Wong contributed to the serum osteocalcin and osteogenic gene array measurements.
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Chapter 1:

Introduction to Bone Physiology and Vitamin E
Introduction to Bone Physiology and Osteoporosis

**Bone Physiology**

Bone is a dynamic, multifunctional organ that plays a significant role in mineral homeostasis, mechanical support, protection of internal organs, and hematopoiesis [1]. Bone is composed of approximately 65% calcium-phosphate crystals (hydroxyapatite), 25% organic material (type 1 collagen and noncollagenous proteins) and 10% water [1]. Bone is organized into two types of structures called cortical and cancellous bone [2]. Cortical bone is made up of densely compact mineral and is located in long and short bone shafts, skull, vertebrae, and iliac crest [1, 3]. Cancellous bone is more porous and made up of trabeculae (thin projections of bone) that are located in long and short bone ends, vertebrae, ribs, and iliac crest [1, 4]. Both structures play critical physiological roles. Cortical bone provides structural support, protection of internal organs, and serves as a mineral depot [3], whereas, cancellous bone responds to mechanical load and serves as a region of metabolic activity [1].

**Bone Physiology: Cells**

Bone consists of two main cell types, osteoblasts and osteoclasts. Osteoblasts are responsible for secreting type 1 collagen, which serves as a matrix for hydroxyapatite mineralization and bone formation [1, 5]. Osteoblasts are derived from mesenchymal stem cell lineage. Differentiation and proliferation of osteoblast precursors are regulated through a variety of mechanisms, including Wnt and transforming growth factor-beta (TGF-beta) signaling [1]. Wnts are a family of proteins that control osteoblast differentiation and proliferation. During canonical signaling, Wnts bind to a receptor complex made up of frizzled and low density lipoprotein 5/6 (LRP5/6), which leads to phosphorylation of a protein complex that contains beta-catenin [5]. Beta-catenin is released from the protein complex and translocates from the cytosol to the nucleus of a cell, and induces transcription of genes involved in osteoblast proliferation and differentiation [5]. In
humans with a defective LRP5 and disrupted Wnt signaling, low bone mass is observed due to decreased formation of mature osteoblasts [6]. In addition to Wnt signaling, TNF-beta signaling also induces osteoblast precursor proliferation and differentiation. TNF-beta activates the Smad-dependent pathway, which induces the transcription of runt-related transcription factor 2 (RUNX2), and activation of osteoblast formation [7]. In humans, loss of function mutations in RUNX2 causes cleidocranial dysplasia, or severe skeletal malformations [8].

Osteoclasts are bone resorbing cells that are responsible for bone resorption, or the process of removing old or damaged bone so that new bone can be formed [1]. Osteoclasts are derived from hematopoietic monocyte-macrophage lineage. Osteoclast differentiation and proliferation are controlled primarily by macrophage colony-stimulating factor 1 (M-CSF) and receptor activator of the NF-κB ligand (RANKL), which are produced by stromal osteoblastic cells and osteocytes (mineralized osteoblasts) [9, 10]. These activators bind to receptors on osteoclast precursors or on the basolateral membrane of mature osteoclasts and initiate signaling cascades that control osteoclast proliferation, differentiation, and survival. Specifically, M-CSF binds to the receptor tyrosine kinase, c-Fms, which induces the phosphorylation of several signaling proteins, including extracellular signal-regulated kinase (ERK), Src, and signal transducer and activator of transcription 1 (STAT1) [9]. In rodent knockout models of Src, an abnormal high bone mass phenotype occurs due to reduction in osteoclast formation and bone resorption [11]. In addition to M-CSF, RANKL also plays a regulatory role in osteoclast formation. RANKL binds to the RANK receptor, which activates nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1), and induces osteoclast differentiation [10]. In rodent models, deletion of genes encoding for NFATc1 lead to osteoclast dysfunction, impaired osteoclastogenesis, and high bone mass phenotype [12].
Bone Remodeling in Adults

Bone remodeling is a lifelong and continuous process that is required for skeletal maintenance. During bone remodeling, old or microdamaged bone is removed and replaced with new bone to improve strength and quality of the skeleton [1]. The bone remodeling process consists of 5 distinct stages: activation, resorption, reversal, formation, and quiescence [1, 13]. During activation, osteoclast precursors are recruited to mineralized bone surfaces and undergo differentiation and maturation to form mature osteoclasts. Once mature osteoclasts are formed, osteoclast microfilaments adhere to bone surfaces and form a sealing zone compartment in which resorption takes place [14]. During resorption, V-ATPase pumps and chloride channel-7 secrete protons and chloride ions, respectively, forming hydrochloric acid in the resorption compartment [15]. Hydrochloric acid, along with cathepsin K [16], matrix metalloproteinases [17], and tartrate-resistant acid phosphatase [18], dissolve mineralized bone over a period of weeks, leaving behind exposed bone matrix.

Once mineralized bone is completely dissolved by osteoclasts, reversal is initiated. The reversal stage is characterized by osteoclast termination and osteoblast recruitment [13]. The exact mechanism which controls reversal is not well understood; however, it is theorized that bone lining cells create a barrier between osteoclasts and osteoblasts during resorption, which is then removed when resorption is complete [19]. Additionally during reversal, bone lining cells deposit a thin layer of new bone matrix, preparing the bone for osteoblastic bone formation [19].

During formation, osteoblast precursors are recruited to the resorption pit compartment and differentiate into mature osteoblasts. Mature osteoblasts are responsible for secreting bone matrix (type I collagen), which serves as a template for hydroxyapatite mineralization [13]. Once new bone matrix is formed, the bone enters a quiescence stage, where the bone is covered with bone lining cells and rests while it continues to mineralize for up to a year or more [13].
Osteoporosis
Disruptions in bone remodeling which favor resorption are known to induce bone loss and osteoporosis. Osteoporosis is the most common metabolic bone disease in humans, and is characterized by a reduction in bone mass, which increases an individual's risk of fracture [20]. In the United States, more than 10 million individuals over 50 years-old have osteoporosis and over 43 million individuals have low bone mass, or osteopenia [20]. A variety of risk factors contribute to the development of osteoporosis. These factors include age [21], gender [22], race [22], diet [23], physical activity [24], and medications [25]. Additionally, one of the largest risk factors for osteoporosis is loss of estrogen in postmenopausal women [26]. In women >65 years-old, lower levels of serum estradiol are associated with low bone mineral density and increased fracture risk [27]. *In vitro*, estrogen plays a role in suppressing osteoclastogenesis by inhibiting the production of tumor necrosis factor-alpha (TNF-alpha) and RANKL [28, 29]. In humans, treatment of osteoporosis with estrogen derivatives improves bone mineral density and fracture risk [30].

Factors Contributing to Osteoporosis: Oxidative Stress
In addition to the factors listed above, high oxidative stress is a known contributor to osteoporosis, bone loss, and increased fracture risk. A meta-analysis of 19 studies found a significant increase in number of fractures between current smokers and non-smokers, which became more severe with age [31]. Additionally, human cross-sectional studies have associated low bone mineral density and osteoporosis with increased serum levels of oxidative stress markers, 8-iso-prostaglandin F2-alpha and lipid hydroperoxides [32, 33]. Furthermore, *in vitro* studies using isolated mouse bone marrow cells reported increased osteoclast differentiation and expression of RANKL from treatment with reactive oxygen species generated by hydrogen peroxide or by xanthine oxidase [34].
Factors Contributing to Osteoporosis: Low Antioxidant Status

In addition to high oxidative stress, low antioxidant status is associated with osteoporosis, bone loss, and fracture risk. A human cross-sectional study reported a significant decrease in plasma vitamin C and vitamin E in postmenopausal osteoporotic women compared with healthy postmenopausal controls [35]. Additionally, low dietary vitamin E is associated with increased risk of fracture, as reported by the Swedish Cohort Study [36]. In this study, 61,433 postmenopausal women completed food frequency questionnaires to determine daily vitamin E intake. The women were followed for 19 years and self-reported hip fractures. Daily vitamin E intake of less than 5 mg/day was associated with an increased risk of hip fracture; however, vitamin E intakes of over 15 mg/day were associated with decreased risk of hip fracture in postmenopausal women, suggesting that vitamin E may be beneficial for bone.

