

RESEARCH ARTICLE

High-Dose α -Tocopherol Supplementation Does Not Induce Bone Loss in Normal Rats

Shunji Kasai^{1*}, Akemi Ito², Kaori Shindo³, Tohru Toyoshi⁴, Masahiro Bando⁵

1 Vitamin E Information and Technology Section, Customer Joy Department, Eisai Co. Ltd., Tokyo, Japan, **2** Ito Bone Histomorphometry Institute, Niigata, Japan, **3** Canon Lifecare Solutions Inc., Tokyo, Japan, **4** Study Department, Nihon Bioresearch Inc., Gifu, Japan, **5** Eisai Product Creation Systems, Eisai Co. Ltd., Tsukuba, Japan

* s-kasai@hmc.eisai.co.jp



OPEN ACCESS

Citation: Kasai S, Ito A, Shindo K, Toyoshi T, Bando M (2015) High-Dose α -Tocopherol Supplementation Does Not Induce Bone Loss in Normal Rats. PLoS ONE 10(7): e0132059. doi:10.1371/journal.pone.0132059

Editor: Dominique Heymann, Faculté de médecine de Nantes, FRANCE

Received: March 3, 2015

Accepted: June 9, 2015

Published: July 6, 2015

Copyright: © 2015 Kasai et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper.

Funding: This study was financially supported by Eisai Co., Ltd. Ito Bone Histomorphometry Institute, Canon Lifecare Solutions Inc., and Nihon Bioresearch Inc. did support in the form of salaries to authors (IS, KS, and TT) but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the 'author contributions' section.

Abstract

Oxidative stress affects bone turnover. Preventative effects of antioxidants such as vitamin E on reduced bone mineral density and fractures associated with aging, osteoporosis, and smoking have been examined in animals and humans. The effects of vitamin E (α -tocopherol; α T) on bone health have yielded conflicting and inconclusive results from animal studies. In this study, to determine the bone effects of α T, we investigated the in vivo effects of α T on the bone mineral density, bone mass, bone microstructure, bone resorption, and osteogenesis through peripheral quantitative computed tomography (pQCT) measurements, micro-computed tomography (micro-CT) analyses, and bone histomorphometry of lumbar vertebrae and femurs in normal female Wistar rats fed diets containing α T in different quantities (0, 30, 120, or 600 mg/kg diet) for 8 weeks. To validate our hypotheses regarding bone changes, we examined ovariectomized rats as an osteoporosis model and control sham-operated rats in parallel. As expected, ovariectomized rats had reduced bone mineral density in lumbar vertebrae and the distal metaphyses of their femurs, reduced bone mass and deteriorated microstructure of cancellous bones in the vertebral body and distal femur metaphyses, and reduced bone mass due to resorption-dominant enhanced bone turnover in secondary cancellous bones in these sites. In comparison, α T administered to normal rats, even at the highest dose, did not induce reduced bone mineral density of lumbar vertebrae and femurs or a reduced bone mass or fragile microstructure of cancellous bones of the vertebral body and distal femur metaphyses. Instead, α T-fed rats showed a tendency for an osteogenesis-dominant bone mass increase in secondary cancellous bones in the vertebral body, in which active bone remodeling occurs. Thus, α T consumption may have beneficial effects on bone health.

Introduction

Osteoporosis is associated with reduced bone strength due to decreased bone mineral density (BMD) and deteriorated bone quality [1]. Osteoporosis is also a major risk factor for fractures. Fractures caused by osteoporosis increase not only mortality rates [2–4] but also care

Competing Interests: All authors are employees of Eisai Co. Ltd., Ito Bone Histomorphometry Institute, Canon Lifecare Solutions Inc., or Nihon Bioresearch Inc. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

requirements. Secondary skeletal deformities due to fractures are often followed by patients becoming bedridden or developing chronic lumbar spondylosis. These conditions limit their functions of daily living. Preventing osteoporosis and maintaining bone health are key factors that both improve individuals' quality of life (QOL) and help to resolve serious social problems in an aging society, such as care for the elderly.

Free radicals suppress osteoblast differentiation [5] and facilitate bone resorption by activating osteoclasts [6, 7]. As such, oxidative stress has been suggested to create an imbalance between osteogenesis and bone resorption. For example, a study of middle-aged males (mean age, 55.7 years) and females (mean age, 55.9 years) revealed that the urinary 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-PGF $_{2\alpha}$) level, a biomarker of oxidative stress, was negatively correlated with BMD [8]. A comparative study between female patients ≥ 60 years with post-menopausal osteoporosis (mean age, 70.4 years) and control subjects (mean age, 68.8 years) revealed marked reductions in the levels of blood antioxidants (vitamins C and E and uric acid) and an anti-oxidative enzyme (superoxide dismutase) in the females with osteoporosis [9].

Oxidative stress affects bone turnover. The preventative effects of antioxidants such as vitamin E on reduced BMD and fractures associated with aging, osteoporosis, and smoking have been studied in animals and humans. Most studies using osteoporosis animal models and normal animals [10–14] as well as epidemiological studies in humans [15–20] have supported the beneficial effects of α -tocopherol (α T), a vitamin E homolog. Fujita et al., however, have recently proposed that α T may have adverse effects on bone health in humans, as it reduced the bone mass in mice and rats by promoting osteoclast fusion [21]. However, they did not assess the effects of different α T doses and did not include an appropriate positive control group to ensure the validity of their bone assessments. To determine whether α T has any adverse effects on bones, we used a rat model to study the *in vivo* effects of α T on various parameters. Using several techniques, we conducted a detailed analysis of BMD, bone mass, bone microstructure, bone resorption, and osteogenesis. Techniques included peripheral quantitative computed tomography (pQCT) measurements, micro-computed tomography (micro-CT) analyses, and bone histomorphometry of the lumbar vertebrae and femurs in normal rats that received different doses of α T. Our results demonstrated that α T did not adversely affect bone health in normal rats and actually suggested that it has beneficial effects.

Materials and Methods

Vitamin E

dl- α -tocopheryl acetate (α T) was synthesized by Eisai Co., Ltd. (Tokyo, Japan).

Diets

[Table 1](#) shows the composition of the α T-deficient diet. α T-supplemented diets were prepared by the addition of 30, 120, or 600 mg/kg of α T to the α T-deficient diet. These diets were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan).

Animal experiments

Animal experiments were approved by animal experiment committees of Nihon Bioresearch Inc. (Gifu, Japan) and Eisai. All animal treatments and experimental procedures were conducted in accordance with the pertinent institutional regulations.

Five-week-old female Wistar rats were purchased from CLEA Japan (Tokyo, Japan). These rats were allowed to acclimatize to the standard experimental conditions of 23°C–25°C, 47%–70% humidity, and 12-h day and night cycles. The animals had free access to water and the

Table 1. Composition of a α T-deficient diet.

