Vitamin D supplementation protects against bone loss associated with chronic alcohol administration in female mice.

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Abstract

Chronic alcohol abuse results in decreased bone mineral density (BMD) which can lead to increased fracture risk. In contrast, low levels of alcohol have been associated with increased BMD in epidemiological studies. Alcohol’s toxic skeletal effects have been suggested to involve impaired vitamin D/calcium homeostasis. Therefore, dietary vitamin D supplementation may be beneficial in reducing bone loss associated with chronic alcohol consumption. Six week old, female C57BL/6J mice were pair-fed ethanol (EtOH)-containing liquid diets (10% or 36% total calories) for 78 days. EtOH exposure at 10% calories had no effects on any measured bone or serum parameter. EtOH consumption at 36% of calories reduced BMD and bone strength (p<0.05), decreased osteoblastogenesis, increased osteoclastogenesis, suppressed 1, 25 hydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) serum concentrations (p<0.05), and increased apoptosis in bone cells when compared to pair-fed controls. In a second study, female mice were pair-fed 30% EtOH diets with or without dietary supplementation with cholecalciferol (VitD) for 40 days. VitD supplementation in the EtOH diet protected against cortical bone loss, normalized alcohol-induced hypocalcaemia, and suppressed EtOH-induced expression of receptor of NFκB ligand (RANKL) mRNA in bone. In vitro, pre-treatment of 1,25(OH)$_2$D$_3$ in osteoblastic cells inhibited EtOH-induced apoptosis. In EtOH/VitD mice, circulating 1,25(OH)$_2$D$_3$ was lower compared to mice receiving EtOH alone (P<0.05), suggesting increased sensitivity to feedback control of VitD metabolism in the kidney. These findings suggest dietary VitD supplementation may prevent skeletal toxicity in chronic drinkers by normalizing calcium homeostasis, by prevention of apoptosis and by suppressing EtOH-induced increases in bone resorption.
Introduction

Chronic alcohol consumption has profound effects on the skeleton, interfering with bone growth, quality, and remodeling. Epidemiological studies in alcoholics of both genders report significant decreases in bone mineral density (BMD), and increased bone fractures and osteoporosis risk compared to nondrinkers (Clark et al., 2003; Malik et al., 2009; Pasoto et al., 2011; Wurmser et al., 2011) which is related to changes in bone turnover, particularly decreased bone formation and increased bone resorption (Alvisa-Negrin et al., 2009; Callaci et al., 2010; Dai et al., 2000; Diez-Ruiz et al., 2010). Previously, we have reported significant decreases in tibial and femoral BMD following chronic ethanol (EtOH) consumption in cycling female rats receiving isocaloric diets via intragastric infusion (total enteral nutrition, TEN) (Chen et al., 2011; Shankar et al., 2006). In these rats, detailed bone histomorphometric analysis demonstrated decreased osteoblast numbers and increased numbers of mature osteoclasts associated with the bone perimeter which correlated with elevated biochemical markers of bone resorption. Further analysis demonstrated that EtOH exposure increases reactive oxygen species in bone tissue which stimulates bone resorption while inhibiting bone formation (Chen et al., 2006; Chen et al., 2010; Chen et al., 2011; Shankar et al., 2006; Shankar et al., 2008b).

Chronic EtOH consumption also disrupts calcium homeostasis resulting in hypocalcaemia, which appears to be caused in part by disturbances in vitamin D metabolism in addition to EtOH’s inhibitory effect on intestinal calcium resorption (Keiver et al., 1996; Krawitt, 1975; Sampson, 1997). In humans, although findings have been quite variable depending on study population, age, sex and drinking status, chronic alcohol abuse has been associated with reduced plasma concentrations of 1, 25 hydroxyvitamin D3 (1,25(OH)2D3) and 25 hydroxyvitamin D3 (25OHD3) (Gonzalez-Reimers et al., 2011; Sampson, 1997). A recent comprehensive study in alcoholic patients reported that nutritional status and low 1,25(OH)2D3 plasma concentrations are independently related to increased prevalence of rib and vertebral fractures (Gonzalez-Reimers et al., 2011). In rodent models, we and others have reported similar decreases in circulating 1,25(OH)2D3 following chronic EtOH exposure (Keiver et al., 1996; Shankar et al., 2006; Turner et al., 1988). Additionally, we have reported that this decrease in serum 1,25(OH)2D3 is
associated with a significant increase in renal cytochrome P450 24A1 (CYP24), the enzyme responsible for the conversion of 1,25(OH)2D3 to inactive 1,24,25 hydroxyvitamin D3 metabolite (Shankar et al., 2008b). Interestingly, we and others have also reported increases, decreases or no change in serum concentrations of 25OHD3 after EtOH consumption in rodents (Shankar et al., 2007).

Vitamin D3 supplementation (cholecalciferol, VitD) alone or combined with calcium is well tolerated, and has shown great promise in reducing fracture risk in men and in women (Bischoff-Ferrari et al., 2010; Dawson-Hughes et al., 1997). Skeletal benefits associated with VitD supplementation are attributed to endocrine actions related to the regulation of calcium homeostasis, and/or paracrine/autocrine actions within bone cell populations. The direct effects on bone include stimulation of osteoblast differentiation and bone mass accrual while reducing osteoclast activity and inhibiting bone resorption (Atkins et al., 2007; Kogawa et al., 2010). To our knowledge, only one group has published a study investigating the benefit of vitamin D supplementation in protecting against EtOH-mediated bone loss (Wezeman et al., 2007). In that study, the authors administered a daily s.c. injection of cholecalciferol (5,000 IU/kg/daily s.c.) to male rats in combination with binge alcohol regimen (3g/kg i.p.) for three weeks, which resulted in a significant amelioration of the loss of trabecular bone and bone strength in VitD/EtOH treated rats compared to EtOH-treated baseline controls. However, no additional mechanistic information was reported.

Contrasting with the clinical findings of chronic alcohol abusers, there is epidemiological evidence supporting a positive association between moderate alcohol consumption (1-2 drinks/day) and increased BMD in pre- and post-menopausal women, suggesting a bimodal effect of EtOH on bone health in both men and women (Berg et al., 2008; New et al., 1997). The biological mechanisms underlying the benefits associated with long-term moderate drinking are unclear and require further investigation. In the present study, the Lieber-DeCarli liquid feeding model was used to feed EtOH diets to cycling female C57Bl/6J mice at two doses: 10% and 36% of total calories, corresponding respectively to moderate and heavy drinking, for 78d, to determine the effects of moderate alcohol consumption on BMD and bone turnover in comparison to the effects observed with higher EtOH intakes. Additionally, a separate study...
was conducted in which an EtOH diet, 30% of total calories, was supplemented with 2000 IU/kg body weight of cholecalciferol to determine if normalizing vitamin D homeostasis would protect against chronic EtOH-mediated bone loss.