Introduction to Vitamin E

Vitamin E Forms

Vitamin E is a family of eight naturally occurring molecules, consisting of four tocopherols (alpha, beta, gamma, delta) and four tocotrienols (alpha, beta, gamma, delta) [37]. Tocopherols contain a fully saturated phytol tail, whereas tocotrienols have an isoprenoid tail with three unsaturated double bonds. Although all eight forms of vitamin E are obtained from diet, alpha-tocopherol is the most biologically active because it reverses vitamin E deficiency symptoms in humans [38, 39]. Additionally, alpha-tocopherol is the most abundant form of vitamin E found in nature [40], and is also preferentially secreted into circulation [41]. Once absorbed in the intestine, vitamin E is packaged into chylomicrons for delivery to the liver through the lymphatic system [42]. In the liver, alpha-tocopherol transfer protein (aTTP) assists in transporting alpha-tocopherol into circulation for delivery to tissues [41]. In rodent models with a defective aTTP, tissue and plasma alpha-tocopherol concentrations are significantly decreased.
Additionally, familial mutations of aTTP in humans causes decreased alphatocopherol status, ataxia, and peripheral neuropathy [44, 45], which can be overcome by large doses (>800 mg/day) of vitamin E [46].

**Vitamin E Antioxidant Function**

Vitamin E is an essential lipid-soluble vitamin that functions as a chain-breaking antioxidant, preventing propagation of lipid peroxidation and damage to cellular membranes [47]. Vitamin E reacts with peroxyl radicals, reducing radicals to lipid hydroperoxides. *In vivo* studies have demonstrated that vitamin E decreases concentrations of F$_2$-isoprostanes, which are formed during lipid peroxidation. Pratico et al. [48] supplemented apoE-deficient mice (model of atherosclerosis) with vitamin E, resulting in a reduction of F$_2$-isoprostanes and atherosclerotic lesions. Additionally, humans taking vitamin E supplements have decreased urinary excretion of 8-epi-prostaglandin F$_2$-alpha, which is an abundant F$_2$-isoprostane [49]. Furthermore, in rodents with vitamin E deficiency, plasma and tissue F$_2$-isoprostanes are increased [50]. Thus, high vitamin E status is associated with low levels of biomarkers of oxidative stress, while the opposite is also true: low vitamin E status is associated with high oxidative stress biomarkers.

Alpha-tocopherol is the biologically active form of vitamin E; however, all forms of vitamin E have antioxidant activity. Ouchi et al. [51] completed biokinetic analyses of each vitamin E form using stopped-flow spectrophotometry. Tocopherols or tocotrienols were individually mixed with an aroxyl radical ethanolic solution, and scavenging activity was measured. Alpha-tocopherol had the greatest antioxidant activity, as evidenced by highest scavenging rate. Alpha-tocotrienol had the second greatest antioxidant activity, followed by beta-tocopherol > beta-tocotrienol > gamma-tocopherol > gamma-tocotrienol > delta-tocopherol > delta-tocotrienol. Thus, alpha-tocopherol has both the highest biological and antioxidant activities of all of the vitamin E forms.
Vitamin E Metabolism

All forms of vitamin E are metabolized to the water-soluble metabolite, carboxyethyl hydroxy chromanol (CEHC) [52], which was discovered from urinary analysis in humans after vitamin E supplementation [53, 54]. Conversion of vitamin E to CEHC is a xenobiotic process that includes three steps: phase I omega-hydroxylation, phase II conjugation, and phase III hepatic transport. During phase I of xenobiotic processing, vitamin E is omega-hydroxylated by the enzyme, cytochrome P4F2 (CYP4F2), and oxidized to form 13'-hydroxycromanol and 13'-carboxycromanol [55]. 13'-carboxycromanol is further metabolized by beta-oxidation to shorten the phytol or isoprenoid tail of vitamin E, forming CEHC [53, 55]. In mice with a disrupted gene equivalent of CYP4F2, vitamin E metabolism is impaired, as evidenced by accumulation of tissue vitamin E [56]. Additionally, humans with genetic variants of CYP4F2 have increased circulating levels of vitamin E, suggesting its critical role in vitamin E metabolism [57].

To increase water solubility for excretion, CEHC or its precursor, 13'-carboxycromanol, undergoes phase II conjugation. 13'-carboxycromanol is conjugated to sulfate [58], and CEHC is conjugated to sulfate [59], glucuronide [60], or glycoside [61] in plasma. Additionally metabolomic analysis of human urine has reported conjugation of CEHC to glycine, glycine glucuronide, and taurine [62]. Once conjugation is completed, CEHC is secreted into circulation or bile by phase III transporters. Multidrug resistance protein 1, 2, and 4 (MDR 1, 2, 4) [63-65], solute carrier family 22, organic anion transporter, member 5 (slc22a5) [63], and breast cancer resistance protein 1 (BCRP1) [65] are reported to increase in response to high vitamin E, suggesting their role in hepatic CEHC secretion.
Vitamin E Sources and Requirements

The current adult recommended daily allowance for vitamin E is 15 mg (22.5 IU) alpha-tocopherol per day [66]. Alpha-tocopherol is found in dietary sources including nuts (almonds), seeds (sunflower), vegetable oils (sunflower, safflower, wheat germ), and green, leafy vegetables. Although alpha-tocopherol is the active form of vitamin E, gamma-tocopherol is the major form consumed in the United States [67]. High concentrations of gamma-tocopherol are found in soybean and corn oil, which are two common oils used and consumed in the American diet [68]. Additionally, tocotrienols are also consumed from sources such as palm oil, however, to a much lesser extent. As reported in the 1999-2000 NHANES, the median dietary intake of alpha-tocopherol was 7.6 mg/day for men and 5.8 mg/day for women [69]. Additionally, 11.3% of adults consume single vitamin E supplements (>400 IU/day) [69], and over 30% of adults (>20 years-old) consume multi-supplements containing vitamin E [70].

