Vitamin D Supplementation Protects against Bone Loss Associated with Chronic Alcohol Administration in Female Mice


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 D₃ [1,25(OH)₂D₃] serum concentrations (P < 0.05), and increased apoptosis in bone cells compared with pair-fed controls. In a second study, female mice were pair-fed 30% EtOH diets with or without dietary supplementation with vitamin D₃ (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 nuclear factor-(B) ligand mRNA in bone. In vitro, pretreatment of 1,25(OH)₂D₃ in osteoblastic cells inhibited EtOH-induced apoptosis. In EtOH/VitD mice circulating 1,25(OH)₂D₃ was lower compared with 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, preventing apoptosis, and 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 with nondrinkers (Clark et al., 2003; Malik et al., 2009; Pasoto et al., 2011; Wuermser et al., 2011), which is related to changes in bone turnover, particularly decreased bone formation and increased bone resorption (Dai et al., 2000; Alvisa-Negrín et al., 2009; Callaci et al., 2010; Diez-Ruiz et al., 2010). Previously, we have reported significant decreases in tibial and femoral BMD after chronic ethanol (EtOH) consumption in cycling female rats receiving isocaloric diets via intra gastric infusion (total enteral nutrition (TEN)).
(Shankar et al., 2006; Chen et al., 2011). In those 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, 2010, 2011; Shankar et al., 2006, 2008b).

Chronic EtOH consumption also disrupts calcium homeostasis, resulting in hypocalcaemia, which seems to be caused in part by disturbances in vitamin D metabolism in addition to EtOH’s inhibitory effect on intestinal calcium resorption (Krawitt, 1975; Keiver et al., 1996; 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 [25(OH)D3] (Sampson, 1997; González-Reimers et al., 2011). 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 (González-Reimers et al., 2011). In rodent models, we and others have reported similar decreases in circulating 1,25(OH)2D3 after chronic EtOH exposure (Turner et al., 1988; Keiver et al., 1996; Shankar et al., 2006). In addition, we have reported that this decrease in serum 1,25(OH)2D3 is associated with a significant increase in renal CYP24A1, the enzyme responsible for the conversion of 1,25(OH)2D3 to inactive 1,24,25-hydroxyvitamin D3 metabolite (Shankar et al., 2008b). It is noteworthy that we and others have also reported increases, decreases, or no change in serum concentrations of 25OHD3 after EtOH consumption in rodents (Turner et al., 1988; Wezeman et al., 2007; Shankar et al., 2008b).

Vitamin D3 (VitD; cholecalciferol) supplementation alone or combined with calcium is well tolerated and has shown great promise in reducing fracture risk in men and women (Dawson-Hughes et al., 1997; Bischoff-Ferrari et al., 2010). 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; Kagawa 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 subcutaneous injection of cholecalciferol (5000 IU/kg/daily) to male rats in combination with a binge alcohol regimen (3g/kg i.p.) for 3 weeks, which resulted in a significant amelioration of the loss of trabecular bone and bone strength in VitD/EtOH-treated rats compared with 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 premenopausal and postmenopausal women, suggesting a bimodal effect of EtOH on bone health in both men and women (New et al., 1997; Berg et al., 2008). 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 78 days, to determine the effects of moderate alcohol consumption on BMD and bone turnover in comparison with the effects observed with higher EtOH intakes. In addition, 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 whether normalizing vitamin D homeostasis would protect against chronic EtOH-mediated bone loss.

