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Vitamin D and Ibandronate Prevent Cancellous Bone Loss Associated with Binge Alcohol Treatment in Male Rats

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Abstract

Decreased bone mass and bone strength can result from excess alcohol consumption in humans and alcohol treatment in the rat. Although the specific mechanism is unknown, the damaging effects of alcohol abuse modulate the bone remodeling cycle and increase bone turnover. Chronic alcohol consumption models have shown an inhibition of bone formation. We previously reported that binge alcohol treatment increases bone resorption and that alcohol-induced damage can be prevented by treatments with intermittent parathyroid hormone and bisphosphonates. In this study we hypothesized that an effective dose of vitamin D (cholecalciferol) or a single dose of ibandronate would prevent bone loss caused by binge alcohol treatment in male rats. Forty-eight adult (450 gram) male Sprague-Dawley rats were randomly assigned to 6 treatment groups (n=8): a) saline, i.p. 3 days/week (C); b) binge alcohol, 3g/kg i.p., 3 days/week (A); c) vitamin D, 5,000 IU/kg daily s.c., (D); d) binge alcohol and vitamin D (AD); e) e) ibandronate, (120 ug, given as a single i.p. injection (I); f) alcohol and ibandronate (AI). After 4 weeks of treatment proximal tibia and L3 and L4 vertebrae were analyzed for bone mineral density (BMD) by quantitative computerized tomography and compressive strength-to-failure using an Instron materials testing machine. Type-I collagen cross-linked c-telopeptide, calcium, and 25-OH vitamin D levels were measured in serum collected at the time of sacrifice. Binge alcohol significantly decreased cancellous BMD by 58% in tibia and 23% in lumbar spine ($p < 0.05$). Binge alcohol treatment decreased L3 and L4 compressive strength-to-failure by 21% ($p < 0.05$). Treatment with vitamin D at 5,000 IU/kg/day prevented alcohol-induced bone loss, significantly increasing both tibial and vertebral cancellous BMD values (161% increase in tibia and 40% increase in vertebra, respectively, $p < 0.05$) compared to alcohol alone groups. Pre-treatment with the single dose of 120 ug ibandronate prevented alcohol-induced bone loss, increasing cancellous BMD by 186% in tibiae and by 46% in vertebrae compared to the alcohol alone group ($p < 0.05$). In summary, binge alcohol-induced tibial and vertebral bone loss can be prevented using an effective dose of vitamin D or a single dose of ibandronate even during high blood alcohol concentrations that have been shown to impair osteoblast functions and increase bone resorption.

Keywords

Alcohol; osteoporosis; vitamin D; ibandronate; bone

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Introduction

We previously tested several therapeutic treatments for the prevention of bone loss and weakening in rat experimental models that mimic binge alcohol consumption by humans. Binge alcohol consumption in humans is characterized by heavy alcohol consumption in a short time frame. The normative consumption level used in alcohol research studies is more than 4 drinks during a single occasion (female) or more than 5 drinks during a single occasion (male) resulting in acute intoxication [1]. Experimentally this can be mimicked by repeated alcohol injections separated by periods of no alcohol administration [2]. This investigation is based on those previous studies in which we demonstrated that the bisphosphonates alendronate and risedronate effectively prevent cancellous bone loss and reduced bone strength in intact male and female rats after chronic consumption of alcohol and after binge alcohol treatments [2,3]. In addition, our previous studies demonstrated that parathyroid hormone given intermittently has a similar preventive effect on the loss of cancellous bone mineral density and bone strength after binge alcohol treatment [4]. Excessive alcohol consumption damages bone by inhibiting osteogenesis [5] and, when given in excess in a binge-like pattern, increases osteoclastic resorption [3]. The testing of agents that either promote bone gain or block resorption in alcohol-induced bone disease is focused on the possibility that preventive interventions might be available to individuals who repeatedly consume excessive amounts of alcohol.