The Effects of Vitamin E on Bone

Adequate intakes of antioxidants, such as vitamin E, have been shown to reduce the risk of osteoporosis and hip fracture in humans [35, 36, 71]. Given this relationship between vitamin E and improved skeletal outcomes, many studies have attempted to elucidate the mechanisms by which vitamin E alters bone; however, the current published findings are largely discrepant. Vitamin E has been shown to improve skeletal outcomes in rodent models of oxidative stress [72, 73]; however, vitamin E may have no effect in healthy rodent models [74, 75]. Additionally, tocotrienols appear to have greater effects on the skeleton than other vitamin E forms [76, 77], suggesting that form of vitamin E may be a determinant of skeletal outcomes. By contrast, high levels of dietary vitamin E appear to have adverse effects on bone [78], suggesting that vitamin E in excess may be detrimental to the skeleton. The following sections will highlight studies that support each conclusion.
**Vitamin E is Beneficial for Bone in Models of Oxidative Stress**

Vitamin E has been reported to improve skeletal outcomes in models representative of increased oxidative stress. Arjmandi et al. [72] examined the effects of vitamin E on the skeleton in an aged rodent model. C57BL male mice (24-month-old) were fed diets containing alpha-tocopherol (30 or 500 mg/kg diet) for 4 weeks. Structural and material properties of femurs were assessed using three-point bending tests. Higher dietary intakes of vitamin E significantly improved ultimate load and stiffness of femurs compared with control aged mice. In addition to age, vitamin E has also been reported to improve skeletal outcomes in a model representative of postmenopausal women. Muhammad et al. [73] fed ovariectomized Wistar rats (3-month-old) diets containing alpha-tocopherol (30 or 60 mg/kg diet) or tocotrienol (60 mg/kg diet) for 4 weeks. Higher dietary intakes of vitamin E prevented femoral bone loss that was observed in rats receiving lower doses of vitamin E. Taken together, these reports demonstrate that dietary vitamin E may improve skeletal outcomes in rodent models representative of increased oxidative stress.

**Vitamin E Has No Effect in Healthy Rodent Models**

Although vitamin E has been shown to improve skeletal outcomes in models of oxidative stress, vitamin E seems to have no effect in healthy rodent models. Iwaniec et al. [74] fed 8-month old Sprague Dawley rats diets containing alpha-tocopherol (500 IU/kg) for 13 weeks. Micro-computed tomography analysis of femora showed no effect on bone mass or microarchitecture from vitamin E supplementation. Additionally, Kasai et al. [75] fed Wistar rats diets containing alpha-tocopherol (120 mg/kg) for 2 months, finding no effect on femoral bone mass or microarchitecture from dietary vitamin E.

**Tocotrienols May Have Greater Skeletal Effects than other Vitamin E Forms**

In addition to rodent model studied, form of vitamin E may also be a determinant of skeletal outcomes. Norazlina et al. [76] fed Sprague Dawley rats (4-month-old)
diets containing alpha-tocopherol (0 or 30 mg/kg diet) or mixed-tocotrienol (30 or 60 mg/kg) for 8 months. Bone calcium content in femurs was significantly increased in rats fed higher levels of dietary tocotrienol compared with E deficient rats. Moreover, lumbar vertebrae bone calcium content was significantly increased with higher tocotrienol intakes compared with both E deficient and alpha-tocopherol-fed rats. Additionally, Mehat et al. [77] fed Sprague Dawley rats (3-month-old) diets containing alpha-tocopherol (60 mg/kg) or gamma-tocotrienol (60 mg/kg) for 4 months. Bone formation rates were significantly increased by dietary tocotrienol compared with tocopherol. Furthermore in vitro, tocotrienols have been reported to inhibit RANKL expression and osteoclast differentiation, thus favoring bone formation [79]. These studies suggest that tocotrienols may have greater effects on the skeleton than other vitamin E forms.

High Levels of Vitamin E May Be Detrimental for Bone
Although vitamin E has been shown to improve bone parameters in vivo and decrease osteoclast activities in vitro, extremely high levels of dietary vitamin E may have adverse effects on bone. Fujita et al. [78] fed C57BL mice (4-week-old) and Sprague Dawley rats (8-week-old) diets containing alpha-tocopherol (600 mg/kg diet) for 8 weeks. High dietary vitamin E decreased cancellous bone mass by 20% in mice and significantly decreased femoral bone mineral density in rats. Additionally in vitro, alpha-tocopherol treatment induced dendritic-cell-specific transmembrane protein (DC-STAMP), which is a promoter of osteoclast fusion and bone resorption. These findings demonstrate that high levels of dietary vitamin E may stimulate bone resorption in rodents, and result in bone loss.

Study Rationale
Over 40% of older adults consume dietary supplements containing vitamin E [70]. Since older adults can be afflicted with osteoporosis, and high intakes of vitamin E are reported to decreased bone mass [78], I sought to investigate the mechanisms for the adverse consequences of high dietary vitamin E on bone.
Specifically, I investigated whether bone loss mediated by vitamin E was specific to alpha-tocopherol or if other vitamin E forms, in excess, also have detrimental effects on the skeleton. Adequate intakes of tocotrienols are reported to have greater effects on bone turnover than other vitamin E forms [76, 77, 79]. I postulated that high intakes of tocotrienols may induce bone loss in rodents. Additionally, I investigated whether the vitamin E metabolite, CEHC, contributed to bone loss. In humans, high intakes of alpha-tocopherol increase urinary excretion of CEHC [80]. I postulated that bone loss mediated by vitamin E may actually be due to increased levels of its metabolite, CEHC.

**Hypothesis and Aims**

This thesis presents a study aimed to examine the effects of high dietary vitamin E on the rodent skeleton. The central hypothesis was that bone loss mediated by high intakes of dietary vitamin E may be due to high levels of its metabolite, CEHC. My study consisted of two specific aims. In Aim 1, I measured the skeletal outcomes of high intakes of dietary tocopherols and tocotrienols. In Aim 2, I investigated the effects of increased concentrations of CEHC on bone turnover.
Chapter 2:

High Dietary Vitamin E has No Effect on Bone Mass, Density, or Turnover in Male Rats

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Abstract

Scope: High vitamin E intakes reportedly decrease cancellous bone mass by 20% in rodents. We hypothesized that these adverse effects are mediated by its metabolite, carboxy ethyl hydroxy chromanol (CEHC).

Methods and results: Male Sprague Dawley rats, 11 w, consumed for 18 w semi-purified diets that contained adequate alpha-tocopherol (A-AT), added 500 mg alpha-tocopherol/kg diet (H-AT), or added 250 mg mixed-tocols/kg diet (H-T3). Vitamin E status was verified by measuring plasma and tissue vitamin E concentrations. Bone density, microarchitecture (cortical thickness, cancellous bone volume fraction, trabecular number, thickness, and spacing), and cancellous bone formation were assessed in tibia using dual energy absorptiometry, micro-computed tomography, and histomorphometry, respectively. In addition, serum osteocalcin was assessed as a global marker of bone turnover. Significant differences among treatment groups were not detected for any endpoints measured.

Conclusions: Vitamin E added to otherwise adequate diets did not induce osteopenia; therefore, we conclude that high intakes of vitamin E do not have a negative impact on the skeleton in young adult male rats.
Introduction

Osteoporosis is the most common metabolic bone disease in humans, and is characterized by a reduction in bone mass, which increases an individual's risk of fracture [20]. In the United States, more than 10 million individuals over 50 years-old have osteoporosis and over 43 million individuals have low bone mass, or osteopenia [20]. A contributing factor to the development of osteoporosis is increased oxidative stress [81]. Increased oxidative stress from aging [82], smoking [31], and chronic inflammatory diseases [83] are associated with low bone mineral density and increased risk of osteoporotic bone loss. In mechanistic studies using isolated mouse bone marrow cells, reactive oxygen species (ROS) generated by hydrogen peroxide or by xanthine oxidase, have been shown to induce expression of the osteoclast differentiation factor, receptor activator of NF-kB ligand (RANKL) [34, 84]. Furthermore, in vitro studies have shown that ROS inhibit bone formation and maturation of osteoblasts [85], while the antioxidant vitamin E suppresses RANKL via induction of the RANKL inhibitor, osteoprotegerin (OPG) [86]. Thus, oxidants and antioxidants appear to have opposing actions on bone turnover.

