Alfacalcidol restores cancellous bone in ovariectomized rats

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Abstract

Active vitamin D metabolites have been demonstrated to reduce vertebral and hip fractures in elderly patients. A number of in vitro and in vivo pre-clinical studies have suggested that vitamin D may effectively stimulate osteoblastic activity and exert an anabolic effect on bone. The current study was designed to further explore the ability of an active vitamin D analog to restore bone in a skeletal site with established osteopenia in ovariectomized (OVX) rats. Female Sprague Dawley rats at five months of age and 8 weeks after sham ovariectomy or ovariectomy were randomly divided into 7 groups with 10 per group. At the beginning of the treatments, one group of sham-operated rats and one group of OVX rats were sacrificed to serve as baseline controls. Another group of sham-operated rats and one group of OVX rats were treated with vehicle for 4 weeks. The OVX rats in the remaining groups were treated with alfacalcidol at 0.05, 0.1 or 0.2 ìg/kg/d by daily oral gavage, 5 days/week for 4 weeks. As expected, estrogen depletion caused high bone turnover and cancellous bone loss in lumbar vertebra of OVX rats. Alfacalcidol treatment at 0.1 or 0.2 but not 0.05 ìg/kg/d increased serum calcium and phosphorus in OVX rats as compared with vehicle treatment. In addition, serum parathyroid hormone was suppressed, whereas serum osteocalcin was increased by alfacalcidol at all dose levels. Furthermore, histomorphometric data of 2nd lumbar vertebral body revealed that cancellous bone volume in OVX rats treated with alfacalcidol at 0.1 or 0.2 ìg/kg/d was increased to the level of sham-operated rats treated with vehicle. This increment in cancellous bone mass was accompanied by increases in trabecular number and thickness and a decrease in trabecular separation. Moreover, osteoclast surface and number were significantly decreased, whereas bone formation variables such as mineralizing surface and bone formation rate were significantly increased in alfacalcidol-treated OVX rats compared with those of vehicle-treated OVX rats. Finally, a linear regression analysis showed that alfacalcidol treatment dose-dependently altered most of the variables measured in the current study. In conclusion, alfacalcidol completely restores cancellous bone by stimulating bone formation and suppressing bone resorption in lumbar vertebra of OVX rats when the treatment is started at an early phase of osteopenia. The evidence of increased bone formation by alfacalcidol treatments further supports the notion that active vitamin D metabolites or their analogs may exert anabolic effects on bone.

Keywords: Alfacalcidol, OVX, Cancellous Bone, Bone Resorption, Bone Formation

Introduction

The active and hormone form of vitamin D, 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃ or calcitriol] and its pro-drug, 1α-hydroxyvitamin D₃ [1α(OH)D₃ or alfacalcidol], have been shown to reduce fracture rates in postmenopausal and senile osteoporosis, as well as glucocorticoid-induced osteoporosis. However, they have not been approved for the treatment of osteoporosis in the United States due to the risk of developing hypercalcemia/hypercalciuria, resulting in a relatively narrow therapeutic window. Therefore, it is important to understand the mechanism of action of vitamin D on bone and develop effective vitamin D analogs without or with minimal calcemic effect.

In vitro and in vivo studies have shown that calcitriol has stimulatory effects on osteoclastogenesis and activity. This effect is believed to be mediated through osteoblastic cells, which possess abundant vitamin D receptors. However, the effects of calcitriol on bone resorption are counteracted in vivo by suppression of parathyroid hormone (PTH) secretion through both a direct inhibitory effect on the parathyroid glands and an indirect effect via stimulation of intestinal calcium absorption and a subsequent rise in serum calcium.

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More importantly, findings from previous in vitro and in vivo studies have suggested that the vitamin D may effectively stimulate osteoblastic activity and exert an anabolic effect on bone. In vitro studies with osteoblast model systems demonstrate that calcitriol stimulates the synthesis of a variety of noncollagenous proteins such as osteocalcin, matrix Gla protein, osteopontin, fibronectin, as well as alkaline phosphatase. In intact rats, high doses of calcitriol upregulate tibial osteocalcin messenger RNA levels and increase the number of osteoblast precursor cells in bone marrow of tibia as well as stimulate cancellous bone formation in lumbar vertebra. Previous studies using the ovariectomized (OVX) rat as a model of postmenopausal bone loss have shown that long-term treatments with calcitriol or alfacalcidol increase bone mass and completely prevent bone loss induced by estrogen deficiency. However, as a potential anabolic agent for the management of osteoporosis, it is important to know if active vitamin D metabolites are capable of rebuilding bone mass in a skeleton with established osteopenia. Preclinical studies designed to address this issue are very limited and the effects of active vitamin D metabolites on bone formation observed in those studies were not consistent. Therefore, the current study was designed to further test the efficacy of active vitamin D metabolites in restoring bone mass to an osteopenic skeleton by using the OVX rat as a model and alfacalcidol as an agent.

