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ORIGINAL PAPER

Histochemical examination of the effects of high-dose $1,25(OH)_2D_3$ on bone remodeling in young growing rats

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Abstract Vitamin D has an anabolic effect on bone developmental processes and is involved in maintaining skeletal integrity. In recent years, pediatric cases of vitamin D intoxication have attracted attention. Therefore, the aim of this study was to investigate the influence of long-term administration of physiologically-high-dose calcitriol $(1,25(OH)_2D_3)$ on bone remodeling in young developing rats. Neonatal rats received once-daily subcutaneous injection of calcitriol (250 ng/kg body weight), or PBS only as a control, for 3 weeks. At 1, 2 and 4 weeks' post-administration, rats were sacrificed and fixed by transcardial perfusion with 4 % paraformaldehyde, following which tibiae were extracted for histochemical analysis. Compared with the control group, the number of tartrate-resistant acid phosphatase- and Cathepsin K-positive osteoclasts were significantly increased, and the expression of alkaline phosphatase in osteoblasts was decreased in trabecular bone of rats administered high-dose $1,25(OH)_2D_3$, leading to decreased trabecular bone volume. In addition, the expression of receptor activator of nuclear factor kappa-B ligand (RANKL) was increased, while that of osteoprotegerin was weaker in osteoblasts in the experimental group compared with the control group.

Moreover, there was weaker immunoreactivity for EphrinB2 in osteoclasts and EphB4 in osteoblasts of trabecular bone in the experimental group compared with the control group. These findings suggest that long-term use of physiologically-high dose calcitriol may result in bone loss through RANKL/RANK/osteoprotegerin and EphrinB2–EphB4 signaling pathways, and that these negative effects could continue after drug withdrawal. Therefore, optimal limits for vitamin D administration need to be established for children and adolescents.

Keywords $1,25(OH)_2D_3$ · Bone loss · Osteoclastogenesis · RANKL/RANK/OPG · EphrinB2–EphB4

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Introduction

Vitamin D is a hormone involved in calcium homeostasis through its actions on the intestine, kidney, parathyroid gland, and bone (van Leeuwen et al. 2001). It is essential for optimal calcium absorption needed for maintaining normal bone mineral density (BMD) (Woeckel et al. 2010). Calcitriol, also called 1,25-dihydroxy vitamin D3 $(1,25(OH)_2D_3)$, is the active metabolite of vitamin D3 and is involved in various physiological processes, including calcium homeostasis, bone metabolism and the immune response (Inada et al. 2008). Vitamin D-deficiency, as a greater risk of osteoporosis, results in poorly mineralized bone, with diminished strength and load-bearing capacity, and is linked to rickets in infants and osteomalacia in adults, both of which are skeletal disorders characterized by defective bone mineralization (Suda et al. 2003; Oku et al. 2016). Moreover, the administration of vitamin D to ovariectomized or rachitic animals can relieve impaired bone mineralization (Inada et al. 2008). Therefore, vitamin

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D is one of the major critical factors involved in bone metabolism.

$1,25(OH)_2D_3$ is widely used as an antiresorptive agent for the management of $1,25(OH)_2D_3$ deficiency and secondary hyperparathyroidism in children and adolescents (Ghergherechi et al. 2012; Alshamsan and Bin-Abbas 2016; Dura-Trave and Gallinas-Victoriano 2016). Though vitamin D is necessary for basal bone homeostasis, excessive concentrations may be harmful. Masayoshi et al. demonstrated that high dose $1,25(OH)_2D_3$ inhibits osteoblast mineralization in vitro (Yamaguchi and Weitzmann 2012). And, high dose of $1,25(OH)_2D_3$ acts on the osteoblasts to induce the formation of osteoclasts in a co-culture system in vitro (Takahashi et al. 2014; Cong et al. 2015). Moreover, high-dose maternal $1,25(OH)_2D_3$ can transferred across the placental barrier and injure fetal development or birth (Lieben et al. 2013). In addition, recently, pediatric cases of vitamin D intoxication (VDI) with dietary supplements have drawn our attention. Rajakumar et al. (2013) described a case study of an infant who had hypervitaminosis D caused by a dosing error in an over-the-counter vitamin D supplement. Cengiz et al. reported seven children aged between 0.7 and 4.2 years were admitted with symptoms of hypercalcemia caused by the consumption of a fish oil supplement containing an excessively high dose of vitamin D caused by a manufacturing error (Kara et al. 2014).