Adequate intakes of dietary vitamin E in humans are associated with a decreased risk of osteoporosis and hip fracture [35, 36, 71]. Moreover, in rodents, tocotrienols appear to have a greater beneficial effect on the skeleton than do other vitamin E forms [76, 77]. Mehat et al. [77] demonstrated that rats fed for 4 months diets containing gamma-tocotrienol (60 mg/kg) have increased bone formation rates compared with rats fed alpha-tocopherol (60 mg/kg). Additionally, Norazlina et al. [76] reported increased femoral and lumbar vertabrae bone calcium contents from rats fed tocotrienol (60 mg/kg diet) compared with vitamin E deficient rats or those fed alpha-tocopherol (30 mg/kg diet). In mechanistic studies in vitro, tocotrienols inhibited RANKL expression and osteoclast differentiation [79, 87]. Thus, both alpha-tocopherol and alpha-
tocotrienol appear to have beneficial skeletal effects by inhibiting factors that increase bone resorption.

Although vitamin E has been shown to decrease osteoclast activities in vitro, extremely high levels of dietary vitamin E may have detrimental effects on bone. Fujita et al. [78] reported that growing mice fed diets fortified with alpha-tocopherol (600 mg/kg diet) for 8 weeks had 20% lower cancellous bone volume. Additionally, they observed that growing rats fed high levels of alpha-tocopherol (600 mg/kg diet) for 8 weeks had significantly lower bone mineral density. Furthermore, in vitro studies using isolated mouse bone marrow cells revealed that alpha-tocopherol induces the expression of dendritic-cell specific transmembrane protein (DC-STAMP), which promotes osteoclast fusion and maturation. These findings suggest that especially high levels of vitamin E intake may stimulate bone resorption.

Since older humans (>70 years-old) can be afflicted with osteoporosis [20] and over 40% of older adults consume dietary supplements containing vitamin E [70], we sought to investigate the mechanisms for the adverse consequences of vitamin E on rodent bones. Vitamin E is not just one molecule, but is a family of lipid-soluble molecules that consists of four tocopherols (alpha, beta, gamma, delta) and four tocotrienols (alpha, beta, gamma, delta). Only alpha-tocopherol meets the requirement for vitamin E in humans [66] because its concentrations are maintained in circulation by the function of the hepatic alpha-tocopherol transfer protein. Although the non-alpha-tocopherols are absorbed and transported in circulation to the liver, they are not maintained in circulation but are instead metabolized to carboxy ethyl hydroxy chromanol (CEHC) [88]. Interestingly, both tocopherols and tocotrienols share the same metabolite, CEHC [89], which was discovered from urinary analysis following vitamin E supplementation [53, 90]. Additionally, consuming high intakes of alpha-tocopherol in humans increases urinary alpha-CEHC excretion [80]. Since
CEHCs are more potent anti-inflammatory agents than are tocopherols [91], CEHCs may potentially be important in bone metabolism.

Given the controversy concerning the potential adverse and beneficial effects of vitamin E on bone pathology, we hypothesized that long-term intakes of alpha-tocopherol or mixed-tocotrienols would be necessary to adequately evaluate overall regulatory effects on bone turnover. Further, we propose that changes in bone status mediated by vitamin E may be due to high concentrations of its anti-inflammatory metabolite, CEHC. Therefore, we assessed skeletal effects including microarchitecture, serum osteocalcin, osteogenic gene expression, and bone formation rates in young adult male rats fed for 18 weeks diets with high levels of alpha-tocopherol or mixed-tocols.
**Materials and Methods**

*Animal study design*

The Oregon State University (OSU) Institutional Animal Care and Use Committee approved all procedures. 11-week male Sprague Dawley rats (Charles River) were housed in plastic cages with hard wood chips, kept on a 12-h light/dark cycle, and maintained on an AIN-93G diet (Harlan) and water ad libitum for 7 days to acclimate to the OSU animal facility. The rats were housed two per cage throughout the study.

Experimental diets were based on AIN-93G (Harlan) including: 1) adequate-dietary alpha-tocopherol (A-AT, 60 IU *all-rac-alpha*-tocopheryl acetate/kg diet, n=6), 2) high-dietary alpha-tocopherol (H-AT, added 500 mg RRR alpha-tocopherol (Archer Daniels Midland)/kg diet, n=6), or 3) high-dietary mixed-tocols (H-T3, added 500 mg 50% tocomin (Carotech) /kg diet n=7). Rats were then randomly assigned into one of three treatments groups. The rats were maintained on their respective diets for 18 weeks.

To label mineralizing bone for assessment of formation, the rats were injected with calcein (15 mg/kg body weight, Sigma Chemical) at 11 and 3 days prior to euthanasia. At necropsy, blood was collected in 10-mL vacutainer tubes containing 1 mg/mL EDTA. Plasma was obtained by centrifugation (1500xg, 15 min) and stored at -80°C until analysis. Livers were excised, frozen in liquid nitrogen, and stored at -80°C. Tibiae and lumbar vertebrae were excised and placed in 70% ethanol for dual energy x-ray absorptiometry (DXA), micro-computed tomography (μCT) and histomorphometry analyses. Femora were removed, cleaned of soft tissue, frozen in liquid nitrogen, and stored at -80°C for mRNA isolation and gene expression analysis. Femora bone marrow was collected and homogenized in PBS, frozen in liquid nitrogen, and stored at -80°C.
**Vitamin E measurements**

Diet, plasma, bone marrow, and liver vitamin E concentrations were determined by high-performance liquid chromatography with electrochemical detection (HPLC-ECD) as previously described [92]. Briefly, diet, plasma, bone marrow, or liver was saponified with ethanolic KOH, extracted with hexane, dried under nitrogen, resuspended in ethanol:methanol (1:1), and injected into the HPLC system. Vitamin E was detected electrochemically in oxidizing mode with 500 mV potential. Peak areas were integrated using Shimadzu Scientific 4.2 Class VP software package. Values were quantitated using calibration standards prepared using authentic compounds.

**CEHC measurements**

Plasma, bone marrow, and liver alpha- and gamma-CEHC concentrations were determined by high-performance liquid chromatography with mass spectrometry (HPLC-MS) as previously described [27]. Using diethyl ether, plasma and bone marrow CEHCs were extracted from acid hydrolyzed samples [93]. Livers were homogenized and then CEHCs were extracted from enzyme hydrolyzed samples [94]. Aliquots of ether fractions were collected and dried under nitrogen. The samples were resuspended in 1:1 (v/v) water:methanol containing trolox (Sigma) as the internal standard, and injected using liquid chromatography-mass spectrometry (LC-MS) with a Waters (Milford, MA) 2695 Separations Module and a Micromass ZQ2000 (Milford, MA) with an electrospray ionization probe (Waters). Concentrations were calculated by comparison to peak areas generated from a standard curve using authentic compounds with correction based on the internal standard, trolox.