Materials and 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 (The Jackson Laboratory, Bar Harbor, ME) were randomly assigned to five 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, and 47% from carbohydrates (Dyets, Bethlehem, PA). For the EtOH groups, EtOH was added to the Lieber-DeCarli diet by substituting carbohydrate calories with EtOH calories (Dyets). The EtOH concentration in the diet was increased slowly in a stepwise manner until 10 or 36% total calories were reached, which constituted final EtOH concentrations of 1.8 and 6.2% (v/v), respectively, and maintained until sacrifice (78 days) as described previously (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 days 0, 48, and 78 of the study, all mice were placed on a La Theta X-ray computed topography (CT) scanner (Alokа Co., Tokyo, Japan), under isoflurane anesthesia to determine body composition as described previously (Shankar et al., 2008a). Densitometric calculations of fat and muscle were performed by using CT software (Alokа Co.) and attenuation number thresholds of −120 to −500 for fat and −120 to 350 for muscle. Indices of percentage of fat mass and percentages of lean mass were calculated. Volumetric BMD was measured by 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, and vertebrae ROI included thoracic vertebrae (T1-T12). 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 four weight-matched groups: EtOH, the Lieber-DeCarli EtOH diet (Dyets), which contains the National Research Council-recommended level of VitD and, based on diet intake, animals received 400 IU VitD/kg body weight; EtOH/VitD, the same...
Liebler-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, trunks were 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 ethanol concentrations (BECs) were analyzed by using an Analox (Huntington Beach, CA) analyzer as described previously (Shankar et al., 2006).

Serum Analysis of Vitamin D Homeostasis and Bone Turnover Markers

Serum 25OHD$_3$ was measured by using a commercially available enzyme immunoassay kit (Immunodiagnostic Systems, Scottsdale, AZ); detection range was 5 to 380 nM, less than 10% CV for interassay variation and less than 5% CV for intra-assay variation. Serum 1,25(OH)$_2$D$_3$ was measured by using a commercially available radioimmunoassay kit (Immunodiagnostic Systems; detection range was 5 to 401 pM, and precision was 19 pM (16% CV), 46 pM (8.8% CV), and 162 pM (8.6% CV) for interassay variation and 20% CV, 13% CV, and 11.9% CV for intra-assay variation, respectively. The intact form of parathyroid hormone (PTH) (Immunotopics, Inc., San Clemente, CA), osteocalcin (Biomedical Technologies, Stoughton, MA), and C-terminal telopeptides (CTXs) of type 1 (Immunodiagnostic Systems) were detected in serum by using commercially available enzyme-linked immunosorbent assay kits. Serum phosphorus and ionized calcium were measured by using colorimetric assay kits (BioVision, Mountain View, CA).

Micro-Computed Tomography Analyses

All μCT analyses were consistent with current guidelines for the assessment of bone microstructure in rodents by using micro-computed tomography (Bouxsein et al., 2010). Formalin-fixed tibiae and femora were imaged by using a MicroCT 40 (Scanco Medical AG, Bassersdorf, Switzerland) and 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 previously (Suva et al., 2008) and using a consistent and predetermined threshold with all data acquired at 70 kVp, 114 mA, and 290-ms integration time. Fractional bone volume [bone volume/tissue volume (BV/TV)] and architectural properties of trabecular bone trabecular thickness (mm), trabecular number (mm$^{-1}$), and trabecular spacing (mm) were calculated by using previously published methods (Suva et al., 2008). Likewise, for cortical bone assessment, μCT slices were segmented into bone and marrow regions by using a visually chosen, fixed threshold for all samples after smoothing the image with a three-dimensional Gaussian low-pass filter (α = 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 and inner perimeter of the cortical midshaft was determined by a three-dimensional triangulation of the bone surface of the 25 slices, and cortical thickness and other cortical parameters were determined as described previously (Suva et al., 2008). Parameters assessed were total cross-sectional area (mm$^2$), total diameter, cortical thickness (mm), medullary area (mm$^2$), periosteal perimeter (mm), and endocortical perimeter (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, MN) as described previously (Brown et al., 2002). Loading point was displaced at 0.1 mm/s until failure, and load displacement data were recorded at 100 Hz. Test curves were analyzed by using TestWorks software (MTS) 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 by using TRI reagent ( Molecular Research Center, Cincinnati, OH) as described previously (Chen et al., 2008). Total RNA was reverse-transcribed by using iScript cDNA synthesis (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions. Subsequent real-time PCR analysis was carried out by using SYBR green and an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA). Gene-specific primers were: renal CYP24A1, forward 5’-GCAAATCGATGACGTGAGTGATGA-3’ and reverse 5’-AGGGCTTCTTCTCTCTGCTTT-3’; renal CYP27B1, forward 5’-ACTACGCTTCTGGCAGCTGACACCA-3’ and reverse 5’-ACAAGTGATTAGGCTGCCAAGGTT-3’; osteocalcin, forward 5’-TTGTGCTGAGTGTGCTCTGAGC-3’ and reverse 5’-CCTACCCCTCCACACTGTGACA-3’; collagen type 1α1, forward 5’-AGGGTCATCGTGCCTTCTC-3’ and reverse 5’-CTTCAAGGAGGCTGTTT-3’; receptor of nuclear factor-κB ligand (RANKL), forward 5’-GGGTTGACACCTGAGCT-3’ and reverse 5’-AAGGTTGTCGAGGACATTG-3’; tartrate-resistant acid phosphatase (TRAP), forward 5’-TGTTCCAGGAGCTTCA-3’ and reverse 5’-CTGAGACTGAGCTTCT-3’; and cathepsin K, forward 5’-GTGGTGGTGTCAAGTCTG-3’ and reverse 5’-GOTGAGTCTCTTCCGTACAC-3’.