One such agent widely available world-wide as an over-the-counter drug and present in food is vitamin D (cholecalciferol). An adequate daily intake of calcium and vitamin D has been shown to be essential for bone maintenance and for treatment-induced bone augmentation [6], and vitamin D itself has captured attention as an important determinant of bone health and fracture prevention in older adults [7–9]. Vitamin D, acting on bone through its final metabolite 1,25 dihydroxycholecalciferol suppresses bone turnover and activation frequency in cancellous bone.[10]. With increasing dosage, vitamin D reduces deoxypyridinoline levels indicative of decreased osteoclast activity and increases bone mineral density and bone mass in the vertebrae of estrogen-deficient osteoporotic rats [10,11]. Vitamin D given at effective non-toxic doses promotes osteoblastic activity and stimulates the proliferation of bone marrow osteoblast precursors in vitro [12]. Based on its efficacy as an agent promoting bone gain, vitamin D is being widely recognized as an excellent, safe, and conveniently available biological agent for increasing bone mass when administered in natural form and below levels associated with side effects or toxicity [13]. The therapeutic effect of 1,25 dihydroxyvitamin D3 has been studied with regard to its additive effect in combination with risedronate on accretion in ovariectomized osteoporotic rats [10]. This is in contrast to the non-additive effect of bisphosphonates during treatment with parathyroid hormone on the restoration of bone mass and strength in ovariectomized osteoporotic rats [14]. Accompanying vitamin D-increased bone mass is a reduction in falls and fractures in osteoporotic human females [15,16]. The bone-sparing effect of adequate dietary vitamin D levels along with adequate calcium intake is now recognized as an important nutritional guideline.

Similarly, the aminobisphosphonates are among several effective agents in the prevention of bone loss associated with up-regulated osteoclastic activity in osteoporosis [17,18]. Bisphosphonates inhibit bone resorption as shown by decreased serum bone turnover markers of bone type I collagen degradation [18]. Ibandronate, a potent nitrogen-containing bisphosphonate with affinity for hydroxyapatite [19], inhibits osteoclast-mediated bone resorption and is effective in the treatment of estrogen-deficient osteoporosis. Its high in vivo potency prevents estrogen-dependent bone loss and it improves bone mass, bone strength and bone architectural parameters in rats [20–22]. In postmenopausal osteoporosis, ibandronate decreases the biochemical markers of bone turnover, including CTX and osteocalcin to levels similar to those before estrogen deficiency.

In this study we examined the effects of a daily effective dose of vitamin D and a single dose of ibandronate on vertebrae and tibiae in male rats given binge-like alcohol treatments. We hypothesized that both agents can be effective in preventing bone loss associated with alcohol-induced osteoporosis in the adult male rat.

Material and method

Binge Alcohol Treatment

The Loyola University Institutional Animal Care and Use Committee (IACUC) approved the animal study used in this investigation. Forty-eight adult (450 g) male Sprague-Dawley rats (Harlan Corp., Indianapolis, IN) were randomly assigned in groups of eight to one of six treatment groups: 1) control (C) saline-injected; 2) alcohol-treated (A); 3) vitamin D (cholecalciferol)-treated (D); 4) ibandronate-treated (I); 5) alcohol plus vitamin D-treatment (AD); 6) alcohol plus ibandronate-treatment (AI). The rats were fed a standard rodent diet (8640 Harlan Teklad 22/5 Rodent Diet) containing 1.13% calcium, 0.94% phosphate, 2.99 IU/gm. vitamin D3 (Harlan Teklad, Madison, Wis.) ad libitum. Alcohol administration was by a single daily i.p. injection of a 20% (vol./vol.) ethanol/saline solution at a dose of 3 g/kg. This dose was chosen to achieve peak BACs of approximately 300 mg/dl [23]. Alcohol injections were given starting at 9:00 a.m., 3 days/week. No injections were given on the remaining 4 days each week to mimic a binge alcohol-drinking pattern. Vitamin D treatment was administered as a single daily s.c. injection of 5,000 I.U./kg of cholecalciferol (Sigma, St. Louis MO) dissolved in ethanol begun one day prior to the start of alcohol treatment and continuing until euthanasia. This dose was chosen based on preliminary data (not shown) indicating that 5,000 I.U/kg was well tolerated and produced no apparent toxic side effects including weight loss. Ibandronate was administered as a single i.p. dose [21] of 120 ug one day prior to the start of alcohol treatments. The total length of the treatment period was 4 weeks. Animals were housed in pairs, with both paired animals assigned to the same treatment group. Animals were weighed twice weekly throughout the study period. At the end of the 4-week treatment period, rats from each treatment group were rendered unconscious by CO₂ inhalation and killed by decapitation from 1 to 3 hours after the final alcohol injection. Blood was collected at time of death for blood alcohol analysis. Tibia and lumbar spine segments were removed, cleaned of all soft tissue and stored in 70% alcohol for subsequent analysis.