**Densitometry**

Total tibia bone mineral content (BMC, mg), bone area (mm²), and bone mineral density (BMD, mg/mm²) were measured using DXA (Piximus 2, Lunar Corporation, Madison, WI).
Micro-computed tomography (μCT) analysis
Tibiae and 2nd lumbar vertebrae were scanned using a Scanco μCT40 scanner (Scanco Medical AG, Basserdorf, Switzerland) at a voxel size of 16 x 16 x 16 μm (55 kVp x-ray voltage, 145 μA intensity, and 200 ms integration time). Filtering parameters sigma and support were set to 0.8 and 1, respectively. The threshold value for evaluation was determined empirically and set at 245 (gray scale, 0-1000). Cortical bone in the tibia diaphysis and cancellous bone in the proximal tibia metaphysis and epiphysis were evaluated (Figure 2.1). Automated contouring was used to delineate cortical bone from non-bone. Following, all cortical slices were examined visually for inclusion of cancellous struts originating from the endocortex (extremely rare at this sight) and manually removed when present. For cortical bone, 62 slices (992 μm in length) were evaluated and cross-sectional volume (cortical and marrow volume, mm$^3$), cortical volume (mm$^3$), marrow volume (mm$^3$), and cortical thickness (μm) were determined. Polar moment of inertia (I$\text{Polar}$) was determined as a surrogate measure of bone strength in torsion. For proximal tibia metaphysis, 50 slices (800 μm in length) of cancellous bone were measured starting 800 μm distal to the growth plate. The entire cancellous compartment (29 ± 1 slices, 465 ± 16 μm, mean ± SEM) was assessed in the tibial epiphysis. Analysis of the lumbar vertebra included the entire region of secondary spongiosa between the cranial and caudal growth plates (282 ± 6 slices, 4,512 ± 96 μm) (Figure 2.1). Cancellous bone measurements in tibia and lumbar vertebra included cancellous bone volume fraction (bone volume/tissue volume, %), trabecular thickness (mean thickness of individual trabeculae, μm), trabecular number (number of trabecular intersects per unit distance, mm$^{-1}$), and trabecular spacing (distance between trabeculae, μm).

Histomorphometry analysis
Preparation of tibiae for histomorphometric evaluation was completed as previously described [95]. Briefly, the proximal tibia was dehydrated in a graded
series of ethanol and xylene and embedded in modified methyl methacrylate. Sections (5 μm thick) were cut with a vertical bed microtome (Leica/Jung 2165) and affixed to slides precoated with a 1% gelatin solution. Sections were mounted unstained for measurement of fluorochrome labels. The sampling site for tibial metaphysis was located approximately 0.75-1.00 mm distal to the growth plate. Fluorochrome-based measurements of bone formation included: 1) mineralizing perimeter (mineralizing perimeter/bone perimeter: cancellous bone perimeter covered with double plus half single label normalized to bone perimeter, %), 2) mineral apposition rate (the distance between two fluorochrome markers that comprise a double label divided by the 8 day interlabel interval, μm/day), and 3) bone formation rate (bone formation rate/bone perimeter: calculated by multiplying mineralizing perimeter by mineral apposition rate normalized to bone perimeter, μm²/μm/yr). All data were collected using the OsteoMeasure System (OsteoMetrics, Inc., Atlanta, GA, USA).

**Serum osteocalcin assay**

Serum gla-osteocalcin was measured using the MK126 rat Gla-osteocalcin High Sensitivity enzyme immunoassay kit from Clontech (Mountain View, CA, USA).

**Gene expression analysis**

Femora were pulverized with a mortar and pestle in liquid nitrogen and then homogenized in Trizol (Invitrogen). Total RNA was isolated according to the manufacturer’s protocol, and mRNA was reverse transcribed into cDNA using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). Osteogenic gene expression was determined using a Rat Osteogenesis RT² Profiler PCR Array (Qiagen) according to the manufacturer’s protocol. Gene expression was normalized to beta-2 microglobulin (B2m), hypoxanthine phosphoribosyltransferase 1 (Hprt1), and lactate dehydrogenase A (Ldha), and relative quantification was determined by the delta delta Ct method using RT² Profiler PCR Array Data Analysis software version 3.5 (Qiagen).
Statistical analysis

One-way ANOVA followed by Tukey post-hoc test was used to evaluate differences among treatment groups. ANOVA assumptions of equal variances between groups were met as evidenced by Bartlett’s test. Pearson Correlation analysis was used to determine the relationship between CEHC and bone parameters. Differences were considered significant at p < 0.05. All data are expressed as mean ± SEM.
Results

_Tocopherol, tocotrienol, and CEHC concentrations in plasma, bone marrow, and liver_

Sprague Dawley rats, 11-weeks-old, were fed semi-purified diets containing A-AT, H-AT, or H-T3 for 18 weeks. Body weights increased similarly in the three treatment groups for the duration of the study (Figure 2.2). Vitamin E analysis of diets revealed that H-AT diets contained 518 mg alpha-tocopherol/kg, while the H-T3 diets contained 234 mg mixed tocopherols and tocotrienols/kg diet (Table 2.1). Thus, the experimental conditions provided a 7-fold range of vitamin E intakes and a variety of vitamin E forms.

Plasma and liver alpha-tocopherol concentrations were significantly elevated in rats consuming the H-AT diet compared with rats consuming the A-AT or H-T3 diets (Figure 2.3 A, C). Additionally, liver gamma-tocopherol concentrations were significantly increased in rats consuming the H-AT diet compared with the H-T3 diet (Figure 2.3 F). Gamma-tocopherol concentrations in plasma and bone marrow were unchanged with dietary treatments (Figure 2.3 D, E). In rats consuming the H-T3 diet, alpha- and gamma-tocotrienols were quantifiable in plasma, bone marrow, and liver (Figure 2.4 G-L). Tocotrienols were not detected in samples from rats fed the A-AT and H-AT diets.

Plasma, bone marrow, and liver concentrations of alpha-CEHC were significantly increased in the H-AT diet group compared with the A-AT and H-T3 diet groups (Figure 2.5 A, B, C). Additionally, liver concentrations of gamma-CEHC were significantly increased in the H-AT and H-T3 diet groups compared with the A-AT group (Figure 2.5 F), and only plasma gamma-CEHC was significantly increased with H-T3 diet (Figure 2.5 D). Bone marrow gamma-CEHC concentrations were unaffected by dietary treatments (Figure 2.5 E).
Assessment of bone mass, bone microarchitecture, and bone formation

Bone microarchitecture was assessed using μCT analysis of tibia and 2nd lumbar vertebrae. The rats consuming A-AT, H-AT, or H-T3 diets did not demonstrate any significant differences with regard to cancellous bone volume fraction in tibia or lumbar vertebrae (Figure 2.6 A, B, D), or in tibial cortical bone volume (Figure 2.6 C). Additionally, there were no significant differences between diet groups with regard to tibial cortical thickness or marrow volume, or in tibial and lumbar vertebral trabecular number, trabecular thickness, and trabecular spacing (Table 2.2). Furthermore, there were no significant differences between diet groups in bone mineral content or bone mineral density (Figure 2.7 A, B). Additionally, no significant differences were observed between diet groups for mineralizing perimeter, mineral apposition rate, or bone formation rate (Figure 2.8 A, B, C).

Assessment of serum osteocalcin and bone marrow osteogenic gene expression

Serum levels of gla-osteocalcin were measured as a marker of bone turnover. Osteocalcin values were not statistically different between diet groups (Figure 2.9). However, serum gamma-CEHC and osteocalcin were significantly correlated (Figure 2.10), with the highest gamma-CEHC and lowest osteocalcin levels in the rats from the H-T3 group.