Component	Amount (g/kg)
Casein (vitamin-free)	200
DL-methionine	3
Corn starch	430
α -Corn starch	120
Sucrose	100
Corn oil (vitamin E-stripped)	50
Cellulose powder	50
AIN-76 Mineral mixture	35
AIN-76 Vitamin mixture (without α T)	10
Choline bitartrate	2

α T: α -tocopherol

doi:10.1371/journal.pone.0132059.t001

standard rodent diet (CE-2, CLEA Japan). At 6 weeks of age, 16 of 48 rats underwent bilateral ovariectomy (OVX; OVX group, $n = 8$) or sham surgery (sham; sham group, $n = 8$) under isoflurane anesthesia. After the surgery, the OVX and sham group rats were fed the α T-deficient diet for 8 weeks and used as the osteoporosis model group and its control counterpart, respectively. The remaining 32 normal rats were randomly divided into 4 groups ($n = 8$ /group), and each group of rats was fed the α T-deficient diet (control group), 30 mg α T/kg diet (low-dose group), 120 mg α T/kg diet (medium-dose group), or 600 mg α T/kg diet (high-dose group) for 8 weeks. These rats had free access to water and diet. Their body weight and food consumption were measured once a week.

Tetracycline hydrochloride (20 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA) and calcein (10 mg/kg) (Sigma-Aldrich) were subcutaneously injected to double-label the bones, 7 days and 2 days before euthanasia, respectively. Blood was collected from the abdominal aorta of rats under anesthesia with isoflurane and centrifuged to obtain the serum. Serum samples were stored at -80°C until analysis. The lumbar vertebrae (L1–L3, L4–L6) and femurs on both sides were resected and fixed with 70% ethanol.

Measurement of serum α T concentration

After extracting serum samples with hexane, the extracts were dried and dissolved in 20 μl of ethyl acetate and 150 μl of ethanol. α T was measured using high-performance liquid chromatography on a Wakosil-II 5C18 column (4.6×250 mm; Wako Pure Chemical Industries, Ltd., Osaka, Japan) using a linear gradient elution system [acetonitrile:dichloromethane:methanol from 85:5:10 to 60:30:10 (v/v)] and a fluorescence detector (excitation, 295 nm; emission, 335 nm).

Measurement of BMD

BMD (mg/cm^3) was measured in the L5 lumbar vertebra (the distal one-fourth of the vertebra) and the distal metaphysis (3.0 mm from the growth plate cartilage) and diaphysial center of the left femur by pQCT using an XCT Research SA+ scanner equipped with XCT 6.20 software (Stratec Medizintechnik GmbH, Prorzheim, Germany).

Micro-CT analysis

The micro-CT system ($\mu\text{CT}40$; Scano Medical, Bassersdorf, Switzerland) was used for measurement of the bone mass and non-destructive 3-dimensional analysis of the cancellous bones

in the L5 lumbar vertebral body and distal metaphysis of the left femur. Three-dimensional analysis was conducted using scanned slice data to compute the following morphometric parameters: trabecular bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), connectivity density (Conn.D), and degree of anisotropy (DA).

Bone histomorphometry

After the removal of soft tissues, the L2 vertebra and the distal part of the right femur were subjected to Villanueva bone staining for 7 days without decalcifying treatment [22], dehydrated with increasing concentrations of ethanol, and embedded in methyl methacrylate (Wako Pure Chemical Industries, Ltd.). Sagittal sections of the vertebral body (5- μ m thick) and slices of the frontal section of the distal femur (5- μ m thick) were prepared using a microtome (LIECA, Germany).

In vertebral body measurements, regions of the secondary cancellous bone located at least 939 μ m away from the growth plate cartilage and at least 313 μ m away from the endosteum surface of the cortical bone were selected on both sides. The measured area was 2.45 mm². In femur measurements, regions of the secondary cancellous bone located 626–1565 μ m proximal to the growth plate cartilage of the distal femur were selected. The measured area was 2.35 mm². The nomenclature and units used were in accordance with the ASBMR Histomorphometry Nomenclature Committee [23]. The following parameters were measured: bone volume/tissue volume (BV/TV), eroded surface/bone surface (ES/BS), osteoid volume/osteoid surface (OV/OS), osteoid thickness (O.Th), mineral apposition rate (MAR), number of osteoclast/bone surface (N.Oc/BS), and number of osteoblast/bone surface (N.Ob/BS). N.Oc was separately measured for mononuclear osteoclast (N.Mo.Oc) and multinuclear osteoclast (N.Mu.Oc).

Statistical analysis

All the data are shown as the mean value \pm standard error of the mean (SEM). SAS version 8.2 (SAS Institute; Tokyo, Japan) was used for statistical analysis. The significant difference was determined by using independent Student's t-test (sham group vs. OVX group) or Dunnett's multiple comparison test (α T-deficient diet-fed normal rat control group vs. α T-supplemented diet-fed normal rat group). A P-value (both sides) of <0.05 was considered statistically significant.

Results

Body weight, food intake, and α T intake

α T supplementation had no significant effect on the body weight in normal rats (Table 2), while OVX rats showed a significant increase in body weight ($P < 0.001$). No significant differences in the mean food intake (g/kg body weight/day) were observed between the sham and OVX groups or between the control and α T-supplemented groups of normal rats over the course of the study period (Table 2). Based on the mean food intake and α T content in the diet, we determined the mean α T intake during the study period. The mean α T intake levels during the study period in the low-, medium-, and high-dose groups of rats were 2.2 ± 0.0 , 9.3 ± 0.1 , and 45.1 ± 0.9 mg/kg body weight/day, respectively.

Effects of α T supplementation on serum α T level

The serum α T levels in normal rats that received low-, medium-, and high-dose supplementation of α T were 11-fold ($P < 0.05$), 19-fold ($P < 0.001$), and 40-fold ($P < 0.001$) higher,

Table 2. Effects of α T supplementation on body weight, food intake, and serum α T levels.

Groups	Body weight (g)		Average daily food intake (g/kg body weight/day)	Serum α T level (μ g/dL)
	initial	final		
Sham rats	136 \pm 2	253 \pm 4	80 \pm 1	59 \pm 4
OVX rats	136 \pm 1	328 \pm 4***	78 \pm 2	50 \pm 2
Normal rats				
Control	136 \pm 1	253 \pm 7	78 \pm 1	54 \pm 2
Low-dose α T	136 \pm 1	252 \pm 7	75 \pm 1	618 \pm 48 [†]
Medium-dose α T	136 \pm 2	256 \pm 5	78 \pm 1	1050 \pm 91 ^{†††}
High-dose α T	136 \pm 1	259 \pm 5	75 \pm 1	2163 \pm 256 ^{†††}

OVX: ovariectomy; α T: α -tocopherol

Values are mean \pm standard error of the mean.

***P < 0.001 vs. sham group (Student's t-test).

[†]P < 0.05

^{†††}P < 0.001 vs. control group (Dunnett's multiple comparison test).

doi:10.1371/journal.pone.0132059.t002

respectively, with statistical significance in comparison with the serum α T level in control rats (Table 2).