To date, though VDI attracted our attention, there is limited data available on the effects of long term administration of high-dose $1,25(OH)_2D_3$ on bone metabolism in children and rodent models in vivo, especially the histological evidence (Fig. 1). Therefore, in the current study, we used neonatal rats administered with physiologically-high-dose $1,25(OH)_2D_3$ to investigate its effects on bone development. Our results indicate that long-term use of high-dose $1,25(OH)_2D_3$, even physiological-high-dose, was harmful to bone, and this negative effects will continue. Therefore, optimal concentrations and protocol for the administration of vitamin D need to be established for humans.
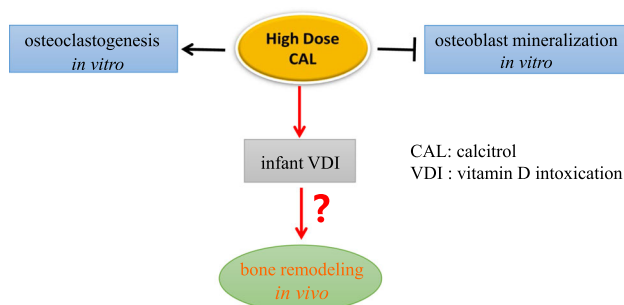
Fig. 1 Schema of research background and aim of this study

Materials and methods

Animal and tissue preparation

All animal experiments in this study were conducted according to the Guidelines for Animal Experimentation of Shandong University. Pregnant rats were obtained from Laboratory Animal Centre of Shandong University (Jinan, China) and kept in plastic cages (1 animal per cage) under standard laboratory. All rats were fed a standard rodent diet ad libitum. Neonatal rats from them were randomly divided into two groups, experimental group and control group (30 rats per group). Calcitriol (Selleck Chemicals, Houston, TX, USA) was dissolved in a small amount of ethanol and diluted with PBS to a final concentration of 1 mg/ml. The newborn rats in the experimental group were further subdivided into three groups (groups A, B, C; ten rats per group), which were then received single, once-daily subcutaneous injections of calcitriol (250 ng/kg body weight) for 1, 2, 3 weeks respectively (group A for 1 week, group B for 2 weeks, group C for 3 weeks). The rats of groups A and B were sacrificed after the indicated administration periods. To determine whether the effects of calcitriol on the development of rats would persist after the drug withdrawal, after 3 weeks of calcitriol administration, the rats of group C were withdrawn from drug treatment and kept for another week followed by execution at the fourth week. The high dose of $1,25(OH)_2D_3$ was utilized as it elicits a maximal physiologic response in normal rats (Ng et al. 2014; Yao et al. 2005). The rats were given only PBS as control (same quantity and frequency with the VD group) and sacrificed at the indicated time points corresponding to their respective experimental groups.

After the above treatment, the rats were anesthetized with an intraperitoneal injection of 10 % chloral hydrate (400 mg/100 g body weight) and fixed with 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) by transcardial perfusion, respectively. After fixation, tibiae were removed and immersed in the same fixative for additional 24 h. Following that, samples were decalcified with 10 % EDTA–2Na solution for 3 weeks at 4 °C. Then the specimens were dehydrated through an ascending ethanol series and then embedded in paraffin using standard procedures. Serial longitudinal 5-µm-thick sections were prepared for following histological analysis using sliding microtome (LEICA SM 2010R, Heidelberger, German).

Histological examination and image analysis

Hematoxylin and eosin (H&E) staining were performed to identify the morphology of metaphysis in both groups.

Stained sections were observed and digital images were taken with a light microscope (Olympus BX-53, Tokyo, Japan). Trabecular bone volume (BV/TV, bone volume/tissue volume × 100 %) were measured by Image-Pro Plus 6.2 software (Media Cybernetics, Silver Spring, MD, USA). Specifically, ten slices of each sample were used for quantitative histomorphometric analysis to get the mean value.