Femoral bones from rats consuming A-AT, H-AT, and H-T3 diets were assessed with regard to osteogenic gene expression using an 84-gene PCR array. Femurs from rats consuming the H-AT diet did not show any significant changes in osteogenic gene expression compared with femurs from rats consuming the A-AT diet (Figure 2.11). In contrast, femurs from rats consuming the H-T3 diet were found to have some alterations in osteogenic gene expression compared with those from rats consuming the A-AT diet (Figure 2.12). Genes that were significantly decreased include annexin V (Anxa5, -0.2-fold), growth
differentiation factor-10 (Gdf10, -1.0-fold), matrix metalloproteinase-8 (Mmp8, -0.5-fold), mothers against decapentaplegic homolog-5 (Smad5, -0.4-fold), and osteopontin (Spp1, -0.6-fold). Genes that were significantly increased include bone morphogenic protein receptor, type 1B (Bmpr1b, 1.8-fold), fibroblast growth factor-2 (Fgf2, 0.5-fold), and noggin (Nog, 1.0-fold). Although eight genes were found to be significantly different between the diet groups, the fold-changes were not large; only Bmpr1b was nearly doubled.
Discussion

In the present study, rats were fed A-AT, H-AT, or H-T3 diets for 18 weeks, but no significant changes between diet groups were observed for bone mass, density, turnover, or bone-related gene expression. Given these findings, we conclude that high dietary levels of vitamin E have no negative impact on the skeleton.

Our findings are in contrast to the study reported by Fujita et al. [78], who fed C57BL mice (4-weeks-old) and Sprague Dawley rats (8-weeks-old) diets containing 600 mg alpha-tocopherol/kg diet for 8 weeks. They observed a 20% lower lumbar vertebra cancellous bone volume in mice and lower bone mineral density in the femora of rats.

Although Fujita and colleagues observed negative skeletal outcomes from elevated vitamin E intakes, the present study, as well as other published reports [72, 74, 75, 96], have shown no adverse effects on the skeleton of rodents fed diets containing high levels of vitamin E. Iwaniec et al. [74], using skeletally mature Sprague Dawley rats, fed high alpha-tocopherol diets (500 IU/kg diet) for 13 weeks, and evaluated femoral bone mass, density, and microarchitecture. No significant differences were observed between control and high vitamin E-fed rats. The authors concluded that high dietary alpha-tocopherol has no effect on the skeleton in skeletally mature rats. Additionally, Arjmandi et al. [72] examined the effects of high dietary vitamin E in adult (6-month-old) and aged (24-month-old) C57BL mice. Mice were fed for 4 weeks with 500 mg alpha-tocopherol /kg diet. The high vitamin E diet had no effect on mechanical bone parameters or serum markers of bone turnover in adult mice. By contrast, high dietary vitamin E significantly improved bone strength in aged mice. The authors concluded that the high dietary vitamin E diet had no effect on the skeleton in adult rodents, and is beneficial for bone in aged rodents [72].
The discrepancy between outcomes relative to the effects of vitamin E on bone status may be due to the age of rodents studied. Iwaniec et al. [74] and Arjmandi et al. [72] used models that were skeletally mature when the high vitamin E-containing diets were initiated, and therefore closely mimicked bone turnover during adulthood [97]. Moreover, these models are more closely representative in age of humans that likely take vitamin E supplements [70]. This is in contrast to the model used by Fujita et al. [78], in which the rats were growing throughout the study. The approach used by Fujita et al. [78] mimics skeletal growth and modeling, which is a site-specific process that involves formation and resorption of bone that occur independently from one another [97]. In growth and skeletal modeling, bone cycles through phases of activation, formation, and resorption. This is in contrast to bone remodeling observed in adults, where bone resorption is coupled with bone formation [97]. The model used by Fujita et al. [78] resembled adolescent skeletal modeling. However, human adolescents are more likely to be consuming diets low rather than high in vitamin E [98].

In the present study, we chose to use 3-month-old Sprague Dawley rats that were slowly skeletally developing and reached skeletal maturity by the end of the study [97]. This particular model was used as an attempt to determine whether the low bone volume observed by Fujita et al. [78], if transient, could be overcome by the time skeletal maturity and normal remodeling was reached. Given our findings, we conclude that if high dietary vitamin E results in reduced bone acquisition during growth, it may be only temporary and not observed once rodents reach skeletal maturity.

In addition to age of rodents, the discrepancy in results may likely be due to unusually high plasma alpha-tocopherol concentrations observed by Fujita et al. [78]. They reported plasma alpha-tocopherol concentrations that were ~6-fold higher in response to their high vitamin E diet, whereas we only observed a ~2-fold increase from feeding similar amounts of alpha-tocopherol. Our results are consistent with previously published data in rodents showing that high oral doses
of vitamin E only increase plasma concentrations of alpha-tocopherol 2-3-fold. Kasai et al. [75] fed rats 600 mg alpha-tocopherol/kg diet for 8 weeks, resulting in a ~2-fold increase in serum alpha-tocopherol compared with control. Additionally, no significant differences in bone mass or bone turnover were detected with high vitamin E. Moreover, only a 2-3-fold increase in plasma alpha-tocopherol has been observed in humans given high oral doses of vitamin E [99].

In addition to the discussed rodent models, high dietary vitamin E has also been examined in human clinical studies, showing no effect or modest beneficial effects on the skeleton. Ruiz-Ramos et al. [100] completed a clinical trial consisting of 135 subjects (mean age = 68) who received a placebo, 500 mg/day ascorbic acid + 400 IU/day alpha-tocopherol, or 1000 mg/day ascorbic acid + 400 IU/day alpha-tocopherol for one year. After one year of supplementation, subjects receiving the high antioxidant dose had increased hip BMD compared with baseline measurements, and subjects receiving placebo or low dose supplementation had no significant change in hip BMD from baseline. Additionally, Chavan et al. [101] examined the effects of high dietary vitamin E on serum markers of oxidative stress in 75 osteoporotic subjects (age range = 45-70). Subjects were given vitamin E (400 IU/day, Evinal), vitamin C (500 mg/day), or a combination for 90 days. Vitamin E supplementation significantly decreased serum levels of malondialdehyde (MDA) in osteoporotic subjects compared with baseline measurements, suggesting an improvement in oxidative status from dietary vitamin E.

In the present study, we fed A-AT, H-AT, or H-T3 diets to assess the role the vitamin E metabolite, CEHC, on bone. As expected, high dietary intakes of vitamin E significantly increased plasma and tissue CEHC concentrations. Plasma, bone marrow, and liver alpha-CEHC were significantly increased with H-AT diet compared with other diet groups. Additionally, plasma gamma-CEHC was significantly increased in H-T3 rats compared with other diet groups. Although H-AT and H-T3 diets had no effect on any bone parameter measured,
higher levels of plasma gamma-CEHC were significantly correlated with lower levels of serum osteocalcin. Studies have associated lower serum osteocalcin with better skeletal outcomes in models of elevated bone turnover, such as postmenopausal women. Singh et al. [102] completed a case-control study of 82 postmenopausal women to examine the relationship between serum osteocalcin, bone mineral density, and osteoporosis. A significant negative correlation was found between serum osteocalcin and bone mineral density. Additionally, women with osteopenia and osteoporosis had significantly higher levels of serum osteocalcin compared with healthy postmenopausal controls. Furthermore, Duggan et al. [103] reported an association between increased serum osteocalcin and lower bone mineral density in patients with chronic pancreatitis, suggesting that increased inflammation may contribute to higher levels of serum osteocalcin and worse skeletal outcomes.