Effects of α T supplementation on BMD

Bone mineral densities in the lumbar vertebra and the distal metaphysis of the femur, which are rich in cancellous bones, in OVX rats were 11% (P < 0.001) and 13% (P < 0.001) lower, respectively, with statistical significance in comparison with those in sham rats. However, there were no significant differences between these groups of rats in terms of BMD of the distal femur diaphysis, which is rich in the cortical bone (Fig 1).

Effects of α T supplementation on bone mass and microstructure of cancellous bone

The effects of OVX treatment and α T supplementation on the bone mass and microstructure were assessed in the vertebral body and distal femur diaphysis by micro-CT analysis.

For the vertebral cancellous bone, OVX rats showed a 28% decreased BV/TV (P < 0.001), 20% decreased Tb.N (P < 0.001), and 29% increased Tb.Sp (P < 0.001) in comparison with sham rats. For the distal femur diaphysis, OVX rats showed a 28% decreased BV/TV (P < 0.01), 55% decreased Tb.N (P < 0.001), 128% increased Tb.Sp (P < 0.001), 61% decreased Conn.D (P < 0.001), and 6% increased DA (P < 0.01) in comparison with sham rats (Figs 2 and 3). Thus, compared with sham rats, OVX rats were confirmed to have a reduced cancellous bone mass and weakened microstructures.

α T supplementation in normal rats had no significant effects on the bone mass or microstructure parameters of cancellous bones in the vertebral body and distal femur metaphysis (Figs 2 and 3).

Effects of α T supplementation on secondary cancellous bone

To assess the effects of OVX treatment and α T supplementation in normal rats on bone turnover, we conducted bone histomorphometry with secondary cancellous bones in the vertebral body and distal femur metaphysis, which have high bone turnover [24].

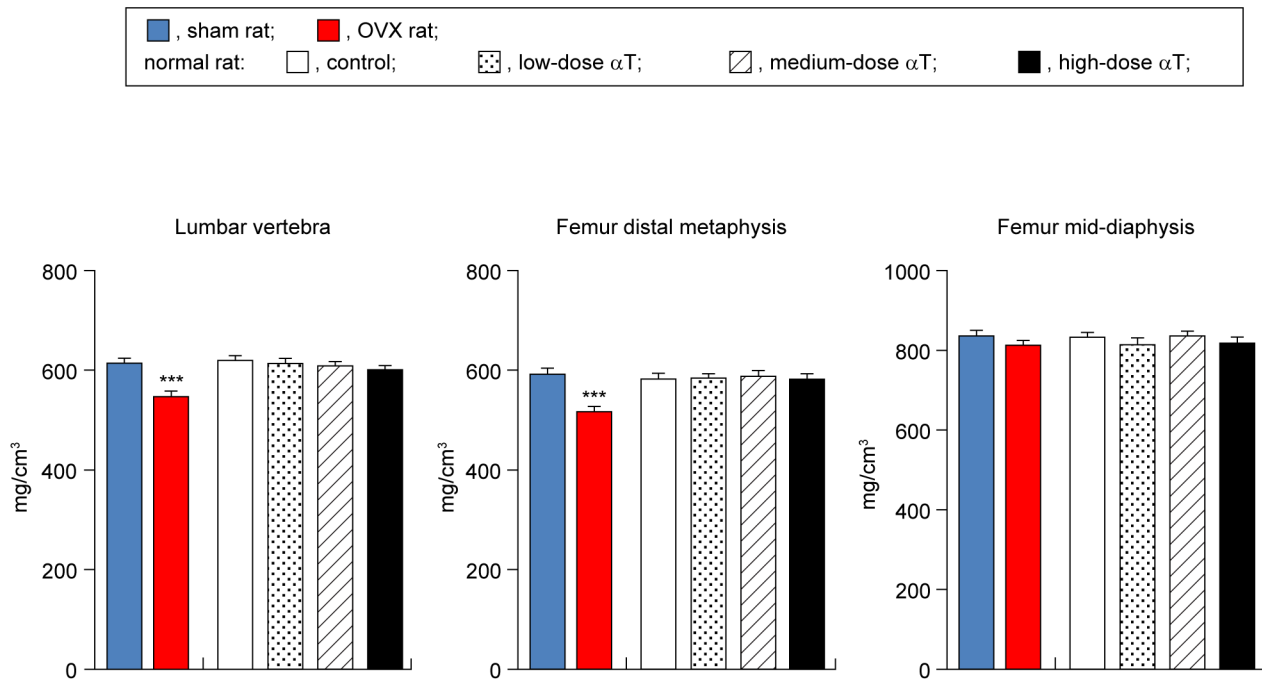


Fig 1. α T supplementation effects on bone mineral density of lumbar vertebra and femur in normal rats. Data are presented as mean \pm standard error of the mean. *** $P < 0.001$ vs. sham rat group.

doi:10.1371/journal.pone.0132059.g001

With regard to the secondary cancellous bone in the vertebral body, a significant 22% reduction in BV/TV ($P < 0.01$) was found in OVX rats in comparison with sham rats; this was accompanied by a 29% increase in the bone resorption parameter ES/BS ($P < 0.05$) and a 50% increase in OV/OS ($P < 0.001$), 50% increase in O.Th ($P < 0.001$), 31% increase in MAR ($P < 0.001$), and 133% increase in N.Ob/BS ($P < 0.001$) as osteogenesis parameters. Similarly, with regard to the secondary cancellous bone of the distal femur metaphysis, OVX rats showed a significant 52% reduction in BV/TV ($P < 0.001$) in comparison with sham rats. This reduction was accompanied by an 18% increase in ES/BS ($P = 0.208$), a 29% increase in OV/OS ($P < 0.001$), a 28% increase in O.Th ($P < 0.001$), a 36% increase in MAR ($P < 0.001$), and a 108% increase in N.Ob/BS ($P < 0.001$). These data confirmed that the bone mass was reduced in OVX rats as a result of enhanced bone resorption and osteogenesis in secondary cancellous bones in the vertebral body and distal femur metaphysis.

α T supplementation had different effects on the bone turnover of secondary cancellous bones in the vertebral body and distal femur metaphysis in normal rats. For the vertebral secondary cancellous bone, α T-supplemented normal rats had a significant increase or a trend for increase in BV/TV in comparison with control rats [low dose, +21% ($P = 0.189$); medium dose, +32% ($P < 0.05$); and high dose, +25% ($P = 0.099$)], which was accompanied by reduced ES/BS [medium dose, -34% ($P < 0.01$); high dose, -30% ($P < 0.01$)], increased OV/OS [medium dose, +14% ($P < 0.01$); high dose, +24% ($P < 0.001$)], increased O.Th [medium dose, +15% ($P < 0.01$); high dose, +25% ($P < 0.001$)], and increased MAR [low dose, +16% ($P < 0.001$); medium dose, +17% ($P < 0.001$); high dose, +22% ($P < 0.001$)]. Although the number of osteoblasts was significantly higher in α T-supplemented normal rats than in control rats [medium dose, +59% ($P < 0.05$); high dose, +82% ($P < 0.01$)], no significant changes were found in the number of osteoclasts, in the mononuclear subpopulation (N.Mo.Oc/BS) or the multinuclear subpopulation [(N.Mu.Oc/BS); Fig 4]. In contrast to the secondary cancellous bone in the