**Materials and methods**

**Animals and Experimental Design.** Female Sprague Dawley rats at five months of age and 6 weeks after sham ovariectomy or ovariectomy were purchased from Taconic Farms Inc. (Germantown, NY). They were acclimated at
assessments right after sacrifice by CO₂ asphyxiation. Blood were then collected by cardiac puncture for biochemical parathyroid hormone (PTH) level. The whole blood samples collected from each rat by tail vein bleeding for the serum necropsy. At the day of necropsy, 300 µl of blood was first alfalcaldol, the animals were fasted 16 hours prior to injections. At the conclusion of 4 weeks of treatment with surfaces that were actively mineralizing at the time of the deposition of a single or double fluorochrome label at bone -12 and -2 days prior to sacrifice. This regimen resulted in a dose of 10 mg/kg (Sigma Chemical Co., St. Louis, MO) both
stock solution with a cottonseed oil vehicle to a given concentration.

The dosing solutions were prepared weekly by diluting the stock solution at 0.1 mg/ml, protected from light, and stored at 4°C.

The LV2 were dehydrated in graded concentrations of ethanol and embedded undecalified in methyl methacrylate28. Longitudinal frontal sections of lumbar vertebra were cut at 4- and 10-µm thickness using a Reichert-Jung Polycut S microtome (Cambridge Instruments, Heidelberg, Germany). The 4-µm sections were stained with modified Masson’s Trichrome stain, and the 10-µm sections remained unstained for measurements of fluorochrome-based indices of bone formation.

All histomorphometric measurements were performed in cancellous bone tissue of the lumbar vertebral body in an area between 0.5 mm distal to the cranial and 0.5 mm proximal to the caudal growth plates using an Image Analysis System (Osteomeasure, Inc., Atlanta, GA). Cancellous bone volume as a percentage of bone tissue area (BV/TV) and osteoclast surfaces as percentages of total cancellous perimeter (Ocs/BS) were measured in 4-µm thick, stained sections. Trabecular number (Tb.N), width (Tb.Th) and separation (Tb.Sp) were calculated as described by Parfitt et al.29. Fluorochrome-based indices of bone formation including the percentage of cancellous bone surface with a double fluorochrome label (mineralizing surface, MS/BS) and mineral apposition rate (MAR) were measured in 10-µm thick, unstained sections. In addition, bone formation rate (bone surface referent, BFR/BS) was calculated by multiplying mineralizing surface by mineral apposition rate. Values for mineral apposition rate were not corrected for obliquity of the plane of section in cancellous bone30.

Statistical analysis. Data are expressed as the mean ± SEM for each group. Statistics were calculated using StatView 4.0 packages (Abacus Concepts, Inc., Berkeley, CA). Statistical differences between groups were evaluated with ANOVA followed by the Fisher PLSD test for multiple comparisons. Probabilities (p) less than 0.05 were considered significant. Dose-dependent responses of alfalcaldol were determined by linear regression analysis using StatView 4.0 packages.

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BW: body weight; Ca: serum calcium; Pi: serum phosphorus; PTH: serum parathyroid hormone; osteocalcin: serum osteocalcin.

Table 1. Dose response for non-histomorphometric variables determined by linear regression analysis.

24°C with a 12h light/12h dark cycle for 2 weeks before initiation of the study. They were allowed free access to water and a commercial diet (Purina Laboratory Rodent Chow 5001, Purina-Mills, St. Louis, MO) containing 0.95% calcium, 0.67% phosphorus, and 4.5 IU/g vitamin D₃. The experiment was conducted according to Pfizer, Inc. animal care-approved protocols, and animals were maintained in accordance with the ILAR (Institute of Laboratory Animal Research) Guide for the Care and Use of Laboratory Animals.