Ex Vivo Osteoblast and Osteoclast Cell Cultures

Bone marrow cells were harvested from the femur of animals fed with chow fed, 10% EtOH-treated, 36% EtOH-treated mice, and the corresponding PF controls from experiment 1 (n = 6/group) and plated for osteoblast and osteoclast differentiation as described previously (Chen et al., 2008). Cells were cultured for osteoblastogenesis in osteogenic media (αMEM supplemented with 10% FBS and 1 μM L-ascorbic acid phosphate) for 10 days and stained for alkaline phosphatase. Separate osteoblast culture were cultured in osteogenic media for 25 days and stained with Von Kossa. Alkaline phosphatase- and Von Kossa-stained cultures were counted under a microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) at 20× magnification. Nonadherent bone marrow cells were plated at a density of 10$^6$ cells per well and cultured in Dulbecco’s modified Eagle’s medium containing L-glutamine, 10% FBS, 100 U/ml penicillin and streptomycin, and 20 nM 1,25(OH)$_2$D$_3$ for 10 days followed by TRAP staining according to the manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO). Mature multinucleated osteoclasts, containing five or more nuclei, were counted under a microscope at 20× magnification.

Measurement of Apoptosis

EtOH-fixed, plastic-embedded tibiae 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 were visualized by using the ApoTag Peroxidase In Situ Apoptosis Detection Kit (Millipore Corporation, Billerica, MA) and terminal deoxynucleotidyl transferase dUTP nick-end 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 six-well plate and maintained in osteogenic media (αMEM supplemented with 10% FBS and 1 mM L-ascorbic acid phosphate) for 20 days, at which time cells were pretreated with 1.25(OH)_2D_3 for 30 min before the addition of increasing concentrations of EtOH. 1,25(OH)_2D_3 (Enzo Life Sciences, Plymouth Meeting, PA) was prepared as a stock solution (60 μM) in absolute EtOH and stored at ~80°C until use. The 1,25(OH)_2D_3 stock was diluted into CO_2-conditioned media (αMEM supplemented with 10% FBS), which was added to the wells for a final concentration of 150 pM 1,25(OH)_2D_3. After the preincubation step, 25 to 100 μl/ml of a 1 M EtOH stock solution made in CO_2-conditioned media was added to the appropriate plates for final EtOH concentrations of 25 to 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% CO_2 for 24 h, at which time cells from each treatment group were lysed in radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA) 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 Technology, Danvers, MA) followed by anti-mouse secondary antibody conjugated with horseradish peroxidase. Blots were developed by using the chemiluminescence detection method according to the manufacturer’s instructions (Thermo Fisher Scientific). Protein bands were quantified by using a densitometer, and band densities were corrected for total protein mass to baseline values obtained in the chow-fed mice (P < 0.05). 