Methods.
Animals and experimental design

All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences. Animals were housed in an Association Assessment and Accreditation of Laboratory Animal Care approved animal facility. Experiment 1: Fifty 6-week-old C57BL/6J female mice (Jackson Laboratories) were randomly assigned to 5 weight-matched diet groups (n=10/group): standard rodent chow diet, a 10% EtOH Lieber-DeCarli liquid diet, a 36% EtOH Lieber-DeCarli diet, and corresponding Pair Fed (PF) controls for each EtOH group. Initially, all mice received the control diet, 35% of energy from fat, 18% from protein, 47% from carbohydrates, (Dyets#710027). For the EtOH groups, EtOH was added to the Lieber-DeCarli diet by substituting carbohydrate calories with EtOH calories (Dyets#710260). The EtOH concentration in the diet was increased slowly in a stepwise manner until 10% or 36% total calories were reached, which constitutes final EtOH concentrations of 1.8%, and 6.2% (v/v), respectively, and maintained until sacrifice (78 d) as previously described (Wahl et al., 2006). All groups had access to water ad libitum. Mice given the control diet were isocalorically PF to their corresponding EtOH group based on the diet consumption of the previous day. Animal body weights were measured weekly. On day 0, 48 and 78 of the study, and all mice were placed on a La Theta x-ray computed topography (CT) scanner (ALOKA CO., LTD., Tokyo, Japan), under isoflurane anesthesia to determine body composition as previously described (Shankar et al., 2008a). Densitometric calculations of fat and muscle were performed using CT software (Aloka, Tokyo, Japan) using attenuation number thresholds of −120 to −500 for fat and −120 to 350 for muscle, and indices of % fat mass, and % lean mass were calculated. Volumetric BMD was measured using the Alcoa software from specific regions of interest (ROI). The femur ROI included distal, midshaft, and proximal
femur bone, tibia ROI included midshaft to proximal tibia bone, vertebrae ROI included thoracic vertebrae (T1-T12), and total body is defined as all bones scanned from clavicle to the lumbar vertebrae which includes, scapula, forelimbs, thoracic vertebrae, pelvic girdle and hind limbs. At sacrifice, trunk blood was collected, and right femurs were harvested and frozen in saline soaked gauze at -80°C for mechanical strength testing. Left femurs were harvested, and bone marrow was used in *ex vivo* osteoblast and osteoclast cultures. Left tibias were fixed in EtOH and embedded in plastic for further analysis.

**Experiment 2:** Twenty-two 6-week-old female C57BL/6J mice were assigned to 4 weight-matched groups, EtOH: the Lieber-DeCarli EtOH diet (Dyets#710260) which contains the NRC recommended level of cholecalciferol/vitamin D$_3$ (VitD) and based on diet intake, animals received 400 IU VitD/kg body weight, EtOH/VitD: the same Lieber-DeCarli EtOH diet supplemented with additional VitD where mice were calculated to receive 2000 IU/kg body weight, and corresponding PF and PF/VitD controls (n=4-6/group). In the EtOH groups, a final EtOH concentration of 5.2% (v/v) was achieved as described in Experiment 1, and constituted 30% of total calories consumed. Both PF groups were isocalorically fed to their corresponding EtOH group based on the diet consumptions of the previous day. EtOH and PF diets were administered for 40 days. At sacrifice, trunk blood was collected, kidneys and femurs were frozen and stored at -80°C, and right tibial bones were formalin fixed for μCT analysis. Left tibial bone was fixed in EtOH and embedded in plastic for further analysis. Blood EtOH concentrations (BEC) were analyzed using an Analox analyzer as previously described (Shankar et al., 2006).

**Serum analysis of vitamin D homeostasis and bone turnover markers**

Serum 25OHD$_3$ was measured using a commercially available EIA kit (catalog# AC-575F1, Immunodiagnostic Systems, Scottsdale, AZ); detection range: 5 nmol/L to 380 nmol/L; less than 10% CV for inter assay variation; less than 8% CV for intra assay variation. Serum 1,25(OH)$_2$D$_3$ was also measured using a commercially available RIA kit (catalog#AA-54F1, Immunodiagnostic Systems); detection range: 5 pmol/L to 401 pmol/L, precision: 19 pmol/L (16% CV), 46 pmol/L (8.8% CV) and 162 pmol/L (8.6% CV) for inter assay variation, and 20% CV, 13% CV and 11.9% CV for intra assay.
variation, respectively. The intact form of parathyroid hormone, PTH (1-84) (Immunotopics, Inc., San Clemente, CA), osteocalcin (Biomedical Technologies, Inc., Stoughton, MA), and C-terminal telopeptides of type 1 (CTX) (Immunodiagnostic Systems, Fountain Hills, AZ) were detected in serum by commercially available ELISA kits. Serum phosphorus and ionized calcium were measured using colorimetric assay kits (BioVision, Mountain View, CA).

**Micro-computed tomography (μCT) analyses**

All μCT analyses were consistent with current guidelines for the assessment of bone microstructure in rodents using micro-computed tomography (Bouxsein et al., 2010). Formalin-fixed tibiae and femora were imaged using a MicroCT 40 (Scanco Medical AG, Bassersdorf, Switzerland) using a 12 μm isotropic voxel size in all dimensions. The region of interest selected for analysis comprised 240 transverse CT slices representing the entire medullary volume extending 1.24 mm distal to the end of the primary spongiosa with a border lying 100 μm from the cortex. Three-dimensional reconstructions were created by stacking the regions of interest from each two-dimensional slice and then applying a gray-scale threshold and Gaussian noise filter as described (Suva et al., 2008) using a consistent and pre-determined threshold with all data acquired at 70 kVp, 114 mA, and 200-ms integration time. Fractional bone volume (bone volume/tissue volume; BV/TV) and architectural properties of trabecular bone trabecular thickness (Tb.Th., mm), trabecular number (Tb.N., mm⁻¹) and trabecular spacing (Tb.Sp., mm) were calculated using previously published methods (Suva et al., 2008). Similarly, for cortical bone assessment, μCT slices were segmented into bone and marrow regions by applying a visually chosen, fixed threshold for all samples after smoothing the image with a three-dimensional Gaussian low-pass filter (σ = 0.8, support = 1.0) to remove noise, and a fixed threshold (245). Cortical geometry was assessed in a 1-mm-long region centered at the tibial midshaft. The outer contour of the bone was found automatically using the built-in Scanco contouring tool. Total area was calculated by counting all voxels within the contour, bone area by counting all voxels that were
segmented as bone, and marrow area was calculated as total area - bone area. This calculation was performed on all 25 slices (1 slice = ~12.5 µm), using the average for the final calculation. The outer and inner perimeter of the cortical midshaft was determined by a three-dimensional triangulation of the bone surface (BS) of the 25 slices, and cortical thickness and other cortical parameters were determined as described (Suva et al., 2008). Parameters assessed were total cross sectional area (Tt.Ar., mm²), total diameter, cortical thickness, (Ct.Th., mm), medullary area, (Me.Ar, mm²), periosteal perimeter, (Ps.Pm, mm), and endocortical perimeter, (Ec.Pm., mm).

