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Eldecalcitol, a second-generation vitamin D analog, drives bone minimodeling and reduces osteoclastic number in trabecular bone of ovariectomized rats☆

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ABSTRACT

To elucidate the histological events that follow administration of eldecalcitol, a second-generation of vitamin D analog currently awaiting approval as a drug for treatment of osteoporosis, we employed the ovariectomy (OVX) rat model. OVX rats received vehicle or 30 ng/kg of eldecalcitol, and sham-operated animals received vehicle only. Rats were sacrificed after 12 weeks and had their femora and tibiae removed and processed for histochemical and histomorphometrical analyses. When compared with OVX group, osteoclastic number and bone resorption parameters were significantly reduced in eldecalcitol-treated rats, accompanied by decreased bone formation parameters. The preosteoblastic layer, with which osteoclastic precursors interact for mutual differentiation, was poorly developed in the eldecalcitol group, indicating less cell-to-cell contact between preosteoblasts and osteoclast precursors. Interestingly, eldecalcitol did promote a type of focal bone formation that is independent of bone resorption, a process known as bone minimodeling. While the number of ED-1-positive macrophages was higher in the bone marrow of treated rats, though osteoclastic number was deceased. Taken together, our findings suggest that eldecalcitol stimulates preosteoblastic differentiation rather than their proliferation, which in turn may prevent or diminish cell-to-cell contact between preosteoblasts and osteoclastic precursors, and therefore, lead to lower osteoclast numbers and decreased bone resorption.

Introduction

Vitamin D metabolism plays an essential role in regulation of mineral and bone homeostasis [1]. The active form of 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$), acts through the vitamin D receptor (VDR) present in target organs such as the intestines, kidney and parathyroid glands. It stimulates calcium absorption and reabsorption while blocking both the synthesis and secretion of another essential regulator of mineral balance, the parathyroid hormone (PTH) [2]. VDR has also been found in osteoblasts and osteoclasts, suggesting that vitamin D may directly affect the skeleton [3,4]. In bone, the hormone is important in at least two different ways: first, it interacts with the VDR in osteoblastic cells and regulates osteoclastic activity via the osteoprotegerin (OPG)/receptor activator nuclear factor kB (RANK)/RANK ligand (RANKL) system [5]; second, it secures a supersaturated state of calcium–phosphorus products in the blood, which indirectly enables osteoid mineralization [6]. Vitamin D deficiency may lead to exacerbated bone resorption as a result of increases in osteoclast number and activity, and may also cause a type of bone mineralization defect known as rickets in children and osteomalacia in adults [7]. Interestingly, 1α,25-$(OH)_2D_3$ was shown to promote osteoclastic bone resorption in culture [8] and *in vivo* [9] and to enhance the expression of RANKL on bone marrow stromal cells [10].

Despite its good acceptance in the management of conditions like psoriasis [11] and cancers [12], the use of vitamin D in the treatment of osteoporosis has been hindered due to its calcemic activity and the notion that the hormone drives osteoclastic bone resorption [13–15]. However, there have been reports showing that the therapeutic effect of active vitamin D can be dissociated from the one on calcium absorption [16] and that it is mostly related to suppression of bone resorption due to decreases in the pool of osteoclast precursors [17,18]. A recent theory based on previous reports of the conversion of 25-hydroxyvitamin D into 1α,25-dihydroxyvitamin D_3 by osteoblasts *in vitro* [19,20] suggests that vitamin D may be metabolized in the

☆ Conflict of interest: HS and ST are employees of Chugai Pharmaceutical Co., Ltd. The other authors have no conflict of interest to disclose.

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skeleton [7]. Evidence that serum calcium increases with the dose of vitamin D administered but calcium absorption reaches a plateau once normalized by a small dose of vitamin D [21] suggests the existence of a safer, side effect-free therapeutic window for vitamin D and its analogs.

Many attempts have been made to synthesize a compound that would retain only the differentiation and anti-proliferative effects of 1α,25-$(OH)_2D_3$, thus allowing for safer usage and less side effects [22,23]. Eldecalcitol, formerly known as ED-71, is an analog of 1α,25-$(OH)_2D_3$ bearing a hydroxypropyloxy residue at the 2β position that was developed in the early 80s and is currently awaiting approval as a drug for treatment of osteoporosis in Japan [24,25]. It has been reported that eldecacitol lowered biochemical and histological parameters of bone resorption in a rat ovariectomized (OVX) model of osteoporosis [26] and that its effects on bone were observed without sustained hypercalcemia or hypercalciuria [27].

Examinations focusing on the effects of vitamin D analogs at the tissue level have been relatively neglected despite the therapeutic potential of these compounds for the treatment of bone diseases. A recent report involving bone marrow ablation showed that eldecalcitol may promote bone formation and angiogenesis in addition to inhibiting bone resorption [23]. Further data on the histological subtleties and on the interplay among bone cells after eldecalcitol treatment are not yet available. In the present study, we used histological, histochemical, histomorphometrical and ultrastructural analyses as tools for investigating the tissue events following the administration of eldecalcitol in OVX rats.