Data and Statistical Analysis

Data are presented as means ± S.E.M. 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 by 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/day, and the mean EtOH intake for the 10% EtOH group was 7.5 g/kg/day. 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 versus 21.0 ± 0.58 versus 21.5 ± 0.58 g, respectively), but body weight was significantly decreased in both the 36% EtOH group and its PF control compared with all other groups (17 ± 0.13 versus 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 versus 10.36 kcal/day, 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 Fig. 1, feeding the high-fat Lieber-DeCarli diet to mice resulted in an increase in fat accumulation (2- to 3-fold), accompanied by a 25% lower percentage of lean mass in both PF control groups compared with chow-fed mice (P < 0.05). It is noteworthy that 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 of 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 by using the La Theta CT scanner as described under Materials and Methods. As seen in Fig. 2a, baseline scans (study day 0) show no differences in total BMD between all groups (P = 0.520). Once maximal EtOH intakes were maintained for several weeks (study day 48), we observed a 4.5 to 6% decrease in total BMD in the 36% EtOH group compared with PF and chow-fed controls, respectively, but it did not reach statistical significance. At study day 78, both PF groups had decreased total BMD compared with the Chow-fed group (P < 0.05). Moreover, at day 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 day 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 with 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; the greatest effect of alcohol on trabecular bone was observed in tibial bone with a 26% decrease in BMD compared with its PF control ($P < 0.001$).

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 compared with 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 Fig. 2b, the loss in BMD observed in the 36% EtOH group was associated with a decrease in load to failure and stiffness (Fig. 2c) compared with 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 with its PF control, 14.2 ± 3.8 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 with its PF control (26.9 ± 6.2 versus 28.8 ± 6.1 ng/ml, respectively). Consistent with these findings, we observed an inhibitory effect on osteoblastogenesis in primary bone marrow cells taken from 36% EtOH-treated

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**TABLE 1**

Cortical and trabecular BMD (mg/cm³) as measured by CT scanning in experiment 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Femur</th>
<th>Tibia</th>
<th>Vertebra</th>
<th>Femur</th>
<th>Tibia</th>
<th>Vertebra</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/cm³</td>
<td></td>
<td></td>
<td>mg/cm³</td>
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</tr>
<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 (7.55)*</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)*#ˆ</td>
<td>466 (5.5)*#ˆ</td>
<td>370 (3.82)*#ˆ</td>
<td>190 (10.95)*#ˆ</td>
<td>216 (9.57)*#ˆ</td>
<td>242 (13.72)*#ˆ</td>
</tr>
</tbody>
</table>

* $P < 0.05$, all groups versus chow-fed animals.  
# $P < 0.001$, 36% EtOH versus corresponding PF control.  
ˆ $P \leq 0.001$, 36% EtOH versus 10% EtOH.
femurs cultured ex vivo. The number of alkaline phosphatase-stained preosteoblasts was lower in cells from bone exposed to 36% EtOH in vivo compared with chow-fed, PF, and 10% EtOH cultures maintained in osteogenic media for 10 days \((P < 0.05)\) (Fig. 3A). As a result, the number of differentiated osteoblasts identified by Von Kossa staining after 25 days in culture was also statistically different \((P < 0.05)\) (Fig. 3b). Chronic consumption of 10% EtOH had no stimulatory or inhibitory effect on osteoblastogenesis. In addition, the number of differentiated, multinucleated TRAP-positive osteoclasts was increased in the 36% EtOH group compared with its PF control and 10% EtOH group \((P < 0.001)\) in ex vivo primary bone marrow cultures (Fig. 3c).