The mechanisms by which gamma-CEHC may influence the skeleton have received little attention. Hypothetically, gamma-CEHC may inhibit pro-inflammatory cytokines and eicosanoids, which could contribute to osteoclast formation and bone resorption. Jiang et al. [104], using a carrageenan-induced inflammation Wistar rat model, measured the effects of increased serum gamma-CEHC concentrations on pro-inflammatory markers. Increased serum gamma-CEHC from gamma-tocopherol diets (33 or 100 mg/kg diet) significantly decreased prostaglandin E2, 8-isoprostone, and tumor necrosis factor-alpha (TNF-alpha) compared with rats receiving alpha-tocopherol diets (33 mg/kg diet). TNF-alpha is an upstream activator of RANKL and Nf-kB, which modulates osteoclast differentiation [87]. Additionally, increased levels of isoprostanes are associated with altered bone metabolism and lower bone mass in humans [105]. Given these findings, we postulate that the correlation between plasma gamma-CEHC and lower serum osteocalcin may be a result of the inhibition pro-inflammatory cytokines and eicosanoids, which may decrease bone resorption and remodeling.
We demonstrate herein that high dietary vitamin E has no effect on the rodent skeleton. We report a significant correlation between plasma gamma-CEHC and low serum osteocalcin; however, we did not observe any skeletal alterations, so the physiological significance of this relationship is unclear.
Figure 2.1: Tibia and lumbar vertebra sampling sites
Figure 2.2: Animal weights

Animal weights increased over the duration of the study. Final average weights were not significantly different between groups (A-AT = 671 ± 35, H-AT = 682 ± 64, H-T3 = 679 ± 58 grams). Individual animal weights are shown.
Figure 2.3: Tocopherol concentrations in plasma, bone marrow, and liver

(A, C) Alpha-tocopherol concentrations were increased in plasma and livers of rats fed H-AT diets. (F) Gamma-tocopherol concentrations were increased in livers of H-AT diet group compared with H-T3 diet group. Statistical significances are shown by columns with different letters. Data are means ± SEM.
Figure 2.4: Tocotrienol concentrations in plasma, bone marrow, and liver

(G-L) Alpha- and gamma-tocotrienol concentrations were increased above the detectable range in plasma, bone marrow, and livers of rats fed H-T3 diets for 18 weeks. Statistical significances are shown by columns with different letters. (ND, not detectable in A-AT and H-AT diet groups). Data are means ± SEM.
Figure 2.5: CEHC concentrations in plasma, bone marrow, and liver

(A, B, C) Alpha-CEHC concentrations were increased in plasma, bone marrow, and livers of rats fed H-AT diets for 18 weeks. (D) Gamma-CEHC concentrations were increased in plasma of rats fed H-T3 diets for 18 weeks. (F) Gamma-CEHC concentrations were increased in livers of rats fed H-AT and H-T3 diets. Statistical significances are shown by columns with different letters. Data are means ± SEM.
Figure 2.6: Tibia cortical volume and cancellous bone volume fractions

High intakes of dietary vitamin E had no effect on cancellous bone volume fraction in tibia (A, B) and lumbar vertebra (D), or on cortical volume in tibia (C). Data are means ± SEM.
**Figure 2.7: Tibia bone mineral content and bone mineral density**

Bone mineral content (A) and bone mineral density (B) were unaffected by high intakes of vitamin E. Data are means ± SEM.
Figure 2.8: Histomorphometry analysis of tibia

High intakes of dietary vitamin E had no effect on mineralizing perimeter (A), mineral apposition rate (B), and bone formation rate (C). Data are means ± SEM.
Figure 2.9: Assessment of serum osteocalcin

High intakes of dietary vitamin E had no effect on serum osteocalcin, a marker of bone turnover. Data are means ± SEM.
Figure 2.10: Correlation between gamma-CEHC and osteocalcin

Plasma gamma-CEHC is negatively correlated with plasma osteocalcin ($p = 0.021$, $r^2 = 0.27$). Black circles are H-T3, gray circles are H-AT, and open circles are A-AT diets. Individual values are shown.
Figure 2.11: High alpha-tocopherol vs. adequate alpha-tocopherol osteogenic gene expression

H-AT diet had no effect on genes from 84-gene osteogenic array.
Figure 2.12: High mixed-tocols vs. adequate alpha-tocopherol osteogenic gene expression

H-T3 diet significantly altered 8 genes from osteogenic array. Significant differences are shown by open circles (p < 0.05). Only Bmpr1b was more than doubled.
**Table 2.1: Vitamin E content in diets**

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Table 2.2: Assessment of cancellous bone microarchitecture

High levels of dietary vitamin E had no effect on tibia and lumbar vertebra cancellous bone microarchitecture. Data are means ± SEM.

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<th>H-T3 (n = 7)</th>
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<td>Trabecular number (1/mm)</td>
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<td>Trabecular spacing (µm)</td>
<td>209 ± 18</td>
<td>225 ± 10</td>
<td>205 ± 10</td>
<td>0.30</td>
</tr>
<tr>
<td><strong>Tibia metaphysis (cancellous bone)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trabecular number (1/mm)</td>
<td>3.14 ± 0.12</td>
<td>3.41 ± 0.20</td>
<td>3.57 ± 0.15</td>
<td>0.17</td>
</tr>
<tr>
<td>Trabecular thickness (µm)</td>
<td>78 ± 2</td>
<td>74 ± 3</td>
<td>74 ± 5</td>
<td>0.71</td>
</tr>
<tr>
<td>Trabecular spacing (µm)</td>
<td>310 ± 12</td>
<td>291 ± 20</td>
<td>273 ± 13</td>
<td>0.24</td>
</tr>
<tr>
<td><strong>Tibia diaphysis (cortical bone)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cross-sectional volume (mm³)</td>
<td>8.26 ± 0.25</td>
<td>8.16 ± 0.31</td>
<td>8.59 ± 0.20</td>
<td>0.46</td>
</tr>
<tr>
<td>Marrow volume (mm³)</td>
<td>1.43 ± 0.10</td>
<td>1.34 ± 0.08</td>
<td>1.37 ± 0.09</td>
<td>0.81</td>
</tr>
<tr>
<td>Cortical thickness (µm)</td>
<td>866 ± 20</td>
<td>899 ± 28</td>
<td>926 ± 23</td>
<td>0.47</td>
</tr>
<tr>
<td><strong>Lumbar vertebra (cancellous)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trabecular number (1/mm)</td>
<td>3.37 ± 0.16</td>
<td>3.67 ± 0.11</td>
<td>3.49 ± 0.07</td>
<td>0.23</td>
</tr>
<tr>
<td>Trabecular thickness (µm)</td>
<td>84 ± 2</td>
<td>81 ± 2</td>
<td>82 ± 2</td>
<td>0.53</td>
</tr>
<tr>
<td>Trabecular spacing (µm)</td>
<td>288 ± 15</td>
<td>257 ± 10</td>
<td>275 ± 7</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Data are means ± SE.
Chapter 3:

Discussion & Conclusion
Introduction to Supplemental Mechanism of Vitamin E-Mediated Bone Loss

In the present study, I examined the effects of high levels of dietary vitamin E and its metabolite, CEHC, on the rodent skeleton. Vitamin E had no effect on bone volume or bone microarchitecture in rats fed high vitamin E diets for 18 weeks. Additionally, high levels of vitamin E did not alter serum osteocalcin, bone formation rates, or osteogenic gene expression. These findings are in contrast to those of Fujita et al. [78] who reported significant bone loss in rodents fed high dietary vitamin E. In the present study, I proposed that this discrepancy in outcomes may be due to age of rodent model studied and level of plasma alpha-tocopherol. The rodents from the study by Fujita et al. [78] were young (<8 weeks-old), skeletally growing, and resembled adolescent skeletal modeling, which was not representative of bone remodeling during adulthood. Also, the rodents had plasma alpha-tocopherol concentrations that were ~6-fold higher in response to their high vitamin E diet, whereas previously published data in rodents consuming high oral doses of vitamin E only report ~2-3-fold increases in plasma alpha-tocopherol concentrations.