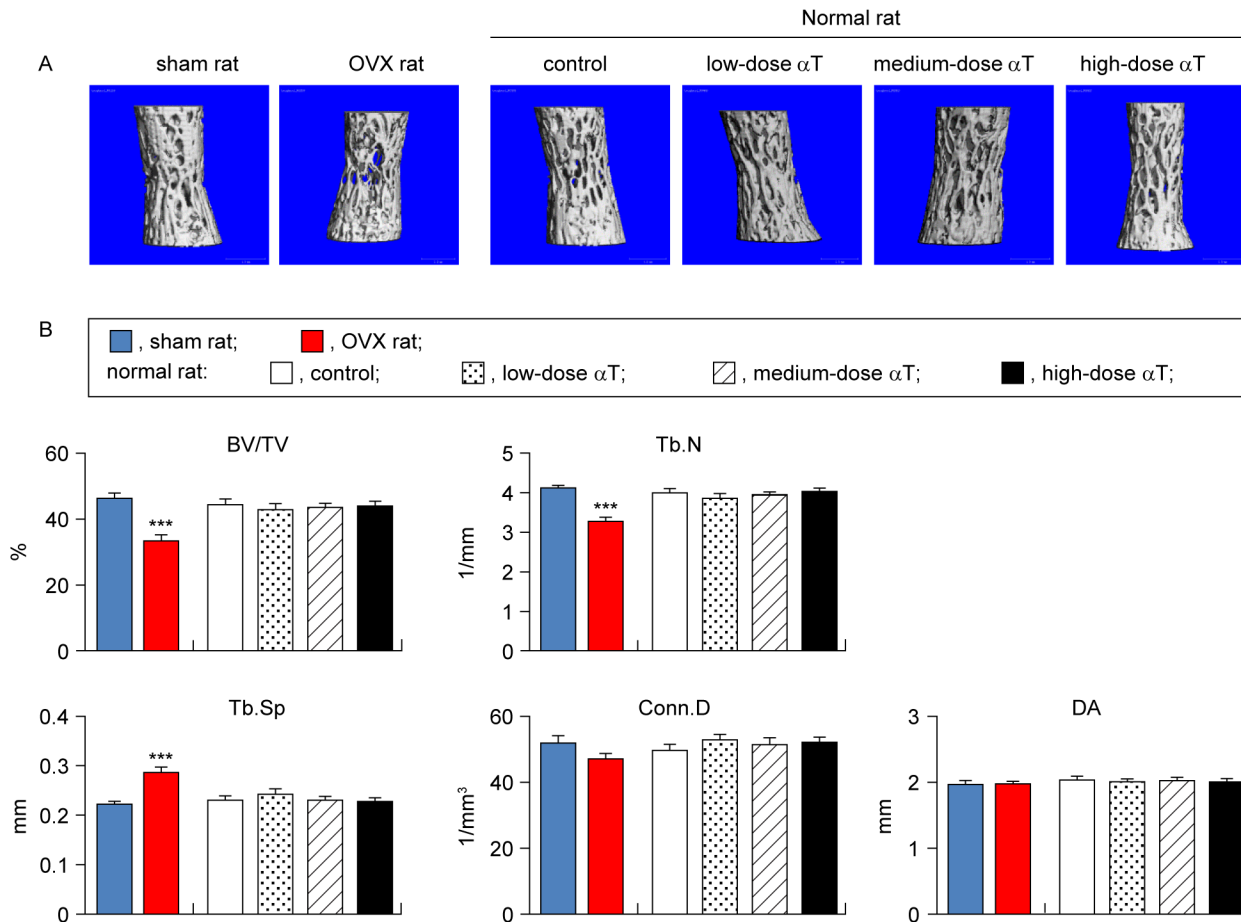


Fig 2. Micro-CT analysis of trabecular bone in vertebral body. (A) Representative 3-dimensional micro-CT images of trabecular bones in vertebral bodies are shown. (B) Microstructural parameters (BV/TV, trabecular bone volume fraction; Tb.N, trabecular number; Tb.SP, trabecular separation; Conn.D, connectivity density; DA, degree of anisotropy) of trabecular bones in vertebral bodies are shown. Data are presented as mean \pm standard error of the mean. ***P < 0.001 vs. sham rat group.

doi:10.1371/journal.pone.0132059.g002

vertebral body, α T supplementation did not significantly alter any of the osteogenic or bone resorption parameters in bone histomorphometry for the secondary cancellous bone of the distal femur metaphysis, except for MAR (Fig 5). These results indicate that α T supplementation in normal rats resulted in a trend for increased bone mass in the vertebral secondary cancellous bone through reducing bone resorption and enhancing osteogenesis but had virtually no effect on the secondary cancellous bone in the distal femur metaphysis.

Discussion

In the present study, we assessed the effects of α T on bones in normal rats fed diets containing different levels of α T. We found that α T did not induce BMD reduction in the lumbar vertebrae or distal femur metaphysis even at the highest dose tested; it did not induce bone mass reduction and microstructure deterioration in the cancellous bones in the vertebral body or distal femur metaphysis. Rather, α T supplementation resulted in a tendency for an osteogenesis-dominant bone mass increase in the vertebral secondary cancellous bone, in which active bone remodeling occurs.