Mechanical strength testing

Whole femur mechanical strength testing was done by three-point bending using a MTS 858 Bionix test system load frame (MTS, Eden Prairie, MH) as described (Brown et al., 2002). Loading point was displaced at 0.1 mm/second until failure, and load displacement data was recorded at 100 Hz. Test curves were analyzed using TestWorks software (MTS, Eden Prairie, MH) to determine measures of whole-bone strength, which are peak load and stiffness. Load-to-failure was recorded as the load after a 2% drop from peak load.

Analysis of gene expression

Total RNA was isolated from kidney and femur shaft using TRI reagent (MRC, Cincinnati, OH) as previously described (Chen et al., 2008). Total RNA was reverse transcribed using IScript cDNA synthesis (Bio-Rad Laboratories, Hercules, CA) according to manufacturer’s instructions. Subsequent real-time PCR analysis was carried out using SYBR green and an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA). Gene specific primers were: renal CYP24A1 F 5’GCAAATCGATGAGCTGCGTGATGA 3’, R 5’ AGGGCTTCTTCCTCTCTGTGCTTTT3’; renal CYP27B1 F 5’ACTCAGCTTCTGCTGACTCTT 3’, R 5’ ACAAAGTGATGGGTCGGCAACGTAA 3’; osteocalcin F 5’ TTGTGCTGGAGTGTTAGGTCAGCAACTG 3’, R 5’CACGCTCTCCCACAC 3’.
TACA 3’; collagen type 1a F 5’ AGGGTCATCGTCGCTTCTC 3’, R 5’ CTCCAGAGGGGCTTTGTT 3’; and RANKL F 5’ GGTTTCGACACCTGAATGCT 3’, R 5’ AACTGGTCGGCAATTCTGG; TRAP F 5’ TGGTCCAGGAGCTTAACGC 3’, R 5’ GCTAGGAGTGGGAGCCATATG; cathepsinK F 5’ GTGGGTGTTCAAGTTTCTGC 3’, R 5’ GGTGAGTCTTCTTCCATAGC 3’.

Ex vivo osteoblast and osteoclast cell cultures.

Bone marrow cells were harvested from the left femur of chow fed, 10% EtOH and 36% EtOH-treated mice, and the corresponding PF controls from Experiment 1 (n=6/group) and plated for osteoblast and osteoclast differentiation as previously described (Chen et al., 2008). Cells were cultured for osteoblastogenesis in osteogenic media (αMEM supplemented with 10% FBS and 1mM L-ascorbic acid phosphate) for 10 d and stained for alkaline phosphatase. Separate osteoblast cultures were cultured in osteogenic media for 25 d and stained with Von Kossa. Alkaline phosphatase and Von Kossa stained cultures were counted under a microscope at 20X magnification.

Non adherent bone marrow cells were plated at a density of 10^5 cells per well and cultured in DMEM containing L-glutamine, 10% FBS, 100 U/ml penicillin and streptomycin and 20 nM 1,25(OH)2D3 for 10 d followed by tartrate-resistant acid phosphatase (TRAP) staining as per manufacturer’s instructions (Sigma-Aldrich). Mature multinucleated osteoclasts, containing 5 or more nuclei, were counted under a microscope at 20X magnification.

Measurement of apoptosis

EtOH-fixed, plastic embedded tibias from the 36% EtOH and PF groups in Experiment 1, and the EtOH and EtOH/VitD groups in experiment 2 were sectioned and apoptotic cells visualized using ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Billerica, MA) utilizing terminal deoxynucleotidyl transferase labeling (TUNEL) of fragmented DNA and immunoperoxidase staining for qualitative detection of apoptotic cells in vivo as described in the manufacturer’s instructions. In addition, neonatal calvaria-derived osteoblastic cells from untreated mice were seeded at a density of 10^5 cells/well in a 6 well plate and maintained in osteogenic media (αMEM supplemented with 10% FBS and 1mM L-
ascorbic acid phosphate) for 20 d, at which cells were pre-treated with 1,25(OH)₂D₃ for 30 min before the addition of increasing concentrations of EtOH. 1,25(OH)₂D₃ (Enzo Life Sciences, Plymouth Meeting, PA) was prepared as a stock solution (60 μM) in absolute EtOH and store at -80°C until use. The 1,25(OH)₂D₃ stock was diluted into CO₂-conditioned media (αMEM supplemented with 10% FBS), which was added to the wells for a final concentration of 150 pM 1,25(OH)₂D₃. Following the pre-incubation step, 25-100 μl/ml of a 1M EtOH stock solution made in CO₂-conditioned media is added to the appropriate plates for final EtOH concentration of 25-100 mM. To prevent EtOH evaporation in the media, all plates, including control plates without EtOH treatment, were wrapped in paraffin and maintained at 37°C and 5% CO₂ for 24 h, at which cells from each treatment group were lysed in RIPA buffer (Thermo Scientific, Rockford, IL) supplemented with protease and phosphatase inhibitors. EtOH-induced apoptosis in vitro was examined by Western blotting using a mouse monoclonal antibody recognizing the cleaved form of caspase-3 (Cell Signaling, Beverly, MA) followed by anti-mouse secondary antibody conjugated with horseradish peroxidase. Blots were developed using chemiluminescence detection method according to manufacturer’s instructions. Protein bands were quantified using a densitometer and band densities were corrected for total protein loaded by staining with 0.1% amido black (Sigma-Aldrich).

Data and statistical analysis

Data presented as means ± SEM. Comparison between multiple groups was accomplished by one-way ANOVA, followed by Student Newman-Keuls post hoc analysis. The effect of VitD supplementation and EtOH and the interaction thereof were determined using two-way ANOVA, followed by Student Newman-Keuls post hoc analysis. Statistical significance was set at P<0.05. SigmaPlot software package 11.0 (Systat Software, Inc., San Jose, CA) was used to perform all statistical tests.

Results
Effects of EtOH on weight gain and body composition in Experiment 1.