**EtOH Consumption Alters Circulating Vitamin D Metabolites.** As shown in Fig. 4a, serum 1,25(OH)\(_2\)D\(_3\) was drastically reduced after chronic consumption of 36% EtOH compared with its PF control \((P < 0.05)\). Moderate alcohol consumption did not alter 1,25(OH)\(_2\)D\(_3\) concentrations. Despite disruption of vitamin D homeostasis, we did not see significant changes in serum PTH among the EtOH or PF control groups (Fig. 4b), which is consistent with previously published reports demonstrating a lack of development of secondary hyperparathyroidism after 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 29 g/kg/day, 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 showed a decrease in body weight in the EtOH group compared with PF control \((P < 0.05)\) and between the EtOH/VitD and PF/VitD groups \((P < 0.05)\). At sacrifice, right femurs from all groups were collected and used for mechanical strength testing. As expected, chronic EtOH feeding resulted in 23 and 32% decreases in load-bearing strength and stiffness in the EtOH group compared with its isocalorically PF control \((P < 0.05)\) (Fig. 5). CT 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 with 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 with the EtOH/VitD group or the PF/VitD controls. In the cortical compartment, VitD supplementation prevented EtOH-mediated effects on med-
Vitamin D Supplementation Prevents EtOH-Mediated Bone Loss

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 RANKL, TRAP, and cathepsin K in the femur shaft of EtOH-treated mice compared with PF controls ($P < 0.05$) (Fig. 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 with PF controls ($P < 0.05$). Serum concentrations of CTX were reduced by 40% in the EtOH/VitD group compared with EtOH alone ($P < 0.05$). Likewise, mRNA expression of bone resorption biochemical markers was also reduced in the EtOH/VitD group compared with EtOH alone ($P < 0.05$). In addition,

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>BV/TV, %</td>
<td>6.640 (0.004)$a$</td>
<td>4.770 (0.003)$b$</td>
<td>4.550 (0.004)$b$</td>
<td>4.770 (0.003)$b$</td>
</tr>
<tr>
<td>Tb.N, 1/mm</td>
<td>2.826 (0.168)$a$</td>
<td>2.352 (0.098)$b$</td>
<td>2.133 (0.098)$b$</td>
<td>2.260 (0.146)$b$</td>
</tr>
<tr>
<td>Tb.Sp, mm</td>
<td>0.366 (0.035)$a$</td>
<td>0.413 (0.027)$b$</td>
<td>0.459 (0.027)$b$</td>
<td>0.459 (0.027)$b$</td>
</tr>
<tr>
<td>Tb.Th, mm</td>
<td>0.044 (0.001)$a$</td>
<td>0.041 (0.001)$b$</td>
<td>0.044 (0.001)$b$</td>
<td>0.042 (0.001)$b$</td>
</tr>
<tr>
<td>Tt.Ar, mm$^2$</td>
<td>0.153 (0.007)$a$</td>
<td>0.138 (0.005)$b$</td>
<td>0.141 (0.007)$b$</td>
<td>0.134 (0.005)$b$</td>
</tr>
<tr>
<td>Total diameter, mm</td>
<td>0.035 (0.002)$a$</td>
<td>0.026 (0.001)$b$</td>
<td>0.035 (0.002)$b$</td>
<td>0.031 (0.001)$b$</td>
</tr>
<tr>
<td>Ct.Th, mm</td>
<td>0.173 (0.006)$a$</td>
<td>0.149 (0.005)$b$</td>
<td>0.179 (0.006)$b$</td>
<td>0.165 (0.005)$b$</td>
</tr>
<tr>
<td>Me.Ar, mm$^2$</td>
<td>0.104 (0.006)$a$</td>
<td>0.126 (0.006)$b$</td>
<td>0.094 (0.006)$b$</td>
<td>0.100 (0.005)$b$</td>
</tr>
<tr>
<td>Ps.Pm, mm</td>
<td>1.442 (0.070)$a$</td>
<td>1.529 (0.080)$b$</td>
<td>1.367 (0.070)$b$</td>
<td>1.375 (0.060)$b$</td>
</tr>
<tr>
<td>Ec.Pm, mm</td>
<td>0.567 (0.042)$a$</td>
<td>0.647 (0.034)$b$</td>
<td>0.533 (0.042)$b$</td>
<td>0.563 (0.034)$b$</td>
</tr>
</tbody>
</table>