Materials and methods

Animals, protocol for eldecalcitol administration, and tissue processing

All animal experiments were approved by the Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co., Ltd., Niigata University and Hokkaido University, and were conducted in accordance with accepted standards of humane animal care. Eight-months old Crl:CD(SD)(IGS) rats were obtained from Charles River Laboratories Japan, Inc., and acclimated until 11 months of age under standard laboratory conditions (23 ± 3 °C, humidity 35%–75%, light–dark cycle 12 h), with *ad libitum* access to food (1.25% calcium, 1.06% phosphate, CE-2, Clea Japan, Inc., Tokyo, Japan) and water. Rats were then divided in three groups: 1) the Sham group, whose animals were sham-ovariectomized and received only vehicle (medium chain triglyceride, MCT) after the procedure (n = 8), 2) the OVX group, where animals underwent standard ovariectomy but received only MCT after the surgical procedure (n = 8), and 3) the eldecalcitol group, where animals underwent standard ovariectomy and were given eldecalcitol by gavage (n = 8, 30 ng/kg, 5 times per week, Chugai Pharmaceutical Co., Ltd., Tokyo, Japan). At 3 and 13 days prior to killing, tetracycline (20 mg/kg BW) or calcein (8 mg/kg BW) was injected subcutaneously. Urine was collected over a twenty-four hour period after the last administration of either eldecalcitol or vehicle.

Rats were perfused through the left ventricle with 4% paraformaldehyde diluted in 0.1 M phosphate buffer (pH 7.4) under anesthesia with an intraperitoneal injection of chloral hydrate, and had their femora and tibiae extracted, decalcified and embedded in paraffin as previously described [28]. Four μm-thick sections were cut and stained with HE for histological analysis. Specimens were observed under a Nikon Eclipse E800 microscope (Nikon Instruments Inc. Tokyo, Japan). Light microscopic images were acquired with a digital camera (Nikon DXM1200C, Nikon). For transmission electron microscopy (TEM), fixed specimens were decalcified, post-fixed with OsO4, dehydrated, and embedded in epoxy resin (Epon 812, Taab, Berkshire, UK) as previously described [28]. Semi- and ultrathin-sections were observed under light microscopy or under a TEM (Hitachi H-7100, Hitachi Co., Tokyo, Japan) at 80 kV.

Bone mineral density and assessment of bone resorption markers

Femoral bone mineral density (BMD) was measured by dual-energy X-ray absorptiometry (DXA: DCS-600EX, Aloka, Tokyo, Japan). Results are shown in milligrams per square centimeter. Urinary creatinine (Cre) concentrations were measured with an automatic analyzer (7170, Hitachi, Tokyo, Japan). Urinary deoxypylidinoline (DPD) concentration was measured using a Metra DPD EIA kit (Quidel Corporation, San Diego, CA). Data were corrected for urinary Cre concentration, and results are expressed in nmol/mmol.

Immunohistochemistry and tartrate-resistant acid phosphatase (TRAP) detection

Dewaxed paraffin sections were treated for endogenous peroxidase inhibition with 0.3% H_2O_2 in phosphate buffered saline (PBS) for 20 min and nonspecific staining blocking with 1% bovine serum albumin in PBS (1% BSA-PBS) for 30 min at room temperature (RT). Sections were incubated with 1) rabbit antisera against tissue nonspecific alkaline phosphatase (ALP) [28,29], 2) mouse anti-rat ED1 (AbD Serotec, Oxford, UK), and 3) rabbit anti-cathepsin K (Daiichi Fine Chemical Co., Ltd., Toyama, Japan) for 2 h at RT, and then, incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibodies (R&D System Inc., Minneapolis, MN) for 1 h at RT. Immunoreactions were detected with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Dojindo Laboratories, Kumamoto, Japan). A sequential approach was employed for the cathepsin K/ED1 double immunostaining procedure. Cathepsin K immunohistochemistry was performed as described above. ED-1 immunoreactivity was detected as described, only with goat ALP-conjugated anti-mouse IgG (Sigma) as the second antibody and with a visualization procedure described previously [30]. For double detection of ALP/PCNA, immunostaining using anti-mouse PCNA (Oncogene Research products, San Diego, CA) was conducted, and a HRP-conjugated second antibody was used to allow for visualization. Then, ALP detection was conducted using an ALP-conjugated second antibody as described above. TRAP-activity was detected as described previously [28]. All sections were faintly counterstained with methyl green.

Von Kossa's staining

Undecalcified semi-thin epoxy resin sections were incubated with an aqueous solution of 5% silver nitrate (Wako Pure Chemical Industries, Tokyo, Japan) for 60 min at RT under sunlight until they took on a dark brown color. Following a distilled water rinse, sections were incubated with a 5% sodium thiosulfate solution (Wako Pure Chemical Industries) for 5 min. Sections were faintly stained with toluidine blue for observation and image acquisition.

Detection of apoptotic cells through TUNEL

For detection of apoptotic cells in the specimens, the "TACS 2TdT-Blue Label *In Situ* Apoptosis Detection Kit" (TREVIGEN Inc., Gaithersburg, MD) for the terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick end-labeling (TUNEL) method was employed. Dewaxed sections were incubated with 1% proteinase K (TREVIGEN Inc.) diluted 1:200 at 37 °C for 15 min, followed by inhibition of the endogenous peroxidases at room temperature for 5 min. After treatment with TdT enzyme (dilution 1:50) at 37 °C for 1 h, sections were incubated with HRP-conjugated streptavidin at room temperature for 15 min. Reaction was made visible with the blue label solution provided in the kit.