Blood Alcohol Determination

Blood alcohol concentrations were determined by NAD⁺ reduction assay (Sigma Chemical Company, St. Louis MO.) on blood samples collected from experimental animals at the time of death. Peak BAC was measured in 4 rats that were not included in any of the experimental protocol groups. These animals were killed by decapitation 60 minutes after a single i.p. injection of 20% (vol./vol.) ethanol/saline solution. The timing of peak BAC after i.p. injection was estimated based on a previous study that evaluated the pharmacokinetic profile of a 3g/kg i.p. alcohol injection in rats [23].

Serum 25-OH Vitamin D and Calcium Measurements

Serum protein-bound 25-OH vitamin D was measured at the time of sacrifice by competitive protein binding using a commercial kit (ALPCO Diagnostics, Windham, NH). Serum calcium was measured at the time of sacrifice by atomic adsorptiometry.

Bone Mineral Density Measurements

Cancellous and cortical bone mineral density (BMD) of the tibiae and the 3rd and 4th lumbar vertebrae were determined using quantitative computed tomography (QCT). Lumbar vertebral bodies were placed in a Norland Stratec bone densitometer (Orthometrix, Inc., White Plains,

NY). The bones were positioned uniformly on a support so that the instrument-scanning plane was perpendicular to the longitudinal axis of the bone.

For vertebrae three density measurements were performed 1 mm apart at the midpoint of the vertebral body following scout views to determine the midpoint of the vertebral body. Three measurements for each vertebra were performed at a resolution of 70 $\mu\text{m}/\text{voxel}$ 1mm apart at this point. For tibiae, three measurements were performed 1 mm apart, beginning 1.8 mm from the subchondral bone of the proximal endplate and going distally. The cancellous area of the bone was defined as 42 or 45% of the total bone cross-sectional area at the midpoint of the proximal tibia or vertebral body respectively, the remaining fractions cortical. The instrument was set to use the threshold contour mode (soft-tissue threshold set at 220 mg/cm^3) and a concentric peel algorithm (58 or 55% peel-off). Scans were made at 50kV and 0.3mA. Data was stored electronically.

Biomechanical Structural Properties of Lumbar Vertebrae

Compressive strength tests were performed on the L3 and L4 vertebral bodies from each rat using an Instron materials testing machine (model 1122, Canton, MA). The vertebral endplates were potted in bone cement using a previously described method that resulted in two parallel loading surfaces necessary to perform a uniform compression test on individual rodent vertebrae [24]. The specimens were prepared so that the posterior elements of the vertebra did not contact the loading platforms. All compression tests were performed in the displacement-control mode at a crosshead speed of 0.5 mm/min to eliminate any strain rate effects. A 50 kg load-cell was used to monitor the compressive load and a precision sensor was used to measure the axial deformation of the specimen. The load-deformation data was analyzed to obtain the compressive strength, defined as the maximum load sustained before failure.

Bone Resorption Assay

Effects of treatments on bone resorption were measured using the RatLaps™ ELISA kit (Nordic BioSciences, Herlev Hovedgade, Norway) according to the manufacturer's protocol. The assay detects c-terminal collagen type I degradation fragments (CTX) generated during osteoclastic bone resorption. Serum samples from each animal were assayed in triplicate.

Statistical Methods

Statistical analysis was performed using SPSS v.10.0.5 software (SPSS, Inc, Chicago, IL). Mean and standard error of the mean was computed for all parameters. One-way ANOVA and Tukey's posthoc procedure was used to determine significance, which was noted at $p < 0.05$.