TB, trabecular bone trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular spacing; Tt.Ar, total cross-sectional area; Ct.Th, cortical thickness; Me.Ar, medullary area; Ps.Pm, peristeal perimeter; Ec.Pm, endocortical perimeter.
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 with EtOH alone.

**VitD Supplementation Lowers Circulating 1,25(OH)2D3 But Protects against EtOH-Induce Hypocalcaemia.** Dietary supplementation to 2000 IU/kg body weight/day increased serum 25OHD3 concentration by 2.5-fold in PF/VitD mice compared with PF controls (P < 0.05) (Table 3). EtOH alone increased 25OHD3 concentrations compared with PF controls (P < 0.05). EtOH exposure combined with VitD supplementation increased serum 25OHD3 still further compared with EtOH-treated mice (P < 0.01). Concurrently, serum 1,25(OH)2D3 concentrations were decreased by 2-fold in PF/VitD mice compared with 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 with 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 deceased in the EtOH group compared with PF control mice, (P < 0.05). VitD supplementation alone did not change serum calcium concentrations between PF controls, but calcium concentrations comparable with PF controls were maintained in the EtOH/VitD group compared with EtOH alone (P < 0.05). Serum phosphorous concentrations were not statistically different between any treatment groups.

**TABLE 3**

<table>
<thead>
<tr>
<th></th>
<th>25OHD3</th>
<th>1,25(OH)2D3</th>
<th>PTH</th>
<th>Serum Ca2+</th>
<th>Serum Phosphorous</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>nmol/l</td>
<td>pmol/l</td>
<td>pg/ml</td>
<td>mg/dl</td>
<td>mmol/l</td>
</tr>
<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>
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<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>
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<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>
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<tr>
<td>EtOH/VitD</td>
<td>141.1 (4.1)d</td>
<td>29.0 (4.1)b</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>

Fig. 6. a to d, VitD supplementation to EtOH liquid diet prevents cortical bone loss by reducing EtOH-mediated increases in biochemical markers of bone resorption: circulating CTX in serum (a), RANKL (b), TRAP (c), and cathepsin K (d) mRNA expression in femur shaft. e and f, biochemical markers of bone formation, osteocalcin (e) and collagen type 1a (f) mRNA expression increased in the EtOH/VitD group compared with EtOH alone. Gene expression was measured by real-time PCR and normalized to Hmbs mRNA. Data are expressed as mean ± S.E.M. Statistical significance was determined by two-way ANOVA followed by Student Newman-Keuls post hoc analysis. Values with different letters are significantly different from each other (P < 0.05).
Increased Circulating 25OHD₃ by EtOH Treatment and/or VitD Supplementation Enhances Feedback Regulation of Renal Cytochrome P450 Expression Controlling 1,25(OH)₂D₃ Synthesis and Degradation. Chronic EtOH feeding increased renal CYP24A1 mRNA expression 2-fold compared with PF control (P < 0.05) (Fig. 7a). VitD supplementation alone also increased CYP24A1 mRNA expression 2-fold compared with PF controls. Moreover, the addition of EtOH increased CYP24A1 mRNA expression further (63%) compared with either agent alone (P = 0.083). Renal CYP27B1 is the enzyme responsible for the 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 with the EtOH or PF/VitD groups (Fig. 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 (Fig. 7c).