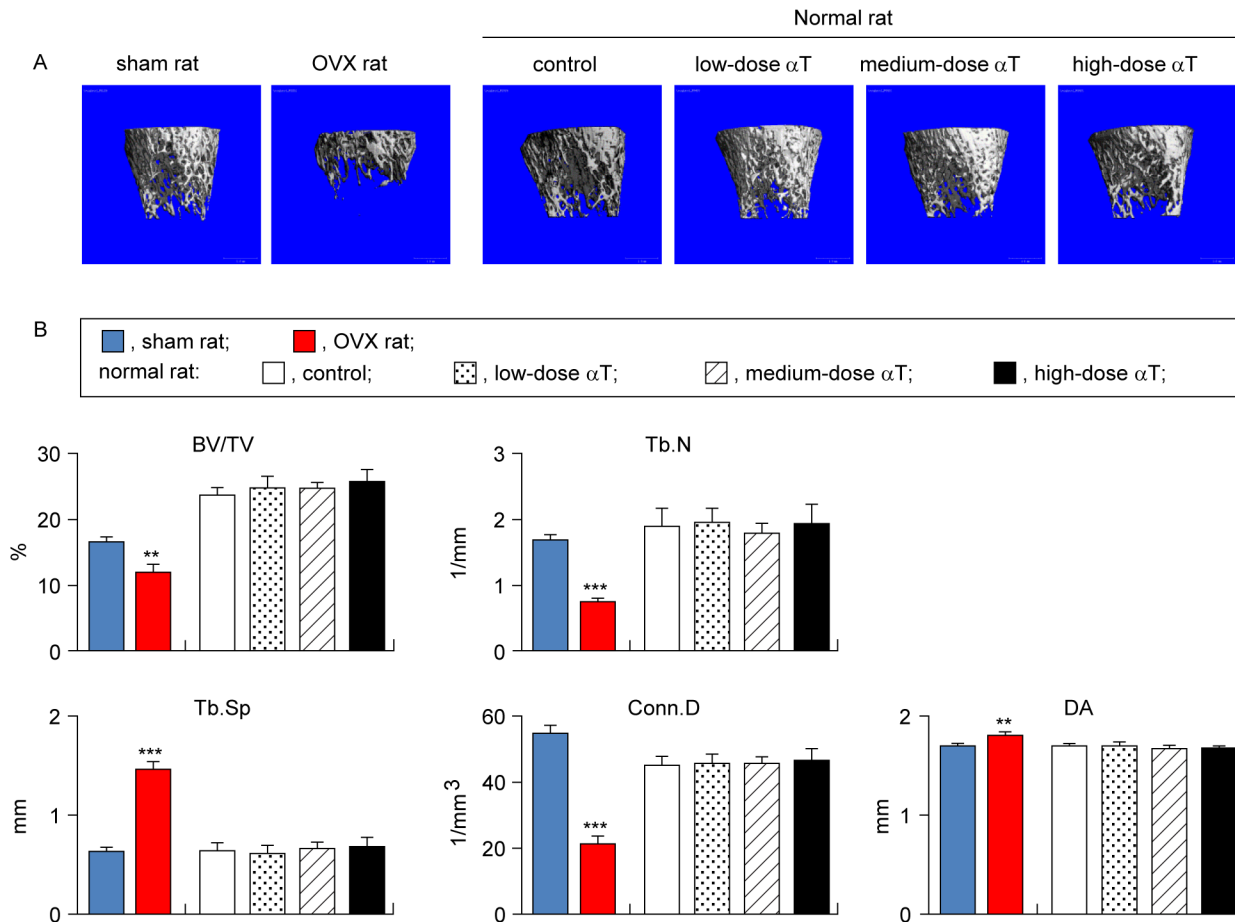


Fig 3. Micro-CT analysis of trabecular bones in distal femur metaphysis. (A) Representative 3-dimensional micro-CT images of trabecular bones in distal femur metaphysis are shown. (B) Microstructural parameters (BV/TV, trabecular bone volume fraction; Tb.N, trabecular number; Tb.SP, trabecular separation; Conn.D, connectivity density; DA, degree of anisotropy) of trabecular bones in distal femur metaphysis are shown. Data are presented as mean \pm standard error of the mean. ** $P < 0.01$, *** $P < 0.001$ vs. sham rat group.

doi:10.1371/journal.pone.0132059.g003

OVX rats are widely used in animal models of osteoporosis. This is a model that offers high reproducibility. It can be characterized by high bone turnover resulting from enhanced bone resorption and osteogenesis due to estrogen deficiency and reduced bone mass because of the bone resorption rate exceeding the osteogenesis rate [24]. To ensure that we correctly assessed bone changes, we performed assessments with the osteoporosis model rats and control sham-operated rats. In agreement with previous studies [25–34], we successfully demonstrated several points: 1) weight gain (Table 2), 2) reduced BMD in the lumbar vertebra and distal metaphysis of the femur (Fig 1), 3) reduced bone mass and deteriorated microstructure in the cancellous bones of the vertebral body and distal femur metaphysis (Figs 2 and 3), and 4) reduced bone mass due to bone resorption-dominant enhanced bone turnover in the secondary cancellous bones of the vertebral body and distal femur metaphysis (Figs 4 and 5). All these indicated that our assessments were valid. In addition to OVX rats, we evaluated the bones of normal rats fed diets containing different levels of α T and found the following characteristics of the effects of α T on normal rat bones: First, α T administered to normal rats, even at the highest dose, did not reduce BMD in the lumbar vertebra or femur (Fig 1). It also did not reduce the bone mass or induce microstructure deterioration of the cancellous bone in the