At 8 weeks post-surgery, the animals weighing an average of 380g were randomly divided into 7 groups with 10 per group. One group of sham-operated rats (BSL + Sham) and one group of OVX rats (BSL + OVX) were sacrificed to serve as baseline controls. Another group of sham-operated rats and one group of OVX rats were treated with vehicle (Sham + VEH and OVX + VEH) for 4 weeks. The OVX rats in the remaining groups were treated with 0.05, 0.1 or 0.2 µg/kg/d body weight of alfalcaldol (Sigma, St. Louis, MO) by daily oral gavage at a volume of 1ml/kg body weight, 5 days/week for 4 weeks. Thus, this study was comprised of the following additional treatment groups: OVX+D₃ (0.05), OVX+D₃ (0.1), and OVX+D₃ (0.2). The stock solution was made by dissolving alfalcaldol in 100% ethanol at a concentration of 0.1 mg/ml, protected from light, and stored at 4°C. The dosing solutions were prepared weekly by diluting the stock solution with a cottonseed oil vehicle to a given concentration.

All rats were subcutaneously injected with calcein at a dose of 10 mg/kg (Sigma Chemical Co., St. Louis, MO) both -12 and -2 days prior to sacrifice. This regimen resulted in deposition of a single or double fluorochrome label at bone surfaces that were actively mineralizing at the time of the injections. At the conclusion of 4 weeks of treatment with alfalcaldol, the animals were fasted 16 hours prior to necropsy. At the day of necropsy, 300 µl of blood was first collected from each rat by tail vein bleeding for the serum parathyroid hormone (PTH) level. The whole blood samples were then collected by cardiac puncture for biochemical assessments right after sacrifice by CO₂ asphyxiation. Blood samples were further centrifuged to obtain the sera, which were stored at -20°C until assay. Finally, 2nd lumbar vertebrae (LV2) were collected for bone histomorphometric analysis.

Serum Biochemistry. Serum calcium (Ca) and inorganic phosphorus (Pi) concentrations were measured with the Cobas Fara 2 analyzer (Roche Diagnostic System, Hoffmann-La Roche Inc., Indianapolis, IN). The serum PTH levels were measured using a rat PTH ELISA kit with a detection limit at 1.6 pg/ml (Immuntopics, Inc., San Clemente, CA) and the serum osteocalcin concentrations were measured with a rat osteocalcin ELISA kit (Biomedical Technologies, Inc., Stoughton, MA).

Cancellable Bone Histomorphometry. The LV2 were dehydrated in graded concentrations of ethanol and embedded undecalcified in methyl methacrylate28. Longitudinal frontal sections of lumbar vertebra were cut at 4- and 10-µm thickness using a Reichert-Jung Polycut S microtome (Cambridge Instruments, Heidelberg, Germany). The 4-µm sections were stained with modified Masson’s Trichrome stain, and the 10-µm sections remained unstained for measurements of fluorochrome-based indices of bone formation.
Results

Effects of ovariectomy in baseline and vehicle-treated OVX rats. Baseline OVX and vehicle-treated OVX rats weighed significantly more than baseline sham controls and vehicle-treated sham controls, respectively (Figure 1A). Serum calcium (Figure 1B), phosphorus (Figure 1C), and PTH (Figure 1D) of OVX rats were not different from sham-operated controls. The mean value for serum osteocalcin (Figure 1E) in OVX rats was increased at 2 months and back to the sham control level at 3 months post-OVX. As expected, OVX caused significant bone loss in the lumbar vertebra of OVX rats as compared with sham controls (Figure 2A). The reduction in vertebral cancellous bone mass in OVX rats was accompanied with a decrease in trabecular number (Figure 2B) and increase in trabecular separation (Figure 2D). Trabecular thickness was not different between OVX and sham-operated rats (Figure 2C). Bone resorption and bone formation variables including osteoclast surface (Figure 2E), mineralizing surface (Figure 2F), mineral apposition rate (Figure 2G), and bone formation rate (Figure 2H) were elevated in baseline OVX rats compared with baseline sham animals. At the end of the experiment, mineralizing surface and bone formation rate but not osteoclast surface and mineral apposition rate in vehicle-treated OVX rats remained higher than those for vehicle-treated sham controls.

Effects of alfalcaldol in OVX rats. The mean body weight of OVX rats treated with the highest dose of alfalcaldol was decreased by 11% compared with that of vehicle-treated OVX rats (Figure 1A). Treatment of OVX rats with 0.1 or 0.2 μg/kg/d of alfalcaldol increased serum Ca (8% and 18%, respectively, Figure 1B) and Pi (24% and 45%, respectively, Figure 1C) significantly relative to vehicle treatment. Serum PTH was decreased by 91% in the rats treated with the lowest dose of alfalcaldol and it was decreased to undetectable levels by the two higher doses of alfalcaldol used in this study (Figure 1D). Treatment of OVX rats with alfalcaldol at all dose levels elevated serum osteocalcin by 47% to 112% relative to vehicle treatment (Figure 1E).