The mean EtOH intake for the 36% EtOH group was 30 g/kg/d, and the mean EtOH intake for the 10% EtOH group was 7.5 g/kg/d. At sacrifice, the mean BEC for the 36% EtOH group was 171.5±27.1 mg/dL (range 104.2-260). This range corresponds to BECs observed in our rat TEN model of chronic alcoholism (Shankar et al., 2006), and falls within the range observed in human alcoholics(Wadstein and Skude, 1979). The mean BEC for the 10% EtOH group was 17.1±1.2 mg/dL. Weights at sacrifice did not differ between the 10% EtOH, its PF control or ad libitum chow-fed groups (21.1 ± 0.61 vs. 21.0 ± 0.58 vs. 21.5 ± 0.58 g, respectively), but body weight was significantly decreased in both the 36% EtOH group and its PF control compared all other groups (17 ± 0.13 vs. 19 ± 0.45 g, p<0.05, Mann-Whitney rank sum test). Diet intakes between the 10% PF and 36% PF control groups differed slightly (11.06 kcal/d vs.10.36 kcal/d, respectively, p<0.05, Mann-Whitney rank sum test), which may explain the 10% increase in body weight between the PF controls. As seen in Figure 1, feeding the control high fat Lieber-DeCarli diet to mice resulted in an increase in fat accumulation (2-3 fold), accompanied by a 25% lower percent lean mass in both PF control groups when compared to chow-fed mice, p<0.05. Interestingly, chronic consumption of EtOH at 36% of total calories, but not at 10% of total calories, reduced the fat accumulation associated with feeding Lieber-DeCarli liquid diets, resulting in an increase in the percentage lean mass to baseline values obtained in the chow-fed mice, p<0.05.

Effects of high alcohol consumption on BMD and mechanical strength

During the study, mice were anesthetized and BMD was measured for each group using the La Theta CT scanner as described in Materials and Methods. The ROI for total body BMD is shown in Figure 2a. As seen in Figure 2b, baseline scans (study d 0) show no differences in total BMD between all groups (p=0.520). Once maximum EtOH intakes were maintained for several weeks (study d 48), we observed a 4.5 to 6% decrease in total BMD in the 36% EtOH group compared to PF and chow-fed controls, respectively, but did not reach statistical significance. At study d 78, both PF groups had
decreased total BMD compared to chow-fed (p<0.05). Moreover, at d 78, we observed a decrease in total BMD between 36% EtOH and its PF control (p≤0.05), but no difference between the 10% EtOH group and its PF control (p=0.9). Further analysis of cortical and trabecular BMD in the femur, tibia, and vertebrae on d 78 showed similar results (Table 1). At both the axial and appendicular skeleton, we observed a 17% decrease in cortical BMD in the 36% EtOH group compared to its PF control group (p<0.001). In trabecular bone, decreases in BMD in both femur and vertebra were observed, a 12% and 18% decrease respectively; although the greatest effect of alcohol on trabecular bone was observed in tibial bone with 26% decrease in BMD compared to its PF control group (p<0.001). Throughout the study, we did not see significant changes in trabecular BMD between the 10% EtOH group and its PF control. Likewise, cortical BMD measurements between 10% EtOH and its PF control did not differ, but both had lower cortical BMD values when compared to chow-fed controls (p<0.05). Mechanical strength testing was also performed on whole femurs taken from EtOH-treated and PF groups. As seen in Figure 2c, the loss in BMD observed in the 36% EtOH group was associated with a decrease in load-to-failure, and stiffness (Figure 2d) when compared to its PF control and 10% EtOH (p<0.001).

EtOH consumption disrupts bone remodeling.

In experiment 1, chronic EtOH consumption decreased circulating osteocalcin by 2-fold in the 36% EtOH group compared to its PF control, 14.2±3.8 ng/ml versus 24.6±8.5 ng/ml, respectively (p<0.001, one-way ANOVA followed by Student-Newman Keuls post hoc analysis). Moderate EtOH consumption had no effect on circulating osteocalcin in the 10% EtOH group compared to its PF control (26.9±6.2 ng/ml versus 28.8±6.1ng/ml, respectively). Consistent with these findings, we observed an inhibitory effect on osteoblastogenesis in primary bone marrow cells taken from 36% EtOH-treated femurs, cultured ex-vivo. The number of alkaline phosphatase-stained pre-osteoblasts was lower in cells from bone exposed to 36% EtOH in vivo compared to chow fed, PF and 10% EtOH cultures maintained in osteogenic media for 10 d (p<0.05) (Figure 3a). As a result, the number of differentiated osteoblasts identified by Von Kossa staining after 25 d in culture was also statistically different; (p<0.05) (Figure 3b).
Chronic consumption of 10% EtOH had no stimulatory or inhibitory effect on osteoblastogenesis. In addition, the number of differentiated, multi-nucleated TRAP-positive osteoclasts was increased in the 36% EtOH group compared to its PF control and 10% EtOH (p<0.001) in ex vivo primary bone marrow cultures (Figure 3c).

*EtOH consumption alters circulating vitamin D metabolites.*

As shown in Figure 4a, serum 1,25(OH)₂D₃ was drastically reduced following chronic consumption of 36% EtOH compared to its PF control (P<0.05). Moderate alcohol consumption did not alter 1,25(OH)₂D₃ concentrations. Despite disruption of Vitamin D homeostasis, we did not see significant changes in serum parathyroid hormone (PTH) among EtOH or PF control groups (Figure 4b), which is consistent with previously published reports demonstrating lack of development of secondary hyperparathyroidism following EtOH-induced hypocalcaemia (Sampson, 1997).

*VitD supplementation prevents EtOH-induced cortical bone loss in Experiment 2.*

The mean EtOH intake for EtOH and EtOH/VitD groups was 29g/kg/d, which corresponds to 30% of total caloric intake. At sacrifice, mean BECs were not significantly different and were, 218.4±48.3 mg/dL (range 63-329.7) for EtOH, and 144.28±28.1 mg/dL (range 71-231) for EtOH/VitD. The amount of diet consumed did not differ between the EtOH and EtOH/VitD groups, but statistical analysis of final body weight using two-way ANOVA followed by Student Newman-Keuls post hoc analysis shows a decrease in body weight in the EtOH group compared to PF control, 21 ± 0.45 and 19.2 ± 0.17, respectively, and between the EtOH/VitD and PF/VitD group, 21.1 ± 0.38 and 20.1 ± 0.2, respectively, p<0.05. At sacrifice, right femurs from all groups were collected and used for mechanical strength testing. As expected, chronic EtOH feeding resulted in a 23% and 32% decrease in load bearing strength and stiffness in the EtOH group when compared to its isocalorically PF control, p<0.05 (Figure 5). μCT analysis of left tibial bone in the EtOH-treated group revealed decreases in trabecular BV/TV,
number, and thickness, p<0.05 (Table 2). Trabecular spacing did not change in the EtOH-treated mice compared to PF controls. In cortical bone, EtOH consumption did not change total cross-section area, but did reduce total diameter and thickness, and increased medullary area, p<0.05. VitD supplementation alone reduced trabecular BV/TV, and number, and increased spacing between PF and PF/VitD controls, p<0.05. However, the addition of EtOH did not result in further reductions in trabecular BV/TV, number, or increases in spacing between the trabeculae compared to the EtOH/VitD group or the PF/VitD controls. In the cortical compartment, VitD supplementation prevented EtOH-mediated effects on medullary area (p<0.05), diameter, and thickness, p=0.08 and p=0.06, respectively, comparing EtOH/VitD and EtOH groups. Consistent with the protective effects of dietary VitD supplementation on cortical bone parameters, VitD supplementation prevented the loss of bone load bearing strength and stiffness produced by EtOH consumption (p<0.05) but no significant difference was observed in the PF/VitD group relative to the PF group (Figure 5).