Bone histomorphometry

Bone histomorphometrical parameters were quantified using the ImagePro Plus 6.2 software (Media Cybernetics, Silver Spring, MD). For determination of structural parameters, HE-stained paraffin sections were used. For kinetic parameters, 10 μm-thick sections embedded in glycol methacrylate (GMA) were stained with the Villanueva method and observed under a fluorescent microscope (Nikon Eclipse E800). Images (region of interest — ROI: a 600 μm^2 portion of metaphyseal region, 400–1200 μm away from the growth plate and excluding the cortical bone) were obtained for all groups (n = 8 per group). Osteoclasts were identified as TRAP-positive multinucleated cells attached to the surface of trabecular bone. Osteoblasts were defined as square- or cone-shaped cells lining the surface of trabecular bone. Abbreviations and calculations were done according to the recommendations of the ASBMR Histomorphometry Nomenclature Committee [31].

Quantification of TUNEL-positive cells, cathepsin K-negative/ED-1 positive cells and ALP/PCNA-double positive cells

Images of TUNEL-positive cells, cathepsin K-negative/ED-1 positive cells and ALP/PCNA-double positive cells (a 400 μm × 400 μm square portion of metaphyseal region, 150 μm below the growth plate, excluding the cortical bone) were taken from eldecalcitol-injected and non-injected samples (n = 8 per group). Stained cells were counted with the aid of the ImagePro Plus 6.2 software (Media Cybernetics, Silver Spring, MD), and the results are shown in cell number per μm^2 of tissue area.

Statistical analysis

All statistical analyses were performed using Microsoft Excel 2003 Analysis ToolPak (Microsoft Corporation, Redmond, WA), with differences among groups being assessed by unpaired Student's *t*-tests or LSD method, and considered statically significant at $p<0.05$.

Results

Eldecalcitol rescues excessive bone loss after ovariectomy

The extent of bone loss following ovariectomy was evident after our initial histological analysis (Figs. 1A, B, D and E) and confirmed by both BMD values (Fig. 1J) and obtained structural histomorphometrical data (Table 1). Statistically significant differences were found between Sham and OVX groups for all structural parameters except for trabecular thickness, which was nevertheless higher in that group. Eldecalcitol successfully rescued the bone loss seen after ovariectomy (Figs. 1C, F), with the treatment group showing histomorphometrical values similar to those of the Sham group (Table 1). Interestingly, there was no obvious difference among the groups with regards to ALP activity as evaluated by immunohistochemistry (Figs. 1G, H, and I). Osteoblastic and bone formation parameters were enhanced in the OVX group accompanied by increased bone resorption parameters (Table 1). However, femoral BMD increased after eldecalcitol treatment in OVX animals, reaching values similar to those obtained from the Sham group (Fig. 1J).

Focal bone formation "bone minimodeling", after eldecalcitol administration

Histological analysis of semithin epoxy sections from eldecalcitol-treated specimens showed an ubiquitous presence of bone "buds" or "boutons" (Figs. 2A–C). The images unveiled a "budding" or "bouton" bone formation pattern characteristic of minimodeling, which is seen when new bone is deposited on previously quiescent surfaces and therefore features smooth cement lines (Figs. 2A–C). Eldecalcitol-treated specimens revealed various bone buds labeled with continuous lines of tetracycline and calcein (Fig. 2A), covered by mature

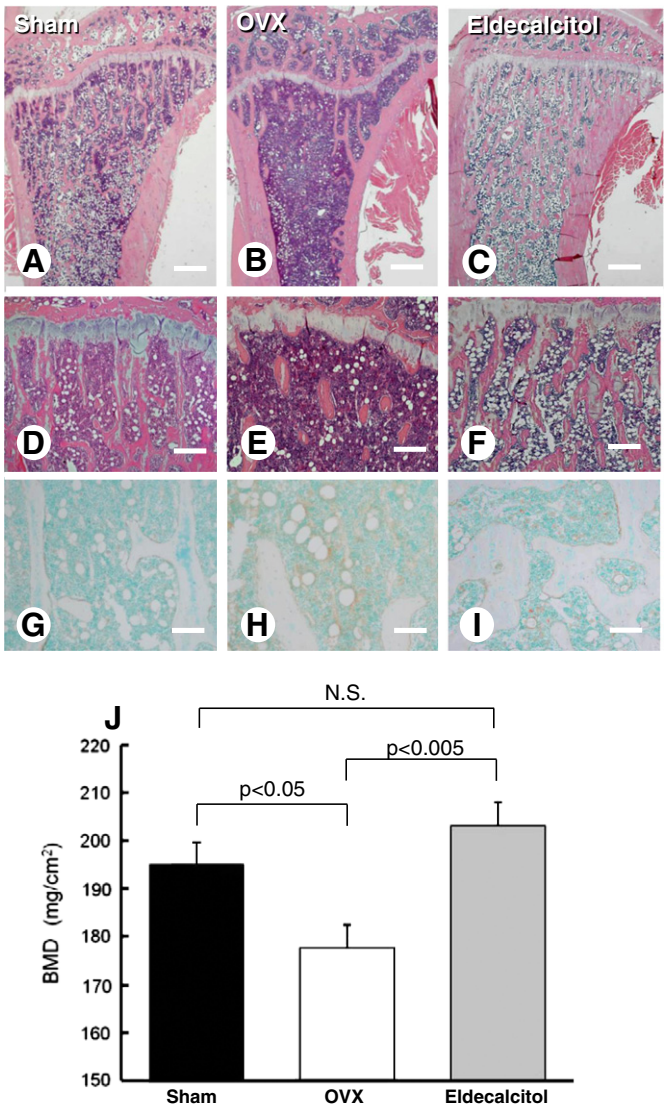

Fig. 1. Histological features of bone specimens from Sham, OVX and eldecalcitol groups and femoral BMD values. Bone loss following ovariectomy is obvious (compare A to B). Eldecalcitol effectively salvaged bone loss after ovariectomy (C) maintaining a bone content similar to that of Sham specimens (compare to A). Panels D–F show highly magnified images of metaphyses. With regards to ALP expression as evaluated by immunohistochemistry, difference among groups was very subtle (G, H, and I). J: Femoral BMD ratified the histological data and showed similar values for Sham and eldecalcitol groups. Bars A–C 1 mm, D–F 50 μm, and D–G 20 μm.