Cancellous bone histomorphometric data from LV2 (Figures 2A to H and Figure 3) showed that alfalcaldol at 0.1 and 0.2 but not 0.05 μg/kg/d significantly increased cancellous bone volume by 33% and 46%, respectively, as compared with vehicle treatment in OVX rats (Figure 2A). At the end of the 4-week treatment with alfalcaldol at these dose levels, the mean values of cancellous bone volume in OVX rats were equivalent to those in vehicle-treated sham controls. The increases in vertebral cancellous bone mass in OVX rats were accompanied by structural improvement of trabecular bone, as revealed by increases in trabecular number (Figure 2B) and thickness (Figure 2C), and decrease in trabecular separation (Figure 2D). In addition, osteoclast surface (Figure 2E) was significantly decreased by alfalcaldol treatments at all dose levels. In contrast to osteoclast surface, mineralizing surface (Figures 2F and 3) was significantly increased in OVX rats treated with 0.1 and 0.2 μg/kg/d of alfalcaldol by 25% and 52%, respectively, compared with that of OVX rats treated with vehicle. Furthermore, mineral apposition rate (Figure 2G) was not altered by alfalcaldol treatment except it was decreased in OVX rats treated with the lowest dose of the hormone when compared with vehicle-treated OVX rats. Finally, bone formation rate (Figure 2H) was increased in OVX rats treated with alfalcaldol at 0.1 and 0.2μg/kg/d dose levels by 14% and 45%, respectively, whereas at the 0.05 μg/kg/d dose level, alfalcaldol decreased bone formation rate in OVX rats as compared with vehicle.

A linear regression analysis showed that alfalcaldol treatment dose-dependently altered most of the variables measured in the current study. There was a dose-dependent decrease in body weight, serum PTH, trabecular separation, and osteoclast surface in OVX rats treated with alfalcaldol. In contrast, alfalcaldol treatment increased the following parameters in a dose-dependent manner: serum Ca, Pi, osteocalcin, cancellous bone volume, trabecular thickness, mineralizing surface, and bone formation rate (Tables 1 and 2).

Discussion

The current study demonstrates that alfalcaldol dose-dependently restores cancellous bone in the vertebra of osteopenic, OVX rats. The restoration of cancellous bone was accompanied with an improvement in trabecular architecture as evidenced by increased trabecular number and thickness and decreased trabecular separation. In accordance with previous studies with active vitamin D metabolites16-27, the current study showed that alfalcaldol dose-dependently suppressed bone resorption in OVX rats. This

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Figure 2. Cancellous bone volume (A), trabecular number (B), trabecular thickness (C), trabecular separation (D), osteoclast surface (E), mineralizing surface (F), mineral apposition rate (G), and bone formation rate (H) of second lumbar vertebra. Data are expressed as mean ± SEM. *p < 0.05 BSL+OVX vs. BSL+Sham; a p < 0.05 vs. Sham + VEH; b p < 0.05 vs. OVX + VEH.
The inhibitory effect of alfacalcidol on bone resorption was accompanied with a dose-dependent increase in serum calcium levels and a decrease in serum PTH levels in OVX rats. The observed hypercalcemia is probably due to increased intestinal absorption of calcium by alfacalcidol treatments. Since PTH is an important physiological regulator of bone resorption and bone turnover, the suppression of bone resorption by alfacalcidol in the current study is considered to be largely a consequence of diminished PTH activity. Although an acute increase in serum calcium leads to the secretion of calcitonin, which in turn inhibits osteoclast activity, studies have shown that long-term treatment of rats with calcitriol decreases calcitonin biosynthesis and secretion. Therefore, it is unlikely that calcitonin contributed to the decreased bone resorption after alfacalcidol treatment in the OVX rats.

Despite the substantial suppression of PTH secretion and bone resorption, alfacalcidol at the lowest dose employed in this study failed to significantly increase bone in OVX rats. This failure may be attributable to the decreases in mineral apposition rate and bone formation rate observed in these animals. The decrease in bone formation may be a coupling phenomenon associated with decreased bone resorption by alfacalcidol treatment at this dose level. Similar results have been reported in OVX rats after 3 months of treatment with alfacalcidol starting 4 days or 2 weeks after surgery. In contrast to the lowest dose, the two higher doses of alfacalcidol applied in this study restored vertebral cancellous bone mass to the control level in OVX rats. Despite the substantial decrease in serum PTH, bone formation indexes such as serum osteocalcin, bone formation rate, and in particular mineralizing surface were significantly increased in these animals. These results suggest that high doses of alfacalcidol stimulate bone formation in the OVX rats. Therefore, the combined effects of alfacalcidol at 0.1 and 0.2 μg/kg/d on stimulating bone formation and inhibiting bone resorption resulted in the restoration of bone in the OVX rats in this study.