**VitD supplementation prevents EtOH-induced changes in biochemical markers of bone remodeling.**

Chronic EtOH exposure significantly increased biochemical markers of bone resorption. We observed a 7-fold increase in circulating CTX in the serum of EtOH treated mice, and 2- to 3-fold increases in mRNA expression of receptor of NFκB ligand (RANKL), TRAP, and cathepsinK in the femur shaft of EtOH-treated mice compared to PF controls, p<0.05 (Figure 6). In contrast, osteoblast genes associated with bone formation such as osteocalcin and collagen type 1a mRNA expression were decreased in the femur shaft of EtOH treated mice compared to PF controls (p<0.05). Serum concentrations of CTX were reduced by 40% in the EtOH/VitD group compared to EtOH alone, p<0.05. Likewise, mRNA expression of bone resorption biochemical markers were also reduced in the EtOH/VitD group compared to EtOH alone, p<0.05. In addition, osteocalcin expression increased, but not significantly (p=0.100), and collagen type 1a mRNA expression increased (p<0.05) in the EtOH/VitD group compared to EtOH alone.
VitD supplementation lowers circulating 1,25(OH)₂D₃ but protects against EtOH-induced hypocalcaemia.

Dietary supplementation to 2000 IU/kg body weight/day increased serum 25OHD₃ concentration by 2.5-fold in PF/VitD mice compared to PF controls, (p<0.05) (Table 3). EtOH alone increased 25OHD₃ concentrations when compared to PF controls (P<0.05). EtOH exposure combined with VitD supplementation increased serum 25OHD₃ still further compared EtOH-treated mice (p<0.01).

Concurrently, serum 1,25(OH)₂D₃ concentrations were decreased by 2-fold in PF/VitD mice compared to PF controls, (p<0.05). EtOH exposure, alone, reduced 1,25(OH)₂D₃ concentrations by 30%, (p<0.05).

However, EtOH exposure combined with VitD supplementation decreased serum 1,25(OH)₂D₃ levels still further in the EtOH/VitD group, compared to EtOH-treated mice, (p<0.01). Serum PTH levels did not change in response to either EtOH or VitD supplementation alone or in combination. However, serum concentrations of ionized calcium were decreased in the EtOH group compared to PF control mice, (p<0.05). VitD supplementation alone did not change serum calcium concentrations between PF controls, but calcium concentrations comparable to PF controls were maintained in the EtOH/VitD group compared to EtOH alone, (p<0.05). Serum phosphorous concentrations were not statistically different between any treatment groups.

Increased circulating 25OHD₃ by EtOH treatment and/or VitD supplementation enhances feedback regulation of renal CYP expression controlling 1,25(OH)₂D₃ synthesis and degradation.

Chronic EtOH feeding increased renal CYP24A1 mRNA expression 2-fold when compared to PF control p<0.05 (Figure 7a). VitD supplementation alone also increased CYP24A1 mRNA expression 2-fold compared to PF controls. Moreover, the addition of EtOH increased CYP24A1 mRNA expression further (63%) compared to either agent alone, p=0.083. Renal cytochrome P450 27 B1 (CYP27B1) is the enzyme responsible for synthesis of 1,25(OH)₂D₃ from 25(OH)D₃(Christakos et al., 2010). Renal CYP27B1 mRNA also decreased in the EtOH/VitD group (p<0.05) compared to EtOH or PF/VitD group,
respectively (Figure 7b). In all groups, we observed no significant changes in bone-specific CYP27B1 mRNA expression in response to EtOH or VitD supplementation alone or in combination (Figure 7c).

**Chronic EtOH consumption induces apoptosis in mouse tibial bone marrow.**

Recently, apoptosis has been reported in rat tibia following chronic consumption of EtOH for 17 wks (Maurel et al., 2011). We observed an increase in apoptosis as measured by qualitative *in situ* TUNEL staining in tibial bone marrow following chronic EtOH exposure (figure 8a,c). Interestingly, VitD supplementation appeared to protect against EtOH-mediated apoptosis in bone marrow cells (Figure 8d). For a more quantitative analysis of the effects of EtOH and VitD on bone cell apoptosis, we measured EtOH-mediated apoptosis *in vitro* using neonatal calvaria-osteoblastic cells derived from control chow-fed C57Bl/6J female mice. In cell culture, we observed a dose-dependent increase in active caspase-3 expression in cell lysates following 24 hrs of EtOH exposure (p<0.05, 0<25mM<50mM). However, pre-treating calvaria cells with 150 pM 1,25(OH)2D3 prior to the addition of EtOH, prevented the increase in active caspase-3 expression (Figure 8e).

**Discussion**

Consistent with our previously published studies using the rat TEN model (Chen et al., 2006;Chen et al., 2010;Chen et al., 2011;Shankar et al., 2006;Shankar et al., 2008b), chronic exposure to high EtOH concentrations significantly decreased trabecular and cortical BMD which resulted in loss of bone strength in the female mouse. In experiment 1, bone loss was associated with decreased bone formation, increased osteoclastogenesis. Serum 1,25(OH)2D3 concentrations were also decreased despite normal levels of PTH. EtOH exposure also decreased body weight and fat accrual in these mice, a phenomena that has been reported in heavy and chronic drinkers (Addolorato et al., 2000). Lower body weights may be, in part, explained by disruption of the GH-IGF1 axis by EtOH as a result of impaired GH secretion (Ronis et al., 2007), or to inefficient utilization of EtOH as an energy source (Lieber, 1991). Alternatively, others have reported that EtOH metabolism in white adipose tissue blocks pre-adipocyte
differentiation to mature adipocytes through inhibition of PPARγ and C/EBPβ expression (Crabb et al., 2011). Chronic EtOH feeding also stimulates lipolysis in white adipose tissue in rats through increased expression of the major adipose lipases, and concurrent down regulation of genes related to adipose tissue fatty acid uptake. These effects were reported to be reversed by treatment with the PPARγ agonist rosiglitazone (Sun et al., 2012).