osteoblasts (Fig. 2C). Despite this uncommon pattern of bone formation characterized by the presence of smooth cement lines, assessment of mineralization by von Kossa's staining ruled out the possibility of defects in mineralization (data not shown). Moreover, TEM imaging permitted the visualization of mature osteoblasts lying on the bone "boutons" (Fig. 2D). Immunohistochemistry for ALP and PCNA demonstrated that preosteoblasts were proliferating less actively in the eldecalcitol group, when compared to the OVX group (Figs. 2E–G; OVX, 10.06 ± 3.84; Eldecalcitol, 3.59 ± 2.48; $p<0.005$). Therefore, eldecalcitol appears to inhibit preosteoblastic proliferation, which may force osteoblast maturation.

Eldecalcitol rescues bone loss essentially by affecting osteoclastic and resorption parameters

TRAP staining allowed for the identification of a higher number of osteoclasts in OVX samples when compared to Sham specimens

Table 1
Histomorphometric parameters in Sham, OVX and eldecalcitol groups.

INDEX	GROUP		
	Sham (n = 8)	OVX (n = 8)	Eldecalcitol (n = 8)
Structural parameters			
Bone volume (BV/TV, %)	23.78 ± 9.12[a]	9.6 ± 3.51	29.2 ± 11.57[a]
Trabecular separation (Tb. Sp, μm)	214.88 ± 83.47[a]	548.56 ± 176.37	190.89 ± 50.92[a]
Trabecular number (Tb. N, mm^{-1})	3.83 ± 0.85[a]	1.79 ± 0.51	3.82 ± 0.55[a]
Trabecular thickness (Tb. Th, μm)	60.55 ± 12.04	52.98 ± 7.62	75.53 ± 24.15[b]
Osteoblast surface (Ob. S/BS, %)	5.51 ± 3.1[b]	11.85 ± 5.22	4.44 ± 3.13[b]
Osteoid surface (OS/BS, %)	8.71 ± 4.89[b]	18.34 ± 8.02	9.06 ± 4.5[b]
Kinetic parameters			
Bone formation rate, tissue level (BFR/BS, $\mu m^3/\mu m^2/d$)	0.04 ± 0.03[b]	0.08 ± 0.04	0.04 ± 0.01[b]
Mineral apposition rate (MAR, μm/d)	1.43 ± 0.21	1.29 ± 0.08	1.19 ± 0.07[b]
Mineralizing surface (MS/BS, %)	6.24 ± 3.39[c]	17.21 ± 7.91	5.85 ± 3.48[c]
Osteoclastic and bone resorption parameters			
Number of mononuclear osteoclasts (N. Mo. Oc/BS, cells/mm)	0.61 ± 0.25[a]	1.35 ± 0.47	0.48 ± 0.3[a]
Number of multinuclear osteoclasts (N. Mu. Oc/BS, cells/mm)	0.47 ± 0.23[a]	1.32 ± 0.52	0.54 ± 0.39[c]
Number of osteoclasts (N. Oc/BS, cells/mm)	1.08 ± 0.46[a]	2.67 ± 0.76	1.02 ± 0.64[a]
Osteoclast surface (Oc. S/BS, %)	3.22 ± 1.65[a]	7.9 ± 2.5	3.59 ± 2.52[c]
Eroded surface (ES/BS, %)	28.7 ± 9.33	37.59 ± 7.02	17.41 ± 11.09[a]

Data are expressed as mean ± SD, p values determined by unpaired student's *t* test.
[a] $p<0.005$ *vs.* OVX.
[b] $p<0.05$ *vs.* OVX
[c] $p<0.01$ *vs.* OVX.

(Figs. 3A–B). After eldecalcitol administration, there were less TRAP-positive osteoclasts (Fig. 3C), a finding verified by histomorphometrical analysis (Table 1). Highly magnified light microscopy images showed that eldecalcitol-treated specimens feature osteoclasts that appear to have an inactive, flattened morphology (compare Fig. 3D to E). TEM imaging consistently showed large active osteoclasts with well-developed ruffled borders in OVX specimens (Fig. 3F), while flattened, inactive osteoclasts with poorly developed ruffled borders were a regular finding in samples from eldecalcitol-treated rats (Fig. 3G). Also, eldecalcitol administration suppressed ovariectomy-induced increment of an important bone resorption marker, urinary DPD (Fig. 3H).

Eldecalcitol administration increases the number of macrophages in the bone marrow

ED1 immunohistochemistry revealed an increased number of immunopositive cells in the marrow of specimens from the eldecalcitol group (compare Fig. 4A to B). To assess to what degree such cells were committed to the osteoclastic lineage, double immunostaining for cathepsin K/ED1 was carried out and made evident the distinction between macrophages and osteoclasts (Figs. 4C–D). Higher-magnified light microscopy revealed that the bone marrow of eldecalcitol-treated specimens has a great number of macrophages with inclusion bodies (Fig. 4E), while TEM further envisioned many lysosomes in these macrophages (Fig. 4F). Quantification of cathepsin K-negative/ED1-positive cells identified a statistically significant increase after eldecalcitol administration when compared to OVX group (Fig. 4G).