The increase in mineralizing surface and bone formation rate by 4 weeks of treatment with alfacalcidol shown in this experiment is consistent with the results from studies in which the rats received only short-term treatment with calcitriol. In those studies, percent trabecular bone surface covered by mature osteoblast cells was increased following 3 or 13 days of calcitriol treatments. Increase in serum markers of bone formation such as osteocalcin and procollagen type I C-terminal propeptide (PICP) has also been reported in humans having received 4- or 7-day treatments with calcitriol. Interestingly, the increased bone formation by alfacalcidol in this study does not agree with the data from studies in which the animals received long-term (12 weeks) treatment with calcitriol or alfacalcidol. In those long-term studies, bone formation parameters generated by histomorphometric measurements in the OVX rats treated with calcitriol or alfacalcidol were not different or lower than those in the OVX rats treated with vehicle at the end of the 12-week treatment period. Taken together, active vitamin D metabolite, calcitriol, may transiently stimulate bone formation during the initial phase of treatment. Such increased bone formation by active vitamin D metabolites may be overridden by the suppression of bone formation coupled with the inhibition of bone resorption during the long-term treatment period.

The study clearly showed that alfacalcidol at higher doses (0.1 and 0.2 μg/kg/d) completely restored cancellous bone to the lumbar vertebra of OVX rats. This finding is somewhat superior to the previous findings in OVX rats treated with calcitriol. A study by Erben et al. showed that 3 months of treatment with calcitriol only partially restores cancellous bone in OVX, osteopenic rats. When comparing the current study with Erben’s study, it is notable that the magnitude of bone...
lost in the lumbar vertebra of OVX rats at the beginning of the treatments is different, a 19% decrease for the current study (2 months post-OVX) and a 37% decrease (3 months post-OVX) in Erben's study. The partial restoration at the same bone site by calcitriol in Erben's study is most likely due to the greater bone loss at the start of the treatment, although other factors such as the age of the rats and pharmacokinetics of pro-drug vs. active hormone cannot be excluded. The even weaker effect seen in the proximal tibia, a bone site that had lost 74% of its cancellous bone as compared with 37% in the lumbar vertebra at the beginning of the treatment in the same study further supports the aforementioned assumption. A similar phenomenon has been seen in the OVX, osteopenic rats treated with PTH, i.e., PTH failed to completely restore cancellous bone to a skeletal site with severe bone loss. The observations in the current study, in concert with previous findings, suggest that in order to achieve the maximal efficacy, treatment with calcitriol or alfacalcidol needs to be initiated at an early stage of bone loss. However, the maximum effect of calcitriol or alfacalcidol may not have been established due to the inability to expand the dose of the compounds because of adverse effects. Therefore, it is not known if an effective vitamin D analog, without affecting calcium homeostasis, would achieve superior efficacy in restoring bone to the osteopenic skeleton. Nevertheless, the increased bone formation and increased bone mass by alfacalcidol treatments in OVX rats, as demonstrated in our study, furthers the notion that active vitamin D metabolites or its analogs may exert an anabolic effect on bone by directly up-regulating osteoblastic cells.

In agreement with previous reports, alfacalcidol at all dose levels tested in the current study caused hypercalcemia in rats under calcium and vitamin D-replete condition. This undesirable effect has complicated the understanding of the direct effects of alfacalcidol on bone resorption and formation in vivo, and limited the usage of these agents for the prevention and treatment of osteoporosis. In recent years, efforts have focused on identifying potent and tissue selective vitamin D analogs to address this issue. Ro-26-9228, a vitamin D analog, has been recently reported to decrease urinary pyridinoline excretion and increase osteoblast number as well as bone mass in OVX rats at doses that have no significant effects on serum and urine calcium levels. In addition, the osteoblast-specific gene products osteocalcin and osteopontin as well as bone growth factors TGF β1 and β2 mRNA were up-regulated in trabecular bone by Ro-26-9228. These findings suggest the possibility of developing bone-selective vitamin D analogs with wider range of therapeutic window.

In conclusion, alfacalcidol completely restores cancellous bone by stimulating bone formation and indirectly suppressing bone resorption in lumbar vertebra of OVX rats when the treatment is started at an early phase of bone loss. The findings of increased bone formation and bone mass by alfacalcidol treatments further support the notion that active vitamin D metabolites or their analogs may exert an anabolic effect on bone.

References


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