Interestingly, moderate EtOH exposure (10% EtOH) had no adverse or positive effects on body composition, cortical and trabecular BMD, or bone strength. These findings suggest there is no gain in bone health in response to moderated EtOH exposure in this mouse model. Aside from alcohol other components found in alcoholic beverages have been reported to have bone anabolic effects, and these, as opposed to EtOH, may contribute to the positive correlation between light-moderate drinking and improved BMD observed in several epidemiological studies (Berg et al., 2008; Jugdaohsingh et al., 2004; New et al., 1997).

In our rat and mouse models of chronic EtOH exposure, heavy alcohol consumption is associated with a significant decrease in serum 1,25(OH)2D3 (Shankar et al., 2006; Shankar et al., 2008b). Given the importance of hormonal VitD regulation in maintaining bone density, we conducted an additional experiment to test if correcting for the loss in 1,25(OH)2D3 through dietary VitD supplementation would provide protection against EtOH-induced bone loss. In Experiment 2, μCT analysis of 30% EtOH-treated tibias revealed significant changes in both cortical and trabecular compartments. These changes were associated with decreased bone strength. However, in the EtOH/VitD group, increasing daily VitD intake from 400 IU/kg to 2000 IU/kg body weight protected against EtOH-dependent losses of cortical bone and bone strength. This protection coincided with decreases in circulating CTX and gene expression of bone resorption markers, and increases in expression of bone formation markers in EtOH/VitD mice compared to EtOH mice. Interestingly, VitD supplementation alone reduced trabecular bone in the PF/VitD controls (Table 2). In the literature, it has been reported that VitD supplementation in 10 wk-old male rats fed low calcium, vitamin D depleted diets produce a positive correlation between trabecular BV/TV and
serum 25OHD₃ concentrations ≥ 80 nmol/L (Anderson et al., 2008). However, it has also been reported that dietary VitD supplementation in 6 wk-old female mice, significantly decreased trabecular BV/TV through an overall increase in bone turnover in that compartment (Iwamoto et al., 2003). Therefore, we suspect that the reduction trabecular volume observed in the PF/VitD group is a sex and/or age-dependent, compartment-specific effect associated with vitamin D receptor (VDR) signaling.

Most interesting was that serum calcium in the EtOH/VitD group was normalized to PF controls despite further decreases in serum 1,25(OH)₂D₃ concentrations. The reduction in serum 1,25(OH)₂D₃ coincided with a significant reduction in renal CYP27B1, and increased renal CYP24 expression in the EtOH/VitD group relative to the EtOH group. These findings support the current view that endocrine regulation of renal VitD synthesis and metabolism is subject to a feedback loop via serum calcium (Christakos et al., 2010). Our data suggest that circulating 1,25(OH)₂D₃ is not important in mediating the normalization of serum calcium associated with dietary VitD supplementation in the current model and is consistent with studies suggesting that CYP27B1 in extra-renal tissues is responsible for 1,25(OH)₂D₃ synthesis and autocrine/paracrine VDR signaling (Fleet and Schoch, 2010; Geng et al., 2011; Heaney et al., 2003; Morris and Anderson, 2010). Although controversial, support for intestinal synthesis of 1,25(OH)₂D₃ comes from clinical studies by Heaney et al. in which calcium absorption was increased 25% after 4 weeks of treatment with 50 μg/d of 25OHD₃ despite no changes in serum 1,25(OH)₂D₃ (Heaney et al., 2003) and from studies localizing CYP27B1 in the intestinal villus (Balesaria et al., 2009). Alternatively, 25OHD₃ may have direct biological actions to stimulate VDR-dependent signaling in the intestine since it was significantly elevated in the EtOH/VitD group (Rowling et al., 2007; Zhang et al., 2011).

The protection from EtOH-mediated bone loss observed in the EtOH/VitD mice may be secondary to the restoration of calcium homeostasis. In VDR KO mice, it has been reported that a “rescue diet” containing 2% calcium, 20% lactose, and 1.25% phosphorous, is capable of reversing the abnormal mineral homeostasis, rickets and osteomalacia associated with this genotype (Li et al., 1998). However, in CYP27B1 KO mice, the “rescue diet” did not completely restore the impaired bone
phenotype in particular the inhibition of longitudinal bone growth (Dardenne et al., 2003). In these same mice, daily injections of 1,25(OH)₂D₃ normalized serum calcium and rescued the bone deficiencies, including the aberrant bone growth (Dardenne et al., 2003), which emphasizes the importance of VDR signaling in bone growth and maintenance and the likelihood that a calcium “rescue diet” in the absence of additional VitD may not completely alleviate EtOH’s effect on bone.

In the EtOH/VitD mice, bone turnover is normalized, which could also be related to increased VDR signaling as a result of local conversion of 25OHD₃ to 1,25(OH)₂D₃ in bone cells by CYP27B1 (Geng et al., 2011). In the present study we have shown that in vivo EtOH exposure is associated with the presence of apoptotic cells in the bone marrow (Figure 8). Moreover, VitD supplementation minimized the in vivo apoptotic response to EtOH exposure, and pre-treatment of osteoblastic cells with a physiological relevant dose of 1,25OH₂D₃ completely suppressed the EtOH-mediated apoptosis in vitro. We suspect that in the EtOH/VitD-treated mice, increased VDR signaling prevents or counteracts the effects of EtOH-mediated oxidative stress in osteoblasts.

In summary, dietary VitD supplementation normalizes serum calcium, prevents cortical bone loss and subsequent loss of mechanical strength associated with chronic EtOH exposure. In the intestine, local VitD synthesis may be responsible for increased calcium absorption which results in normalization of serum calcium. In bone, direct actions of locally produced 1,25(OH)₂D₃ on bone cells may suppress EtOH-induced oxidative stress signaling pathways involved in reducing osteoblastogenesis, increasing bone resorption and stimulating apoptosis. Since the study described here increased VitD in the diet around 5-fold, it is plausible that increasing average daily dietary VitD intake by a similar amount would be a feasible approach to prevent the deleterious effects of EtOH on bone in heavy or chronic drinkers.
Authorship Contributions

Participated in research design: Mercer, Chen, Badger, Ronis

Conducted experiments: Mercer, Wynne, Lazarenko, Hogue

Contributed new reagents or analytic tools: Mason

Performed data analysis: Mercer, Lazarenko, Suva, Chen, Ronis

Wrote or contributed to the writing of the manuscript: Mercer, Lumpkin, Suva, Mason, Chen, Badger, Ronis
References


Footnotes

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\(^b\) SOT 2010

\(^c\) Reprint requests: Martin J. Ronis, Ph.D., Department of Pharmacology and Toxicology, Department of Pediatrics, University of Arkansas for Medical Sciences, Arkansas Children’s Nutrition Center, 15 Children’s Way, Little Rock, AR. 72202. Phone (501)-364-2796, Fax (501)-264-2818, email RonisMartinJ@uams.edu
Figure legends

Figure 1. Female mice were chronically fed EtOH diets for 78d. Peak EtOH concentrations for 10% EtOH (7 g/kg/d) and 36% EtOH (30 g/kg/d) were reached on day 25 and maintained for the length of the study. Body composition, (a) lean mass and (b) fat mass was assessed for each group using in vivo CT scanning encompassing the entire visceral region of the animal as described in Materials and Methods, and expressed as a percentage of total body weight. Statistical significance was determined using One-way ANOVA followed by Student-Newman Keuls post hoc analysis. *P<0.05 versus chow fed animals, #P<0.001 36% EtOH versus corresponding PF control, ^P≤0.001 36% EtOH versus 10% EtOH.