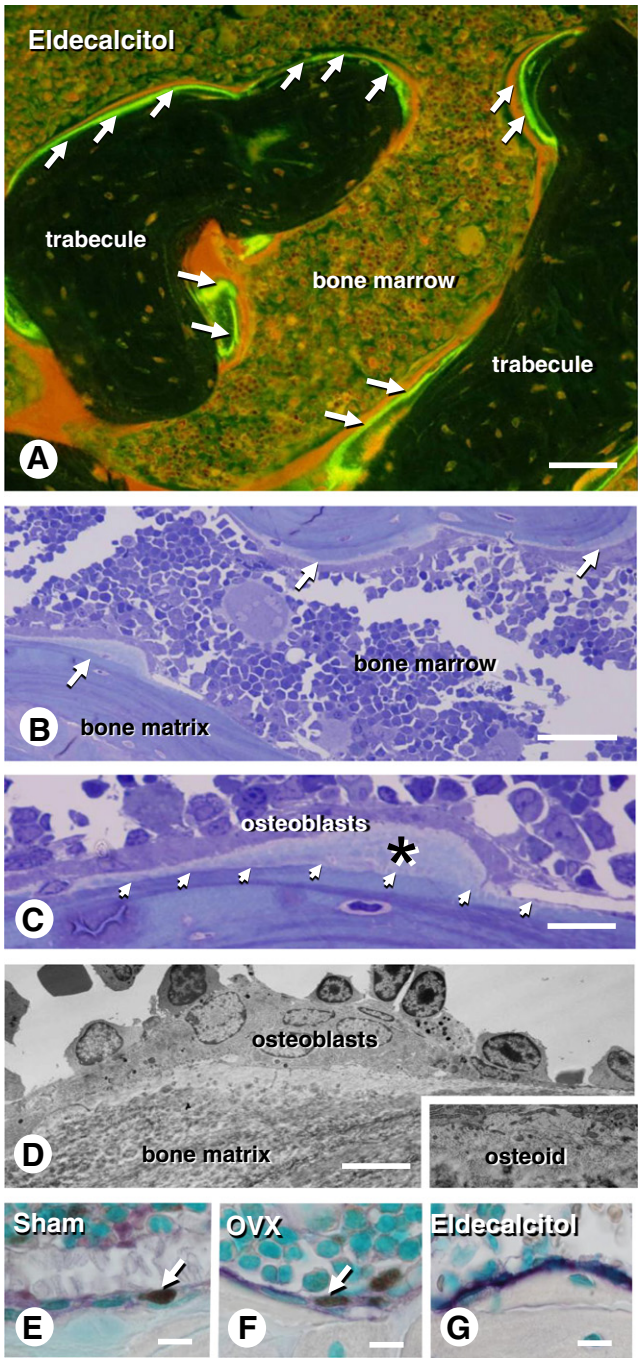

Fig. 2. Bone minimodeling in a specimen treated with eldecalcitol. Eldecalcitol-treated specimens revealed continuous tetracycline and calcein labeling on many bone buds (white arrows in A). In a low magnified image of toluidine blue staining, the "budding" or "bouton" bone formation pattern can be seen (B). This pattern can be characterized as minimodeling, since bone (asterisk) is laid out on top of smooth cement lines (white arrows in C). TEM imaging (C) depicts mature osteoblasts lying on top of a bone "bouton" whose normal osteoid appears to be mineralizing (inset, D). Despite the presence of many mature osteoblasts, there is hardly any preosteoblastic cell layer overlying mature osteoblasts (C, D). Panels E–G show double staining for ALP (blue color) and PCNA (brown color) in Sham (E), OVX (F) and eldecalcitol-administered (G) groups. Bars A–B 50 μm, C 20 μm, and D–G 10 μm.

Eldecalcitol administration does not increase apoptosis in the bone marrow

In order to investigate whether the increased presence of macrophages in the marrow was due to enhanced apoptosis after eldecalcitol administration, we conducted TUNEL staining. Quantification of TUNEL-