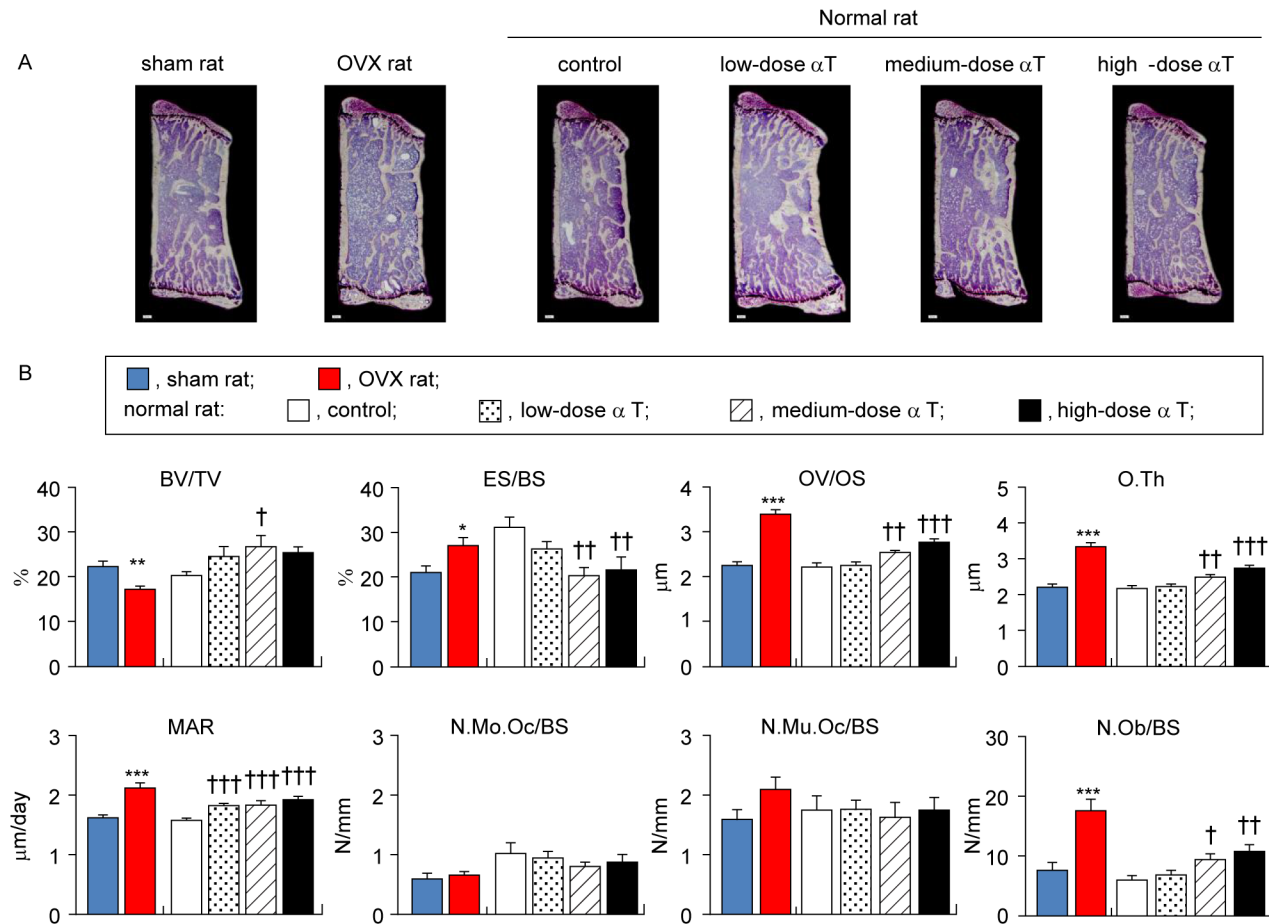


Fig 4. Histomorphometric analysis of secondary cancellous bones in vertebral body. (A) Representative micrographs of sagittal sections of vertebral bodies stained with Villanueva bone staining without decalcifying treatment are shown. (B) Histomorphometrical parameters (BV/TV, bone volume/tissue volume; ES/BS, eroded surface/bone surface; OV/OS, osteoid volume/osteoid surface; O.Th, osteoid thickness; MAR, mineral apposition rate; N.Mo.Oc/BS, number of mononuclear osteoclast/bone surface; N.Mu.Oc/BS, number of multinuclear osteoclast/bone surface; and N.Ob/BS, number of osteoblast/bone surface) of secondary spongiosa in vertebral bodies are shown. Data are presented as mean \pm standard error of the mean. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. sham rat group; † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$ vs. normal rat control group.

doi:10.1371/journal.pone.0132059.g004

vertebral body or distal metaphysis of the femur (Figs 2 and 3). Second, α T supplementation resulted in a tendency for increased bone mass in the secondary cancellous bone in the vertebral body in normal rats; however, it had no effect on the secondary cancellous bone in the distal femur metaphysis (Figs 4 and 5). In the present study, rats were sacrificed at 14 weeks of age. At this age, remodeling dominates bone turnover in the cancellous bone of the vertebral body, whereas modeling is dominant in the cancellous bones in distal metaphyses of the long bones [35–37]. Based on these findings, the vertebral body-selective effects of α T may be due to the specificity of α T effects to remodeling. The third feature of α T effects on the bones of normal rats was that an α T-induced tendency for increased bone mass in the vertebral secondary cancellous bone involved the suppression of bone resorption and promotion of osteogenesis (Fig 4).

With regard to the effects of α T on bone health, conflicting results have been reported for animal experiments. Most of these reports supported the beneficial effects of α T on bone health. Hermizi et al. [10] reported that in a nicotine-induced osteoporosis rat model, α T ameliorated the nicotine-induced bone mass reduction, bone resorption enhancement, and

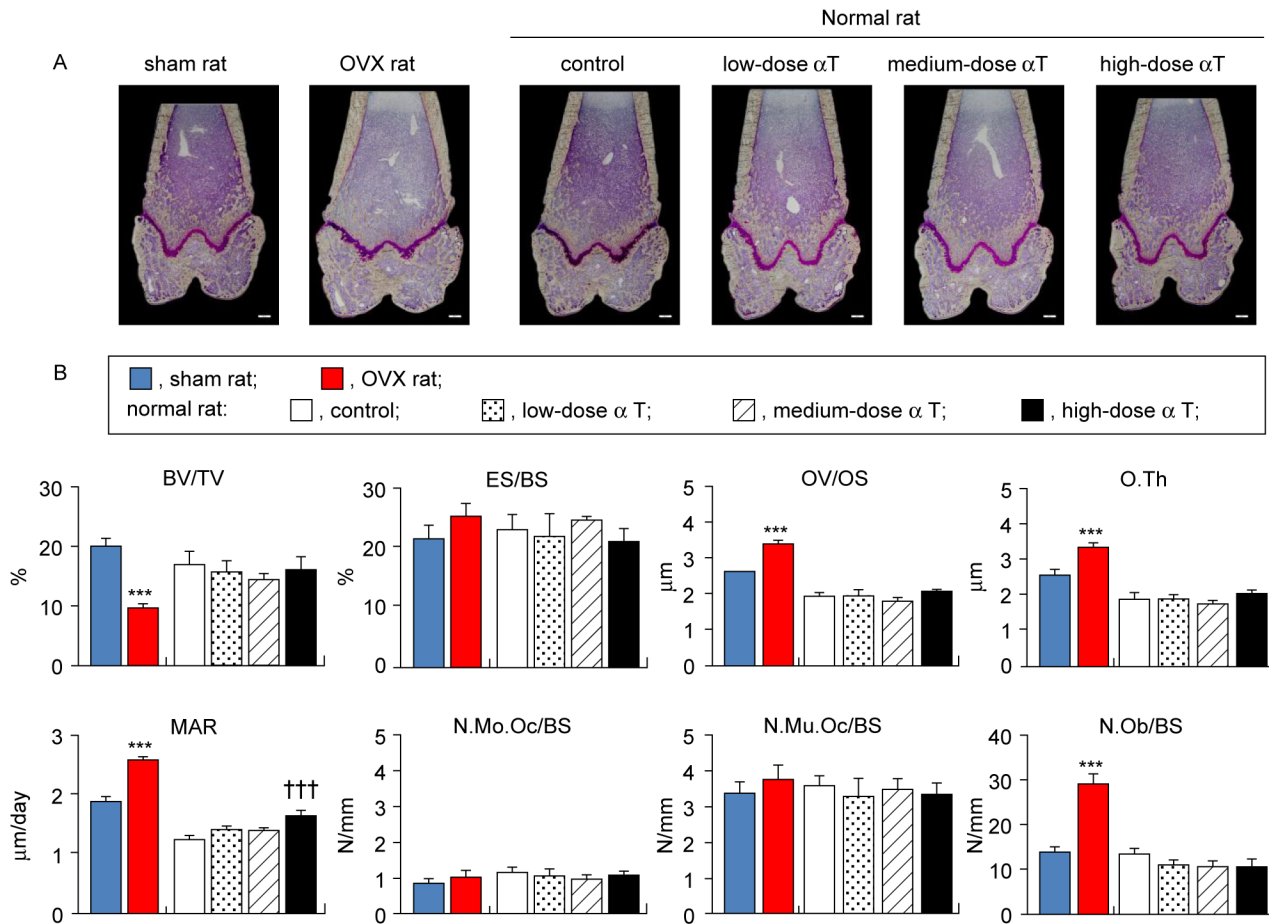


Fig 5. Histomorphometric analysis of secondary cancellous bones in distal femur metaphysis. (A) Representative micrographs of slices of frontal section of distal femur stained with Villanueva bone staining without decalcifying treatment are shown. (B) Histomorphometrical parameters (BV/TV, bone volume/tissue volume; ES/BS, eroded surface/bone surface; OV/OS, osteoid volume/osteoid surface; O.Th, osteoid thickness; MAR, mineral apposition rate; N.Mo.Oc/BS, number of mononuclear osteoclast/bone surface; N.Mu.Oc/BS, number of multinuclear osteoclast/bone surface; and N.Ob/BS, number of osteoblast/bone surface) of secondary cancellous bones in distal femur metaphysis are shown. Data are presented as mean \pm standard error of the mean. *** $P < 0.001$ vs. sham rat group; ††† $P < 0.001$ vs. normal rat control group.