Figure 2. CT scanning was used to assesses total BMD for chow fed, EtOH-treated and PF control groups in vivo as described in Materials and Methods on study d0, 48, and 78; (a) the ROI used for total BMD determinations and (b) total BMD. At sacrifice, mechanical strength of whole femurs, (c) peak load and (d) stiffness, was assessed by 3-point bending. Statistical significance was determined using One-way ANOVA followed by Student-Newman Keuls post hoc analysis. *P<0.05 versus chow fed animals, #P<0.001 36% EtOH versus corresponding PF control, ^P≤0.001 36% EtOH versus 10% EtOH.

Figure 3. Chronic EtOH consumption alters both osteoblastogenesis and osteoclastogenesis in primary bone marrow cells cultured in osteogenic media for 10 days (a) followed by alkaline phosphatase staining or 25 days (b) followed by Von Kossa staining, or (c) cultured in αMEM containing 10 nM 1,25(OH)2D3 and 15 ng/well recombinant RANKL for 10 days. Data represent mean ± SEM for n=6 animals per group plated in triplicate. Statistical differences were determined using Mann-Whitney Rank Sum Test. *P≤0.05 versus chow, #P≤0.05 36% EtOH versus its corresponding PF control, ^P≤0.05 36% EtOH versus 10% EtOH.

Figure 4. Vitamin D₃ and PTH serum concentrations in mice chronically fed EtOH, (a) 25OHD₂, (b) 1,25 (OH)₂D₃, (c) PTH. Statistical significance was determined using One-way ANOVA followed by Student-
Newman Keuls post hoc analysis.  *P<0.001 36% EtOH versus corresponding PF control,  ^P≤0.001 36% EtOH versus 10% EtOH.

Figure 5. Mechanical strength testing of whole femurs from EtOH and EtOH/VitD treated mice (n=7/group) and corresponding PF and PF/VitD controls (n=4/group), (a) peak load and (b) stiffness. Statistical significance was determined by two-way ANOVA followed by Student Newman-Keuls post hoc analysis. Values with different letter subscripts are significantly different from each other (P<0.05).

Figure 6. VitD supplementation to EtOH liquid diet prevents cortical bone loss by reducing EtOH-mediated increases in biochemical markers of bone resorption, (a) circulating CTX in serum, (b) RANKL (c) TRAP, and (d) CathepsinK mRNA expression in femur shaft. Biochemical markers of bone formation (e) osteocalcin and (f) collagen type 1a mRNA expression increased in the EtOH/VitD group compared to EtOH alone. Gene expression was measured by real-time PCR and normalized to Hmbs mRNA and data expressed as mean ± SEM. Statistical significance was determined by two-way ANOVA followed by Student Newman-Keuls post hoc analysis. Values with different letter subscripts are significantly different from each other (P<0.05).

Figure 7. VitD supplementation enhances EtOH-induced regulation of renal CYP24A1 (a) and CYP27B1 (b) expression, but not CYP27B1 mRNA expression in femur bone (c). Gene expression was measured by real-time PCR and normalized to Pkg1 mRNA and data expressed as mean ± SEM. Statistical significance was determined by two-way ANOVA followed by Student Newman-Keuls post hoc analysis. Values with different letter subscripts are significantly different from each other (P<0.05).

Figure 8. Representative pictures of in situ TUNEL staining in tibial bone marrow taken from 36% EtOH (a) and its corresponding PF control (b), 30% EtOH (c) and 30% EtOH/VitD-treated mice (d) as described in Materials & Methods. Arrows indicated apoptotic cells. EtOH exposure (0-50 mM)
increased cleaved caspase-3 expression in differentiating osteoblastic cells (e) in a dose-dependent manner. Pre-treatment with 1,25(OH)₂D₃ protected against EtOH-induced apoptosis in osteoblastic cells treated with EtOH. Apoptosis was assessed by Western blot with caspase-3 expression quantified by densitometry. Band densities were corrected for total protein loaded by staining with 0.1% amido black. Bars indicated mean ± SEM of triplicate determinations. Statistical differences were determined by one-way ANOVA followed by Student-Keuls post hoc analysis. P<0.05 for EtOH treatment a<b<c.
Table 1. Cortical and Trabecular BMD (mg/cm³) as measured by CT scanning in experiment 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Femur BMD (SEM)</th>
<th>Tibia BMD (SEM)</th>
<th>Vertebrae BMD (SEM)</th>
<th>Femur BMD (SEM)</th>
<th>Tibia BMD (SEM)</th>
<th>Vertebrae BMD (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>638 (8.42)</td>
<td>604 (4.26)</td>
<td>433 (3.42)</td>
<td>256 (13.88)</td>
<td>318 (10.63)</td>
<td>286 (15.93)</td>
</tr>
<tr>
<td>PF to 10% EtOH</td>
<td>610 (9.68)*</td>
<td>577 (5.93)*</td>
<td>415 (6.28)*</td>
<td>258 (15.15)</td>
<td>317 (16.35)</td>
<td>310 (12.78)</td>
</tr>
<tr>
<td>10% EtOH</td>
<td>599 (7.65)*</td>
<td>556 (5.20)*</td>
<td>410 (4.63)*</td>
<td>237 (7.69)</td>
<td>315 (16.89)</td>
<td>284 (16.023)</td>
</tr>
<tr>
<td>PF to 36% EtOH</td>
<td>591 (7.66)*</td>
<td>567 (4.02)*</td>
<td>410 (4.76)*</td>
<td>216 (9.74)</td>
<td>296 (13.55)</td>
<td>295 (19.3)</td>
</tr>
<tr>
<td>36% EtOH</td>
<td>493 (11.00)**</td>
<td>466 (5.5)**</td>
<td>370 (3.82)**</td>
<td>190 (10.95)**</td>
<td>216 (9.57)**</td>
<td>242 (13.72)**</td>
</tr>
</tbody>
</table>

Following 78d of chronic EtOH consumption, BMD measurements were obtained using whole-body CT scanning as described in the *Materials and Methods*. Statistical differences between treatment groups were determined using one-way ANOVA followed by Student-Newman Keuls *post hoc* analysis.