Results

General Observations

All animals gained weight during the four-week treatment protocol. The only significant factor affecting weight gain during the protocol was alcohol ($p < 0.05$); no significant differences in weight gain were observed with other treatments. A once daily treatment regimen was chosen to avoid alcohol withdrawal symptoms that can occur when high doses of alcohol are administered twice daily [25]. All alcohol-treated animals were monitored throughout the study period and no obvious symptoms of alcohol withdrawal were observed during the three-day period each week when alcohol was not administered. Rats exhibited a short (approximately 1–2 hour) period of acute alcohol intoxication immediately following each i.p. injection. Necropsy performed on each animal revealed neither apparent internal injury from i.p. alcohol injections nor subcutaneous inflammation from vitamin D injections. Abdominal organs (including liver) of alcohol-treated animals appeared normal by gross inspection.

Bone Mineral Density

The proximal tibia and the third and fourth lumbar vertebra (L3, L4) of each rat were analyzed for effects of treatment on bone mineral density. Since vertebral cancellous BMD did not differ between L3 and L4, these values were averaged for each animal. Binge alcohol-treatment significantly lowered both tibial and vertebral cancellous BMD (Fig. 1a). Both vitamin D and ibandronate treatments significantly increased cancellous BMD at both sites over baseline control values. Both vitamin D and ibandronate treatment prevented the alcohol-induced loss of cancellous BMD in the tibiae and vertebrae returning values to control levels.

Treatment effects of vitamin D and ibandronate on cortical BMD were also examined (Fig. 2a, b). In contrast to its effect on cancellous bone, binge alcohol treatment did not significantly affect tibial cortical BMD values but did significantly decrease vertebral cortical BMD. Vitamin D did not significantly change cortical BMD at either site when administered without alcohol treatment. Vitamin D improved vertebral cortical BMD by attenuating the effect of alcohol resulting in values not significantly different from control. Ibandronate treatment significantly increased cortical BMD in both tibia and vertebra when administered to non-alcohol treated animals, and significantly increased vertebral cortical BMD in alcohol-treated animals.

Vertebral Compressive Strength

Vertebral L3 and L4 segments from each rat were analyzed for strength by determining the maximal compressive load (N)-to-failure (Fig. 3). Binge alcohol-treatment significantly decreased compressive strength compared to control animals. Both vitamin D and ibandronate significantly increased compressive strength compared to controls. Vitamin D and ibandronate treatment prevented binge-alcohol-related decreases in compressive strength, maintaining strength at control levels. The size of vertebral bodies as determined by cross-sectional area measurement obtained during QCT was equivalent across treatment groups (data not shown), suggesting that the decreased vertebral compressive strength values observed after binge alcohol treatment were the result of altered biomechanical properties of the vertebrae and not due to treatment-induced differences in vertebral body area.

Serum CTX Analysis

Effects of experimental treatments on bone resorption were analyzed by measuring bone-specific collagen I c-terminal cross-linked degradation products found in serum. Due to the observed intra-group variability in serum CTX levels, no statistically significant changes between groups were observed (data not shown).

Serum Calcium and 25-OH Vitamin D Analysis

Serum calcium levels remained unaffected by vitamin D administration in all groups. Serum calcium levels averaged 11.5 ± 0.4 mg/dL in all groups with no statistically significant difference between control and vitamin D-treated animals. Rats treated with vitamin D and rats treated with vitamin D plus alcohol had increased serum 25-OH vitamin D levels at the time of sacrifice which was significantly different from control, ibandronate-treated, alcohol-treated, and alcohol plus ibandronate-treated rats ($C = 16.4 \pm 3.8$ nmol/L, $I = 14.4 \pm 4.3$ nmol/L, $A = 14.4 \pm 4.9$ nmol/L, $AI = 15.5 \pm 3.1$ nmol/L vs. $D = 186.8 \pm 71.4$ nmol/L, and $AD = 229.2 \pm 77.0$ nmol/L; $p < 0.01$ for D and AD vs. C, I, A, AI. No significant difference exists between D and AD).