doi:10.1371/journal.pone.0132059.g005

osteogenesis suppression in the femur. Norazlina et al. [11] reported that in OVX rats, α T suppressed BMD decrease in the femur and lumbar vertebra associated with OVX. Similarly, Muhammad et al. reported that α T improved OVX-associated bone mass decrease and bone microstructure deterioration in this osteoporosis model rats [12]. In addition, Muhammad et al. reported that α T suppressed bone resorption and facilitated osteogenesis in normal rats and increased bone strength [13, 14]. In contrast to these results, Fujita et al. recently proposed a hypothesis that α T reduces bone mass via the facilitation of osteoclast fusion and thereby exerts an adverse effect on bone health [21]. Their hypothesis was based on 3 experimental results: 1) When normal mice and rats in their growth periods were fed diets containing α T at 600 mg/kg diet for 8 weeks, their bone mass decreased by 20% compared with that of animals fed normal diets; this was accompanied by enhanced bone resorption and enlarged osteoclasts. 2) The bone mass of mice with low α T levels in their bodies due to deletion of the α T transfer protein (α TTP) was higher than that of wild-type mice; α T supplementation in these α TTP knockout mice reduced the bone mass. 3) In vitro experiments demonstrated that α T facilitated osteoclast fusion and formation of multinuclear osteoclasts, which have a high bone

resorption capability. One of our aims in the present study was to assess the hypothesis proposed by Fujita et al. using normal rats. Our study with normal rats in their growth period was conducted under the same conditions as those used by Fujita et al., including the strain and age in weeks of the rats, α T contents in the diet, and feeding period. The only exception was the gender of the rats. In the present study, young normal rats that received α T-supplemented diet did not show an increased number of multinuclear osteoclasts, reduced cancellous bone mass, deteriorated cancellous bone microstructure, or enhanced bone resorption. Thus, our experimental results did not support the hypothesis proposed by Fujita et al. Recently, Iwaniec et al. also assessed the hypothesis of Fujita et al. using normal adult rats with mature skeletons and old α TTP knockout rats [38]. They reported that their results also did not support the hypothesis.

In the present study, the mean α T intake levels in normal rats in the low-, medium-, and high-dose groups were 2.2, 9.3, and 45.1 mg/kg body weight/day, respectively, which are equivalent to 132, 558, and 2,708 mg/day, respectively, in a 60-kg human. The highest dose used in the present study exceeded 1,000 mg/day [39]. This is the tolerable upper level of regular daily vitamin E intake for an adult that is likely to pose no risk of adverse health effects for most people in the general population, as established by The Food and Nutrition Board (FNB) of the Institute of Medicine, National Academies (United States). Thus, the results of the present study suggest that α T consumption by humans below the tolerable upper intake level is unlikely to adversely affect bones.

The present study had some limitations. We used young female rats that received α T for 8 weeks. Further studies should be conducted in rats with a different gender, ages, and duration of α T administration. In addition, in the present study, we noted that α T tended to increase the secondary cancellous bone mass in the vertebral body, in association with suppressed bone resorption and enhanced osteogenesis; however, the underlying mechanism remains to be determined. This mechanism should be elucidated at the molecular level in the future.

In conclusion, α T supplementation was demonstrated to cause no adverse effects on the bone at a high dose but tended to induce an osteogenesis-dominant bone mass increase in the vertebral secondary cancellous bone, in which active bone remodeling occurs. Therefore, α T consumption may have beneficial effects on bone health.

Acknowledgments

We thank M. Ito (Nagasaki University Hospital) for micro-CT analysis.

Author Contributions

Conceived and designed the experiments: SK. Performed the experiments: AI KS TT. Analyzed the data: MB SK. Wrote the paper: SK.

References

1. NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy (2001) Osteoporosis prevention, diagnosis, and therapy. *JAMA* 285: 785–795. PMID: [11176917](#)
2. Ensrund KE, Thompson DE, Cauley JA, Nevitt MC, Kado DM, Hochberg MC, et al. (2000) Prevalent vertebral deformities predict mortality and hospitalization in older women with low bone mass. Fracture Intervention Trial Research Group. *J Am Geriatr Soc* 48: 241–249. PMID: [10733048](#)
3. Nguyen ND, Center JR, Eisman JA, Nguyen TV (2007) Bone loss, weight loss, and weight fluctuation predict mortality risk in elderly men and women. *J Bone Miner Res* 22: 1147–1154. PMID: [17635040](#)
4. Suzuki T, Yoshida H (2010) Low bone mineral density at femoral neck is a predictor of increased mortality in elderly Japanese women. *Osteoporos Int* 21: 71–79. doi: [10.1007/s00198-009-0970-6](#) PMID: [19499274](#)