Chronic EtOH Consumption Induces Apoptosis in Mouse Tibial Bone Marrow. Recently, apoptosis has been reported in rat tibia after chronic consumption of EtOH for 17 weeks (Maurel et al., 2011). We observed an increase in apoptosis as measured by qualitative in situ TUNEL staining in tibial bone marrow after chronic EtOH exposure (Fig. 8, a and c). It is noteworthy that VitD supplementation seemed to protect against EtOH-mediated apoptosis in bone marrow cells (Fig. 8d). For a more quantitative analysis of the effects of EtOH and VitD on bone cell apoptosis, we measured EtOH-mediated apoptosis in vitro by 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 after 24 h of EtOH exposure (P < 0.05; 0 < 25 < 50 mM). However, pretreating calvaria cells with 150 pM 1,25(OH)₂D₃ before the addition of EtOH prevented the increase in active caspase-3 expression (Fig. 8e).

Discussion
Consistent with our previously published studies using the rat TEN model (Chen et al., 2006, 2010, 2011; Shankar et al., 2006, 2008b), chronic exposure to high EtOH concentrations significantly decreased trabecular and cortical BMD, which resulted in the loss of bone strength in the female mouse. In experiment 1, bone loss was associated with decreased bone formation and 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 phenomenon 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 growth hormone/insulin-like growth factor 1 axis by EtOH as a result of impaired
growth hormone secretion (Ronis et al., 2007) or inefficient utilization of EtOH as an energy source (Lieber, 1991). Alternatively, others have reported that EtOH metabolism in white adipose tissue blocks preadipocyte differentiation to mature adipocytes through the inhibition of peroxisome proliferator-activated receptor γ and CCAAT-enhancer-binding protein β expression (Crabb et al., 2011). Chronic EtOH feeding also stimulates lipolysis in white adipose tissue in rats through the 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 peroxisome proliferator-activated receptor γ agonist rosiglitazone (Sun et al., 2012).

Fig. 8. a to d, representative pictures of in situ TUNEL staining in tibial bone marrow taken from 36% EtOH-treated mice (a) and corresponding PF controls (b), 30% EtOH-treated mice (c), and 30% EtOH/VitD-treated mice (d) as described under Materials and Methods. Arrows indicate apoptotic cells. e, EtOH exposure (0–50 mM) increased cleaved caspase-3 expression in differentiating osteoblastic cells in a dose-dependent manner. Pretreatment 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 indicate mean ± S.E.M. 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.

It is noteworthy that 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 (New et al., 1997; Jugdaohsingh et al., 2004; Berg et al., 2008).

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, 2008b). Given the importance of hormonal VitD regulation in maintaining bone density, we conducted an additional experiment to test whether 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 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 the expression of bone formation markers in EtOH/VitD mice compared with EtOH mice. It is noteworthy that 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-week-old male rats fed low-calorie, vitamin D-depleted diets produced a positive correlation between trabecular BV/TV and serum 25OHD₃ concentrations ≥80 nM (Anderson et al., 2008). However, it has also been reported that dietary VitD supplementation in 6-week-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 protecting the bone metabolism in the EtOH/VitD mice compared with EtOH mice. It was significantly elevated in the EtOH/VitD group (Rowl- ing et al., 2007; Zhang et al., 2011).

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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 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 (Fig. 8). Moreover, VitD supplementation minimized the in vivo apoptotic response to EtOH exposure, and pretreatment of osteoblastic cells with a physiological relevant dose of 1,25(OH)₂D₃ 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 and 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 the normalization of serum calcium. In bone, the 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. Because the study described here increased VitD in the diet approximately 5-fold, it is plausible that increasing average daily dietary VitD intake by a similar amount would be a feasible approach to preventing the deleterious effects of EtOH on bone in heavy or chronic drinkers.

**Authorship Contributions**

**Participated in research design:** Mercer, Chen, Badger, and Ronis.

**Conducted experiments:** Mercer, Wynne, Lazarenko, and Hogue.

**Contributed new reagents or analytic tools:** Mason.

**Performed data analysis:** Mercer, Suva, Chen, and Ronis.

**Wrote or contributed to the writing of the manuscript:** Mercer, Lumpkin, Suva, Badger, and Ronis.

**References**