Discussion

Common and effective therapeutic interventions for osteoporosis principally include treatments with bisphosphonates, calcium and vitamin D nutritional supplementation, hormone administration, and combinations of various effective agents. Working via different mechanisms, these promote mineralization or suppress resorption resulting in improved bone quality and strength. In humans, these therapies reduce vertebral and non-vertebral fracture risk [17]. We have extended ibandronate and vitamin D treatment options to studies in rats with binge alcohol-related bone damage, a condition expressed in the human population. It is well-understood that bone remodeling is altered when blood alcohol concentrations remain significantly elevated for continuous periods of time or during repeated binge cycles in male rats [3,5,26–28]. Consequently, an imbalance is created between formation and resorption characterized by reduced formation and increased resorption. Experimental studies in young and adult rats have demonstrated that excessive alcohol consumption impairs osteogenesis resulting in altered cortical and cancellous architecture, reduced bone mineral density, and reduced bone strength [5,29–32]. More recently we demonstrated the effect of higher blood alcohol concentrations achieved by binge alcohol administration on male rat bone resorption [3]. Taken together, inhibited bone formation and increased bone resorption accompanying elevated blood alcohol concentrations account for the condition of alcohol-induced osteoporosis.

We previously extended the concept of preventive intervention to a binge model of alcohol abuse to determine the efficacy of intermittent parathyroid hormone on the restoration of bone mineral density and bone strength in rat vertebrae [4]. These studies led to the current investigation in which we hypothesized that a continuous and effective dose of vitamin D or a single injection of ibandronate would prevent alcohol-initiated loss of bone mineral density and bone strength. Whereas it is the total administered dose of ibandronate that determines the effects on bone [21], continuous effective dosing of vitamin D determines its outcome [13].

The primary assessment tools used in this investigation were bone mineral density as determined by quantitative computerized tomography of a tibia and vertebral body, and bone strength determined by load-to-failure in a lumbar vertebral body. Bone mineral density accounts for approximately 80% of bone strength [33], and compressive loading to the point of structural collapse is a measure of total bone strength contained in both the cortical and cancellous compartments of individual rat lumbar vertebra [34,35]. Vitamin D was administered by subcutaneous injection at a daily dose of 5,000 IU/kg. begun one day prior to the start of the alcohol injections. This elevated dose showed no signs of toxicity and the vitamin D-treated rats were not hypercalcemic. The animals gained weight throughout the experiment. In theory, the use of elevated doses of vitamin D to induce higher serum 25-hydroxycholecalciferol levels in the rat resulting in increased mineralization is consistent with current recommendations for the use of higher doses in humans to prevent osteoporosis. As expected, serum 25-(OH) vitamin D levels were significantly higher in the vitamin D and alcohol plus D treatment groups than the baseline levels observed in treatment groups that did not receive daily cholecalciferol injections. Although serum 25-(OH) levels achieved in vitamin D and alcohol plus D-treated animals (186 and 229 nmol/L respectively) were approximately 11 times higher than baseline, serum calcium levels were not significantly changed from control levels. This supports our empirical observation that no signs of toxicity were observed in animals receiving daily 5,000 IU/kg. cholecalciferol injections. It is difficult to compare serum 25-(OH) vitamin D levels achieved in this study to clinically relevant human values because of different baseline values between humans and rats. The data presented however, clearly suggests that daily 5,000 IU/kg. cholecalciferol injections can elevate circulating 25-OH vitamin D levels to an effective range that prevents alcohol-induced bone loss, but below levels associated with overt vitamin D toxicity. Doses of vitamin D3

(cholecalciferol) as high as 300,000 IU/kg/day are tolerated by adult male rats but are accompanied by progressive arterial calcification (36).

Elevated vitamin D dosing and a single effective dose of ibandronate resulted in increased vertebral and tibial cancellous and cortical BMD, accompanied by increased vertebral compressive strength over control levels. Binge alcohol treatments accompanied by higher blood alcohol levels result in a very apparent loss of both tibial and vertebral cancellous BMD thereby providing a useful model for the study of preventive interventions. Most notable in this investigation was the effect of ibandronate on tibial and vertebral cortical and cancellous BMD levels during the administration of alcohol in binge cycles. Vitamin D prevented the effect of alcohol on BMD loss in both tibiae and vertebrae and paralleled the outcome of ibandronate treatment.