5. Mody N, Parhami F, Sarafian TA, Demer LL (2001) Oxidative stress modulates osteoblastic differentiation of vascular and bone cells. *Free Radic Biol Med* 31: 509–519. PMID: [11498284](#)
6. Garrett IR, Boyce BF, Oreffo RO, Bonewald L, Poser J, Mundy GR. (1990) Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo. *J Clin Invest* 85: 632–639. PMID: [2312718](#)
7. Suda N, Morita I, Kuroda T, Murota S (1993) Participation of oxidative stress in the process of osteoclast differentiation. *Biochim Biophys Acta* 1157:318–323. PMID: [8323962](#)
8. Basu S, Michaëlsson K, Olofsson H, Johansson S, Melhus H (2001) Association between oxidative stress and bone mineral density. *Boichem Biophys Res Commun* 288:275–279.
9. Maggio D, Barabani M, Pierandrei M, Polidori MC, Catani M, Mecocci P, et al. (2003) Marked decrease in plasma antioxidants in aged osteoporotic women: results of a cross-sectional study. *J Clin Endocrinol Metab* 88: 1523–1527. PMID: [12679433](#)
10. Hermizi H, Faizah O, Ima-Nirwana S, Ahmad Nazrun S, Norazlina M (2009) Beneficial effects of tocotrienol and tocopherol on bone histomorphometric parameters in sprague-dawley male rats after nicotine cessation. *Calcif Tissue Int* 84: 65–74. doi: [10.1007/s00223-008-9190-x](#) PMID: [19020790](#)
11. Norazlina M, Ima-Nirwana S, Gapor MT, Khalid BA (2000) Palm vitamin E is comparable to alpha-tocopherol in maintaining bone mineral density in ovariectomised female rats. *Exp Clin Endocrinol Diabetes* 108: 305–310. PMID: [10961363](#)
12. Muhammad N, Luke DA, Shuid AN, Mohamed N, Soelaiman IN (2012) Two different isomers of vitamin E prevent bone loss in postmenopausal osteoporosis rat model. *Evid Based Complement Alternat Med* 2012:161527. doi: [10.1155/2012/161527](#) PMID: [23118785](#)
13. Mehat MZ, Shuid AN, Mohamed N, Muhammad N, Soelaiman IN (2010) Beneficial effects of vitamin E isomer supplementation on static and dynamic bone histomorphometry parameters in normal male rats. *J Bone Miner Metab* 28: 503–509. doi: [10.1007/s00774-010-0159-2](#) PMID: [20145960](#)
14. Shuid AN, Mehat Z, Mohamed N, Muhammad N, Soelaiman IN (2010) Vitamin E exhibits bone anabolic actions in normal male rats. *J Bone Miner Metab* 28: 149–156. doi: [10.1007/s00774-009-0122-2](#) PMID: [19779668](#)
15. Östman B, Michaëlsson K, Byberg L, Helmersson J, Basu S, Melhus H, et al. (2009) Oxidative stress and bone mineral density in elderly men: antioxidant activity of alpha-tocopherol. *Free Radic Biol Med* 47: 668–673. doi: [10.1016/j.freeradbiomed.2009.05.031](#) PMID: [19500667](#)
16. Zhang J, Munger RG, West NA, Cutler DR, Wengreen HJ, Corcoran CD, et al. (2006) Antioxidant intake and risk of osteoporotic hip fracture in Utah: an effect modified by smoking status. *Am J Epidemiol* 163: 9–17. PMID: [16306312](#)
17. Melhus H, Michaëlsson K, Holmberg L, Wolk A, Ljunghall S (1999) Smoking, antioxidant vitamins, and the risk of hip fracture. *J Bone Miner Res* 14: 129–135. PMID: [9893075](#)
18. Mata-Granados JM, Cuenca-Acebedo R, Luque de Castro MD, Quesada Gómez JM (2013) Lower vitamin E serum levels are associated with osteoporosis in early postmenopausal women: a cross-sectional study. *J Bone Miner Metab* 31: 455–460. doi: [10.1007/s00774-013-0432-2](#) PMID: [23536191](#)
19. Michaëlsson K, Wolk A, Byberg L, Årnlöv J, Melhus H (2014) Intake and serum concentrations of α -tocopherol in relation to fractures in elderly women and men: 2 cohort studies. *Am J Clin Nutr* 99:107–114. doi: [10.3945/ajcn.113.064691](#) PMID: [24225359](#)
20. Holvik K, Gjesdal CG, Tell GS, Grimnes G, Schei B, Apalset EM, et al. (2014) Low serum concentrations of alpha-tocopherol are associated with increased risk of hip fracture. A NOREPOS study. *Osteoporos Int* 25:2545–2554. doi: [10.1007/s00198-014-2802-6](#) PMID: [25062727](#)
21. Fujita K, Iwasaki M, Ochi H, Fukuda T, Ma C Miyamoto T, et al. (2012) Vitamin E decreases bone mass by stimulating osteoclast fusion. *Nat Med* 18:589–594. doi: [10.1038/nm.2659](#) PMID: [22388090](#)
22. Villanueva AR, Lundin KD (1989) A versatile new mineralized bone stain for simultaneous assessment of tetracyclin and osteoid seams. *Stain Technol* 64: 129–138. PMID: [2480003](#)
23. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, et al. (1987) Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* 2: 595–610. PMID: [3455637](#)
24. Turner RT, Vandersteenhoven JJ, Bell NH (1987) The effects of ovariectomy and 17β -estradiol on cortical bone histomorphometry in growing rats. *J Bone Miner Res* 2: 115–122. PMID: [3455160](#)
25. Wronski TJ, Cintrón M, Dann LM (1988) Temporal relationship between bone loss and increased bone turnover in ovariectomized rats. *Calcif Tissue Int* 43: 179–183. PMID: [3141020](#)
26. Wronski TJ, Dann LM, Scott KS, Cintrón M (1989) Long-term effects of ovariectomy and aging on the rat skeleton. *Calcif Tissue Int* 45: 360–366. PMID: [2509027](#)

27. Wronski TJ, Dann LM, Horner SL (1989) Time course of vertebral osteopenia in ovariectomized rats. *Bone* 10: 295–301. PMID: [2803866](#)
28. Wronski TJ, Yen CF (1992) The ovariectomized rat as an animal model for postmenopausal bone loss. *Cells Mater Suppl* 1: 69–74.
29. Kalu DN (1991) The ovariectomized rat model of postmenopausal bone loss. *Bone Miner* 15: 175–191. PMID: [1773131](#)
30. Seedor JG, Quartuccio HA, Thompson DD (1991) The bisphosphonate alendronate (MK-217) inhibits bone loss due to ovariectomy in rats. *J Bone Miner Res* 6:339–346. PMID: [1858520](#)
31. Wronski TJ, Walsh CC, Ignaszewski LA (1986) Histologic evidence for osteopenia and increased bone turnover in ovariectomized rats. *Bone* 7: 119–123. PMID: [3718786](#)
32. Black D, Farquharson C, Robins SP (1989) Excretion of pyridinium cross-links of collagen in ovariectomized rats as urinary markers for increased bone resorption. *Calcif Tissue Int* 44:343–347. PMID: [2496906](#)
33. Shiraishi A, Higasi S, Masaki T, Saito M, Ito M, Ikeda S, et al. (2002) A comparison of alfacalcidol and menatetrenone for the treatment of bone loss in an ovariectomized rat model of osteoporosis. *Calcif Tissue Int* 71: 69–79. PMID: [12073154](#)
34. Otomo H, Sakai A, Ikeda S, Tanaka S, Ito M (2004) Regulation of mineral-to-matrix ratio of lumbar trabecular bone in ovariectomized rats treated with risedronate in combination with or without vitamin K2. *J Bone Miner Metab* 22: 404–414. PMID: [15316861](#)
35. Erben RG (1996) Trabecular and endocortical bone surfaces in the rat: modeling or remodeling? *Anat Rec* 246:39–46. PMID: [8876822](#)
36. Iwaniec UT, Turner RT (2008) Animal models of osteoporosis. In: Marcus R, Feldman D, Nelson DA, Rosen CJ, editors. *Osteoporosis*, 3rd ed. Amsterdam: Elsevier. pp 985–1110.
37. Lelovas PP, Xanthos TT, Thoma SE, Lyritis GP, Dontas IA (2008) The laboratory rat as an animal model for osteoporosis research. *Comp Med* 58:424–430. PMID: [19004367](#)
38. Iwaniec UT, Turner RT, Smith BJ, Stoecker BJ, Rust A, Zhang B, et al. (2013) Evaluation of long-term vitamin E insufficiency or excess on bone mass, density, and microarchitecture in rodents. *Free Radic Biol Med* 65: 1209–1214. doi: [10.1016/j.freeradbiomed.2013.09.004](#) PMID: [24051180](#)
39. Food and Nutrition Board, Institute of Medicine (2000) Dietary reference intakes for vitamin C, vitamin E, selenium, and carotenoids. A report of the Panel on Dietary Antioxidants and Related Compounds, Subcommittees on Upper Reference Levels of Nutrients and Interpretation and Uses of Dietary Reference Intakes, and the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. Washington, DC: National Academy Press.