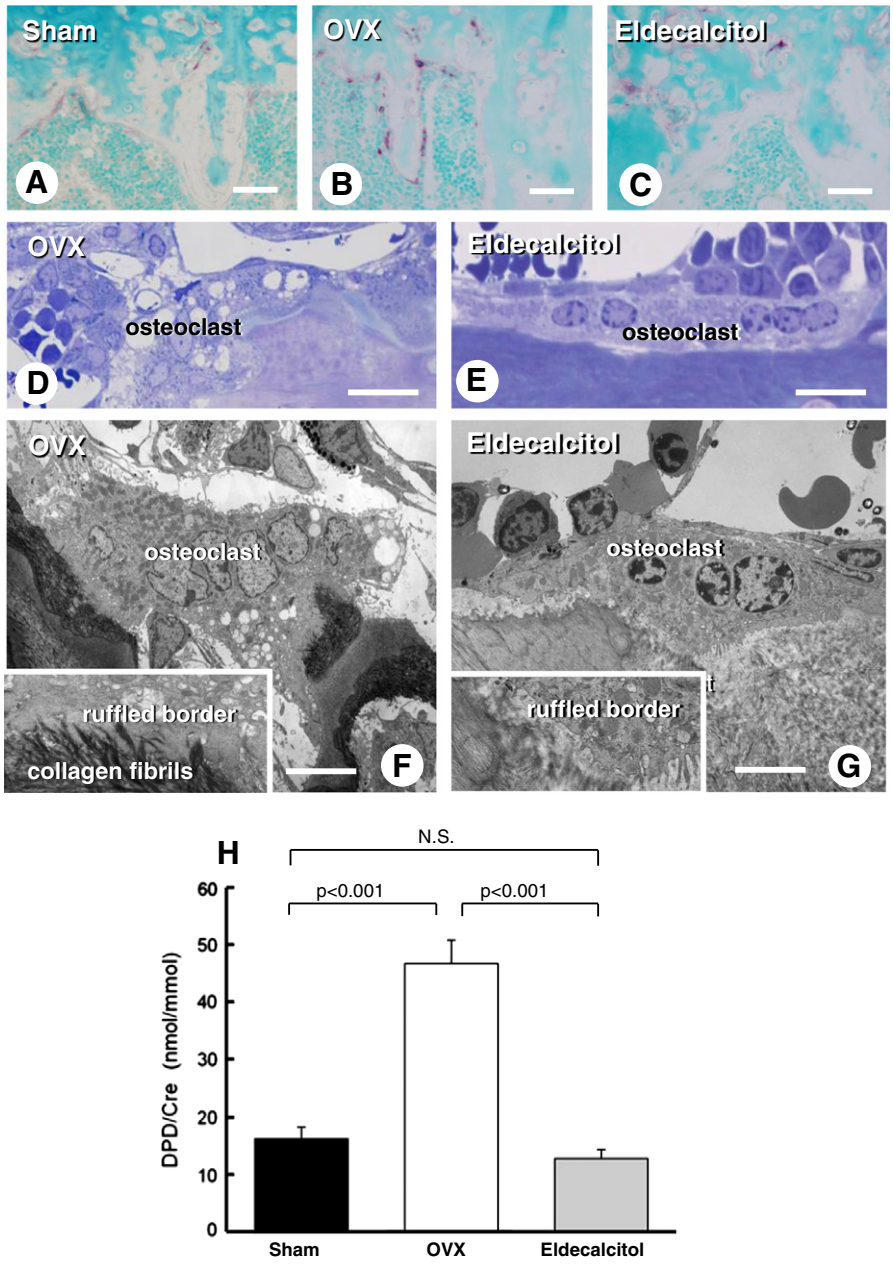

Fig. 3. Presence, morphology and function of osteoclasts in the different groups. TRAP staining shows higher numbers of osteoclasts in OVX samples compared to the other groups (A, B and C). Toluidine blue-stained semithin sections from eldecalcitol-treated specimens feature osteoclasts that seem rather inactive and less exuberant when compared to OVX osteoclasts (compare D to E). TEM imaging unveils large active osteoclasts with well-developed ruffled borders in OVX samples (F), while showing flattened, quiescent osteoclasts in eldecalcitol-treated specimens (G). Eldecalcitol administration suppressed ovariectomy-induced increase of urinary DPD (H). Bars A–C 50 μm, D–E 20 μm, and F–G 10 μm.

stained cells showed that the number of apoptotic cells is the lowest in eldecalcitol group (Fig. 5A).

Discussion

After administering eldecalcitol or vehicle to OVX rats, our main findings were: 1) with eldecalcitol administration, osteoblasts accumulate and synthesize new bone on top of smooth cement lines in process known as bone minimodeling; 2) eldecalcitol appears to affect osteoblastic differentiation and activation instead of stimulating preosteoblastic proliferation; 3) treatment with eldecalcitol lowers osteoclast number and diminishes osteoclastic activity/functionality, without promoting osteoclast apoptosis; and 4) eldecalcitol administration may favor the macrophage differentiation cascade on the expense of cells that would otherwise become osteoclasts. Therefore, eldecalcitol indirectly promotes a bone formation process known as minimodeling, but appears to exert its bone-protective effects mainly by affecting osteoclastic number and function. It may do so by favoring the macrophage lineage while hampering final osteoclastic differentiation, since there is an increased macrophage population in the bone marrow of eldecalcitol-treated specimens that cannot be explained by enhanced apoptosis.

In agreement with previous reports on the action of vitamin D analogs [23,26,32,33], this experiment showed that eldecalcitol can successfully prevent bone loss after ovariectomy. Our histological, histomorphometrical and femoral BMD analyses did demonstrate the recovery of bone structural parameters in OVX rats administered with eldecalcitol (Table 1). Interestingly, neither osteoblast and osteoid surface nor any of the kinetic bone parameters' values were positively affected by eldecalcitol; in fact, the values obtained for eldecalcitol