We also found significance for the individual effects of vitamin D and ibandronate on the maintenance of vertebral compressive strength in binge alcohol-treated animals. Compressive loading of vertebral bodies revealed a loss of bone strength after binge alcohol treatment that was recovered by treatment with both vitamin D and ibandronate. Studies in rats have shown that vitamin D prevents the loss of cancellous and cortical bone in estrogen-deficient osteoporosis and that at higher doses it is anabolic [11,37]. The loss of connectivity and trabecular architecture is not restored but accretion on bone surfaces thickens existing trabecular elements. The microarchitectural outcome of treatment with ibandronate after the development of post-ovariectomy osteoporosis is also trabecular thickening [20]. In the current investigation when vitamin D or ibandronate is co-administered with alcohol vertebral and tibial cancellous BMD remain at control values preventing alcohol-induced bone loss. The resulting improvement in vertebral compressive strength is therefore largely attributable to the increased cancellous bone mass where yield strength has been shown to positively correlate with bone mass, volume, and structure in rat lumbar vertebrae [35]. Vertebrae, which are approximately 70% cancellous, can therefore improve compressive load-to-failure values by increasing BMD in response to both vitamin D and ibandronate.

The measurement of terminal peptides of type I collagen in serum reflects active bone resorption and our previous studies have shown that bone resorption is increased during binge alcohol cycles [3]. In this study we found no significant differences between groups.

The net effect of interventions by either a single injection of ibandronate or continuous elevated doses of vitamin D is to prevent the loss of bone in binge alcohol-induced osteoporosis. This is achieved either by bisphosphonate inhibition of osteoclastic resorption and promotion of secondary mineralization by a reduction in bone turnover and activation frequency [12] and increasing bone age, or the anabolic effects of vitamin D.

Conclusion

Binge drinking [38,39] represents a significant social problem in the adolescent, young adult and mature adult populations and constitutes a major field within alcohol abuse prevention. Its prevalence across various age groups including those under age 18 [40] contributes to those already at risk for fracture from bone loss associated with metabolic bone disease, aging, and drug treatment (e.g. steroids) or abuse. The data presented in this investigation demonstrates that an established response of bone to damaging levels of alcohol (2,4) can be prevented by either a clinically relevant dose of ibandronate, or vitamin D in excess of a normal daily maintenance dose. These treatments contribute significantly to bone mass and strength in animals that would otherwise demonstrate significant alcohol-induced bone loss. We have demonstrated that vitamin D, a proven safe and effective nutritional supplement, can prevent

binge alcohol-induced bone loss in male rats as effectively as the anti-resorptive nitrogen-containing bisphosphonate ibandronate.

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Figure 1. Rat Tibial and Vertebral Cancellous BMD

A) Tibia (B) Vertebrae. Treatment Groups. C: control, A: binge alcohol, D: vitamin D, I: ibandronate, AD: binge alcohol and vitamin D, AI: binge alcohol and ibandronate. For groups not sharing the same superscript; $p < 0.05$, one-way ANOVA and Tukey's multiple comparison procedure.

Figure 2. Rat Tibial and Vertebral Cortical BMD

(A) Tibia (B) Vertebrae. Treatment Groups. C: control, A: binge alcohol, D: vitamin D, I: ibandronate, AD: binge alcohol and vitamin D, AI: binge alcohol and ibandronate. For groups not sharing the same superscript; $p < 0.05$, one-way ANOVA and Tukey's multiple comparison procedure.

Figure 3. Rat Vertebral Compressive Strength

Treatment Groups. C: control, A: binge alcohol, D: vitamin D, I: ibandronate, AD: binge alcohol and vitamin D, AI: binge alcohol and ibandronate. For groups not sharing the same superscript; $p < 0.05$, one-way ANOVA and Tukey's multiple comparison procedure.