*P<0.05 versus chow fed animals, #P<0.001 36% EtOH versus corresponding PF control, ^P≤0.001 36% EtOH versus 10% EtOH.
Table 2. The effect of VitD supplementation on bone loss associated with chronic EtOH consumption.

<table>
<thead>
<tr>
<th></th>
<th>PF</th>
<th>EtOH</th>
<th>PF/VitD</th>
<th>EtOH/VitD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>μCT Trabecular bone parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>6.640 (0.004)</td>
<td>4.770 (0.003)</td>
<td>4.550 (0.004)</td>
<td>4.770 (0.003)</td>
</tr>
<tr>
<td>Tb.N, 1/mm</td>
<td>2.826 (0.168)</td>
<td>2.352 (0.098)</td>
<td>2.133 (0.194)</td>
<td>2.260 (0.146)</td>
</tr>
<tr>
<td>Tb.Sp, mm</td>
<td>0.366 (0.035)</td>
<td>0.413 (0.027)</td>
<td>0.487 (0.035)</td>
<td>0.459 (0.027)</td>
</tr>
<tr>
<td>Tb. Th, mm</td>
<td>0.044 (0.001)</td>
<td>0.041 (0.001)</td>
<td>0.044 (0.001)</td>
<td>0.042 (0.001)</td>
</tr>
<tr>
<td><strong>μCT Cortical bone parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tt.Ar, mm²</td>
<td>0.153 (0.007)</td>
<td>0.138 (0.005)</td>
<td>0.141 (0.007)</td>
<td>0.134 (0.005)</td>
</tr>
<tr>
<td>Total diameter, mm</td>
<td>0.035 (0.002)</td>
<td>0.026 (0.001)</td>
<td>0.035 (0.002)</td>
<td>0.031 (0.001)</td>
</tr>
<tr>
<td>Ct.Th, mm</td>
<td>0.173 (0.006)</td>
<td>0.149 (0.005)</td>
<td>0.179 (0.006)</td>
<td>0.165 (0.005)</td>
</tr>
<tr>
<td>Me.Ar, mm²</td>
<td>0.104 (0.006)</td>
<td>0.126 (0.006)</td>
<td>0.094 (0.006)</td>
<td>0.100 (0.005)</td>
</tr>
<tr>
<td>Ps.Pm, mm</td>
<td>1.442 (0.070)</td>
<td>1.529 (0.060)</td>
<td>1.367 (0.070)</td>
<td>1.375 (0.060)</td>
</tr>
<tr>
<td>Ec.Pm, mm</td>
<td>0.567 (0.042)</td>
<td>0.647 (0.034)</td>
<td>0.533 (0.042)</td>
<td>0.563 (0.034)</td>
</tr>
</tbody>
</table>

Following 40 d of chronic EtOH consumption, tibial bone was analyzed by μCT as described in Materials and Methods. Values are mean±SEM. Statistical differences between treatment-groups were determined using two-way ANOVA followed by Student-Newman Keuls post hoc analysis. Values with different letter subscripts are significantly different from each other (P<0.05).
Table 3. Vitamin D homeostasis parameters in Experiment 2.

<table>
<thead>
<tr>
<th></th>
<th>25 VitD$_3$ (nmol/L)</th>
<th>1,25 VitD$_3$ (pmol/L)</th>
<th>PTH (pg/ml)</th>
<th>Serum iCa$^{2+}$ (mg/dL)</th>
<th>Serum Phosphorous (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF</td>
<td>48.3 (1.09)$^a$</td>
<td>94.4 (9.6)$^a$</td>
<td>184.9 (33.2)$^a$</td>
<td>11.87 (1.48)$^a$</td>
<td>2.76 (0.163)$^a$</td>
</tr>
<tr>
<td>EtOH</td>
<td>99.1 (3.95)$^b$</td>
<td>66.5 (11)$^b$</td>
<td>189.0 (21.7)$^a$</td>
<td>8.00 (1.21)$^b$</td>
<td>3.17 (0.198)$^a$</td>
</tr>
<tr>
<td>PF/VitD</td>
<td>121.5 (2.58)$^c$</td>
<td>45.3 (2.1)$^{b,c}$</td>
<td>162.9 (33.2)$^a$</td>
<td>11.26 (1.48)$^a$</td>
<td>3.20 (0.298)$^a$</td>
</tr>
<tr>
<td>EtOH/VitD</td>
<td>141.1 (4.1)$^d$</td>
<td>29.0 (4.1)$^d$</td>
<td>126.1 (21.7)$^a$</td>
<td>14.63 (1.48)$^a$</td>
<td>3.36 (0.232)$^a$</td>
</tr>
</tbody>
</table>

Statistical significance was determined by two-way ANOVA followed by Student Newman-Keuls post hoc analysis. Values with different letter subscripts are significantly different from each other (P<0.05).
Figure 2.

a.

![Graph showing Total vBMD (mg/cm³) vs. Length of Diet Consumption (d) for different diets: Chow, 10% PF, 10% EtOH, PF 36%, 36% EtOH. The graph includes error bars and statistical symbols.]

b.

![Bar chart showing Peak load (N) for different diets: chow, PF to 10% EIOH, 10% EIOH, PF to 36% EIOH, 36% EIOH. Each bar has error bars.]

c.

![Bar chart showing Stiffness (N/mm) for different diets: chow, PF to 10% EIOH, 10% EIOH, PF to 36% EIOH, 36% EIOH. Each bar has error bars.]

* # ^ Statistical symbols indicate significance.
Figure 4.

(a) 1,25-Dihydroxyvitamin D₃ (pmol/L)

- PF to 10% EtOH
- 10% EtOH
- PF to 36% EtOH
- 36% EtOH

(b) PTH (pg/ml)

- PF to 10% EtOH
- 10% EtOH
- PF to 36% EtOH
- 36% EtOH
Figure 5.

(a) Peak load (N)

(b) Stiffness (N/mm)

PF  | EtOH | PF/VitD | EtOH/VitD
--- | --- | --- | ---
12  | 9   | 12  | 12
9   | 9   | 9   | 9
6   | 6   | 6   | 6
3   | 3   | 3   | 3
0   | 0   | 0   | 0

PF  | EtOH | PF/VitD | EtOH/VitD
--- | --- | --- | ---
60  | 45  | 60  | 60
45  | 45  | 45  | 45
30  | 30  | 30  | 30
15  | 15  | 15  | 15
0   | 0   | 0   | 0
Figure 6.

(a) CTX (ng/ml)

(b) RANKL mRNA fold expression

(c) TRAP mRNA fold expression

(d) Cathepsin K mRNA fold expression

(e) Osteocalcin mRNA fold expression

(f) Col1a1 mRNA fold expression
Figure 7.