In addition to these possible explanations discussed in Chapter 2, I would like to propose an additional mechanism by which vitamin E may mediate bone loss in rodents. I postulate that the rodents from the study by Fujita et al. [78] may have had low circulating levels of vitamin K (as a result of a vitamin K-insufficient diet), which became further depleted by high intakes of vitamin E, resulting in under-carboxylation of vitamin K-dependent bone proteins and alterations in bone turnover.
Introduction to Vitamin K

Vitamin K Forms
Vitamin K is an essential lipid soluble vitamin and exists in three primary forms: phylloquinone (PK), menadione (MN), and menaquinone-4 (MK-4). PK is the major dietary form and accounts for more than 90% of plant-derived vitamin K [106]. In the body, PK is converted to the intermediate, MN, by side chain cleavage and is then further converted to the tissue storage form, MK-4 [107]. MK-4 is formed by geranylgeranylation and catalysis of MN, which is done by UbiA prenyltransferase domain containing 1 (UBIAD1) [108]. The site of conversion of PK to MK-4 is controversial. Okano et al. [107] has reported intestinal conversion of PK to MK-4; however, other reports suggest that conversion occurs in the liver [109].

Vitamin K Function and the Vitamin K Cycle
Vitamin K is a cofactor for proteins involved in coagulation and calcium homeostasis [110], and does so through a recycling process referred to as the vitamin K cycle. The vitamin K cycle is first initiated when vitamin K quinone is reduced to a hydroquinone form by vitamin K oxidoreductase (VKOR) [111]. Vitamin K hydroquinone serves as a substrate for gamma-glutamyl-carboxylase (GGCX) [112], which is an enzyme required for post-translational modification of glutamate to gamma carboxyl glutamate residues on vitamin K-dependent proteins [113]. During this process, vitamin K hydroquinone is oxidized to the vitamin K epoxide form, which is then reduced back to vitamin K quinone by VKOR.

Vitamin K-Dependent Bone Proteins
Vitamin K serves as a cofactor for proteins involved in a variety of physiological functions that include coagulation, bone mineralization, and vascular calcification [110]. Particularly, osteocalcin and protein S are two abundant bone proteins that
play a role in bone homeostasis. Both osteocalcin and protein S are secreted by osteoblasts, bind to bone surfaces, and increase affinity for mineral binding and bone formation [114, 115]. Additionally, in vivo studies suggest that osteocalcin may promote recruitment and differentiation of osteoclast precursors. Glowacki et al. [116], using a bone particle implantation model in rodents, found that osteocalcin-deficient bone particles resorbed only 60% as well as normal bone particles. This suggests that osteocalcin is not only needed for bone formation, but is also involved in bone resorption and overall remodeling.

Matrix Gla protein is another vitamin K-dependent protein found in bone and is also an inhibitor of vascular calcification [117]. Similar to its function in the vasculature, matrix Gla protein has been identified to inhibit calcification of cartilage. In matrix Gla protein-deficient mice, premature calcification of chondrocytes (bone cartilage cells) occurs, along with increased vascular calcification and premature death [118]. Matrix Gla protein has also been reported to influence the activity of bone morphogenetic protein-2 (BMP-2), which is involved in TGF-beta signaling and osteoblast formation. In vitro studies have shown that matrix Gla protein interferes with BMP-2 binding to its receptor, which decreases activation of the Smad-dependent pathway, osteoblast differentiation, and potentially bone formation [119].

Low Vitamin K Status and Poor Skeletal Outcomes

Previous reports have associated low vitamin K status with adverse skeletal outcomes. Vitamin K insufficiency resulting from either malnutrition or vitamin K cycle inhibitors (i.e. anti-coagulants) have been reported to decrease circulating carboxylated levels of osteocalcin and matrix Gla bone proteins [120, 121]. Additionally, low vitamin K status is associated with increased vascular calcification [122], decreased bone mineral density [123], and increased risk of fractures [124]. Moreover, under-carboxylation of these bone proteins serve as
clinical predictors for fracture [125], low bone mineral density [126], and cardiovascular calcification [127] risks in humans.

Vitamin E Decreases Vitamin K Status

Vitamin E supplementation has been reported to decrease vitamin K levels in both human and rodent models. The Women’s Health Study conducted a 10 year randomized clinical trial to test the effects of vitamin E supplements (600 IU) for the prevention of cardiovascular disease in 40,000 women aged 45 years and older [128]. Women receiving vitamin E supplementation reported increased nose bleeds and decreased thromboembolism, which are signs of altered coagulation and decreased vitamin K status. In an additional study, humans receiving vitamin E supplements (1000 IU) had increased levels of serum under-carboxylated prothrombin, which is a marker of inadequate vitamin K status [129].

In rodents, bleeding abnormalities have been observed when administered high levels of vitamin E; however, these bleeding abnormalities were reversed by vitamin K administration [66]. Additionally, high levels of vitamin E and its metabolite, CEHC, are associated with decreased vitamin K status in rodents. Vitamin E was administered subcutaneously for 7 days in rats fed diets containing PK [130]. Plasma and extra-hepatic MK-4 levels were decreased significantly with vitamin E injections compared with injections of the vehicle. Additionally, decreased MK-4 levels in the brain were associated with increased concentrations of CEHC, suggesting that CEHC may play a role in regulation of vitamin K status [130].
Proposed Supplemental Mechanism of Vitamin E-Mediated Bone Loss

I propose that my findings, in contrast to the opposite findings by Fujita et al. [78], are the result of adequate dietary vitamin K in the rodent diet, which protects against vitamin E-mediated bone loss. It is likely that the Fujita diet, from CLEA Japan, used AIN76 vitamin mix, which has been found to be inadequate with regard to vitamin K [131]. Our diets, on the other hand, had adequate dietary vitamin K (0.9 mg PK/kg diet), which not only prevented bone loss from high levels of dietary vitamin E, but also maintained levels of carboxylated osteocalcin in rats given high dietary vitamin E for 18 weeks. A similar protective association has been observed in humans. Booth et al. [129] orally administered alphatocopherol (1000 IU/day) to humans with normal vitamin K status. After 12 weeks, osteocalcin and vitamin K levels remained unchanged.

I postulate that the adverse effects observed by Fujita et al. [78] may be the result of inadequate dietary vitamin K in the rodent diets. It is plausible that the rodents had low levels of circulating vitamin K from vitamin K-insufficient diet, which became further depleted by high dietary intakes of vitamin E, resulting in under-carboxylation of vitamin K-dependent bone proteins and alterations in bone turnover. Given the relationship between low vitamin K status and adverse skeletal outcomes, I postulate that low dietary K is the reason for observed bone loss in rodents fed high levels of vitamin E.

Concluding Remarks

In conclusion, I demonstrate that high intakes of dietary vitamin E have no adverse effects on the rodent skeleton. I postulate that vitamin K status may be a critical determinant of skeletal outcomes mediated by high levels of vitamin E. It will be important to investigate whether low vitamin K status contributes to
vitamin E-mediated bone loss. These studies will be critical for determining a safe and tolerable level of vitamin E supplementation in humans.
Bibliography