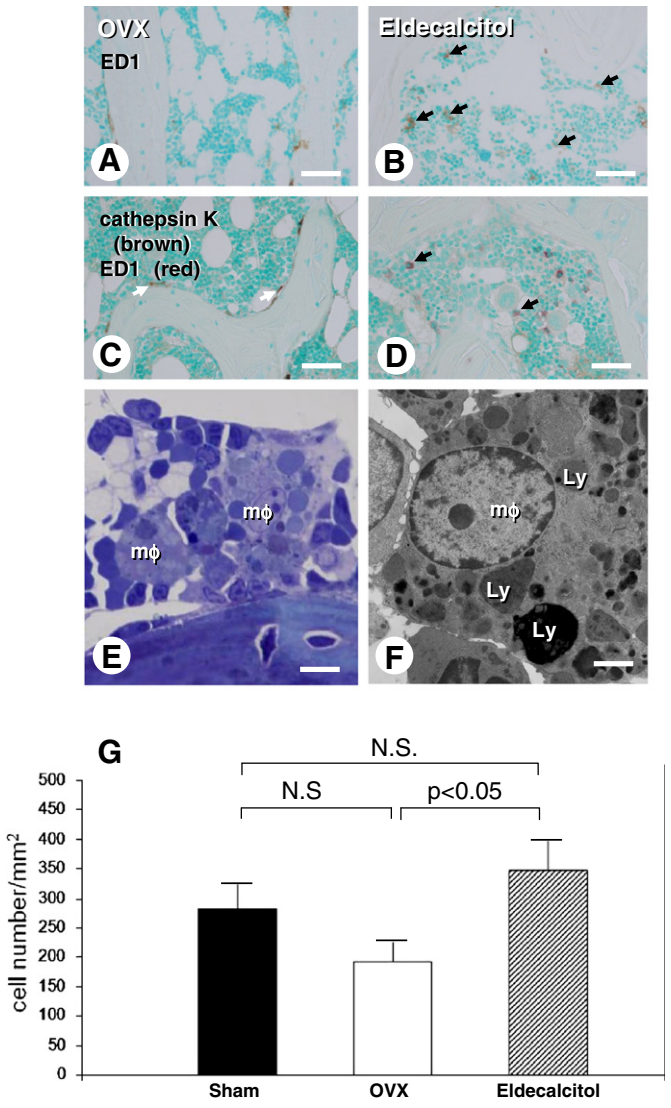

Fig. 4. Findings after ED1 and cathepsin K/ED1 immunohistochemistry and ultrastructural analysis of the bone marrow of eldecalcitol-treated specimens. The abundant presence of ED1-positive cells in the bone marrow of eldecalcitol-treated samples (arrows, B) was an interesting finding, since ED1 is a marker for both macrophages and osteoclasts (compare A to B). To rule out the possibility that these ED1-positive cells were osteoclasts, or osteoclast precursors, we performed cathepsin K/ED1 double immunohistochemistry to create a visual distinction between osteoclasts and macrophages. ED1/cathepsin K-positive cells (white arrows in C) are osteoclasts, which invariably populate bone surfaces, while ED-1-positive/cathepsin K-negative cells (black arrows in D) can be regarded as macrophages (compare C and D). Toluidine blue staining of semithin epoxy sections of eldecalcitol-treated specimens demonstrates macrophages (mϕ) populating the bone marrow (E). TEM imaging shows the ultrastructure of a bone marrow macrophage (F) with its many lysosomes (Ly). Quantification of cathepsin K-negative/ED1-positive cells shows statistically significant higher numbers after eldecalcitol administration when compared to the OVX group (G). Bars A–D 50 μm, E 20 μm, and F 2 μm.

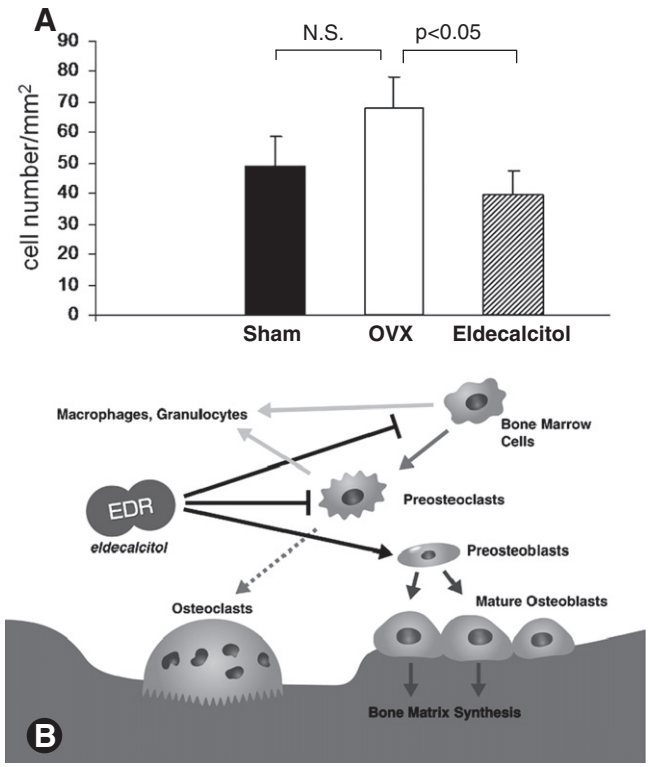

Fig. 5. TUNEL staining for assessment of apoptosis. Quantification of TUNEL-stained cells showed that the number of apoptotic cells is lowest in the eldecalcitol group (A). In panel B, there is a schematic summary of the proposed mechanism of eldecalcitol (EDR)'s action on bone cells: the drug appears to inhibit final differentiation of osteoclastic precursors, which may in turn become macrophages; it also seems to drive osteoblastic differentiation and to promote bone minimodeling.

and Sham groups were very similar. In that case, changes in the pattern of ALP staining are not expected, as seen in Figs. 1G–I. Enhanced ALP staining was anticipated, but not verified, in the OVX group. ALP expression, while expressed consistently seen throughout osteoblastic differentiation, has been demonstrated to be *condition sine qua non* for mineralization as demonstrated in ALP knockout mice [34]. OVX animals suffer from accelerated bone turnover, showing stimulated osteoclastic bone resorption and reactive osteoblastic bone formation with a net result of bone loss. Even though eldecalcitol activates mature osteoblasts and induces minimodeling, the activated osteoclastic status in OVX animals may conceal any surplus in bone formation. Osteoblasts may compensate for the abnormal bone destruction by frantically synthesizing osteoid, while mineralization seems to be slowed down. After ovariectomy, Parameters that refer to non-mineralized bone matrix such as osteoid surface and mineralizing surface show two- and three-fold increases, respectively, when compared to Sham animals. Osteoblasts in the OVX group, therefore, may not show enhanced expression of ALP because their main function, in a scenario of untamed bone destruction, is rapid bone matrix synthesis, not its mineralization. The histological picture seen after eldecalcitol treatment is quite different from the one obtained with an intermittent PTH regimen, in which we showed the clear proliferative and osteoblastic activation effects of that hormone [35]. Alternatively, Okuda et al. [23] have shown that ED-71, the former denomination of eldecalcitol, was capable of promoting enhancement of ALP activity and bone nodule formation in bone marrow cells *in vitro*, where the influence of osteoclastic bone resorption does not exist. Under our experimental circumstances, it seems that eldecalcitol drives osteoblastic differentiation *in vivo* with consequent bone minimodeling without noticeable differences in the pattern of ALP staining.

The histological data in this study unveiled the consistent presence of a rather particular type of bone formation after eldecalcitol treatment: bone minimodeling. Minimodeling is termed so because magnification is needed to visualize it [36], and it basically consists of bone formation not preceded by osteoclastic bone resorption with cement lines that are typically smooth [37]. Minimodeling in bone has been reported after treatment with bone anabolic agents like PTH [38] and prostaglandin E_2 [39]. It has been hypothesized that the mechanism guiding minimodeling-based bone formation is the resumption of osteoblastic activity of bone lining cells [40]. In

our histological samples, we did observe a dominant presence of plump osteoblasts compared to that of resting bone lining cells in eldecalcitol-treated specimens (Figs. 2C–D). The absence of numerical data regarding the amount of minimodeling-based bone formation and the number of active osteoblasts as opposed to bone lining cells are limitations of this study. However, it is evident from the histology shown here that eldecalcitol treatment fosters bone minimodeling.

In contrast with the effect of the drug upon osteoblastic cells seen in our experimental setup, observations on the behavior and morphology of osteoclastic cells have been more elusive of eldecalcitol's main mechanism of bone loss prevention. In our study, osteoclastic, bone resorption parameters and urinary DPD have demonstrated that eldecalcitol is an inhibitor of bone resorption, as previous studies have reported for other vitamin D analogs [17,26]. Eldecalcitol administration lowered osteoclast numbers in OVX rats, and more importantly, significantly lowered the amount of eroded surface (Table 1). Accordingly, our histological data showed inactive osteoclasts on the bone surfaces of eldecalcitol-treated samples, suggesting that not only was the drug able to bring osteoclastic parameters close to those from the Sham group, but it also may have affected the osteoclast's ability to disorganize the bone matrix. This mechanism of action is different from that of bisphosphonates, which drive osteoclastic apoptosis when given in concentrations above 100 μM [41]. Baldock et al. have shown that overexpression of VDR in mature osteoblasts suppresses osteoclastogenesis [42], possibly by an OPG-related mechanism [43]. Also, it has been suggested that increased osteoblast maturation can reduce $1\alpha,25\text{-}(OH)_2D_3$-regulated osteoclastogenesis in bone marrow/osteoblast co-culture [44]. This postulation can be supported by the histological findings of preosteoblasts with a lessened proliferative profile in eldecalcitol-administered specimens (Figs. 2E–G). It is possible that, by forcing osteoblastic differentiation towards the mature phenotype, eldecalcitol indirectly suppresses cell-to-cell contact between osteoclastic precursors/osteoclasts and preosteoblastic cells, thereby affecting osteoclastogenesis and osteoclastic activity.

The increased number of cells of the macrophage phenotype in the bone marrow of eldecalcitol-treated samples was another interesting finding of our study. It is now common knowledge that the osteoclast is a member of the monocyte/macrophage family and that final osteoclastic differentiation is influenced by many different molecules [45]. $1\alpha,25\text{-}(OH)_2D_3$ stimulates osteoclast formation indirectly through bone marrow stroma cells [46]. The hormone is regarded as a fusion factor for monocytes/macrophages, as well [47]. Our results have shown that the increase in macrophage numbers is not related to increased apoptosis, which would implicate a need for more phagocytic cells, and therefore indicated facilitated macrophage differentiation by eldecalcitol. Based on our data, it is fair to infer that complete osteoclastic differentiation is blocked somewhere along the differentiation cascade; instead, the precursors might be guided towards differentiating into the macrophage phenotype, probably because of lessened interaction between preosteoblastic cells and preosteoclasts.

In summary, our findings showed that eldecalcitol prevents bone loss following ovariectomy in rats mainly by suppressing bone resorption (Fig. 5E). The increased macrophage numbers seen in eldecalcitol-treated specimens may be a reflection of a block in the osteoclastic differentiation cascade. Bone minimodeling, although not quantified, was ubiquitous in samples from the eldecalcitol group. Taken together, the evidence presented here unveiled the histological aspects of eldecalcitol's action upon bone, qualifying it as a promising drug for the treatment of osteoporosis.

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