Histochemistry (TRAP staining) for osteoclasts

To evaluate the osteoclasts, tartrate-resistant acid phosphatase (TRAP) staining was performed as previously showed (Li et al. 2013). In brief, dewaxed paraffin sections were submerged in a mixture of 3.0 mg of naphthol AS-BI phosphate, 18 mg of red violet LB salt, and 100 mM L(+) tartaric acid (0.36 g) diluted in 30 ml of 0.1 M sodium acetate buffer (PH 5.0) for 15 min at 37 °C. The sections were then faintly counterstained with methyl green for assessing by light microscopy (Olympus BX-53, Tokyo, Japan). Osteoclasts numbers were counted in three randomly selected non-overlapping microscopic fields in each section by Image-Pro Plus 6.2 software (Media Cybernetics, Silver Spring, MD, USA). At least ten sections from each sample were analyzed.

Immunohistochemistry for ALP, CK, RANKL, OPG, EphrinB2 and EphB4

To evaluate the activities of osteoblasts and osteoclasts, immunohistochemistry of (alkaline phosphatase) ALP and (Cathepsin K) CK were performed respectively. RANKL/RANK/OPG pathway and EphrinB2/EphB4 pathway are both involved in the normal coupling process between osteoclasts and osteoblasts, thus the sections were examined for RANKL, OPG, EphrinB2 and EphB4 immunohistochemistry staining after calcitriol administered. Briefly, after xylene treatment, dewaxed paraffin sections were pretreated with 0.3 % hydrogen peroxide for 30 min, and then with 1 % bovine serum albumin (BSA; seologicals proteins Inc. Kankakee, IL, USA) in PBS (1 % BSA-PBS) for 20 min to reduce non-specific staining. The sections were then incubated for 2 h at room temperature with: (1) rabbit antiserum against rat tissue nonspecific ALP, generated by Oda et al. (1999) at a dilution of 1:100; (2) anti-CK antibody (Abcam, Cambrige, MA, USA) at a dilution of 1:100; (3) anti-RANKL antibody (Santa Cruz Biotechology, Santa Cruz, CA, USA) at a dilution of 1:50; (4) anti-OPG antibody (Abcam, Cambrige, MA, USA) at a dilution of 1:50; (5) Mouse EphrinB2 antibody (R&D Systems, Minneapolis, MI, USA) at a dilution of 1:25; (6) Mouse EphB4 antibody (R&D systems, Minneapolis, MI, USA) at a dilution of 1:50 in 1 % BSA-PBS. After rinsing

with PBS, the sections were incubated with horseradish peroxidase-conjugated swine anti-rabbit IgG (DaKo, Glostrup, Denmark) for ALP, CK, RANKL and OPG; rabbit anti-Goat IgG (Jackson ImmunoResearch Labortories, Inc, Baltimore, MD, USA) for EphrinB2 and EphB4 1 h at room temperature. The immunoreaction was visualized with diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO, USA). All sections were counterstained faintly with methyl green and observed under a light microscope (Olympus BX-53, Tokyo, Japan). Immunostaining intensities (optical density, OD) were also analyzed using Image-Pro Plus 6.2 software. Positive reaction areas of ALP, CK, RANKL, OPG, EphrinB2 and EphB4 were manually selected through color cube based manner. At least ten sections from each sample were analyzed. In addition, metaphysis of each tissue section was selected to be evaluated at 4× magnification, in which the metaphysis was totally included.

Statistical analysis

All statistical analyses were performed using SPSS software. All values are presented as mean ± standard deviation (SD) in the results and scatter plots of individual data points in the figures. The differences between $1,25(OH)_2D_3$ administered group and control were assessed by student's t test, and considered statistically significant at $*p < 0.05$ and $**p < 0.01$.

Results

Histological alterations of tibial metaphysis and statistical analysis

At 1, 2 and 4 weeks after $1,25(OH)_2D_3$ administration, high-dose $1,25(OH)_2D_3$-treated rats showed obvious decreases in trabecular bone volume compared with that of the control group (Fig. 2a–f). Statistical analysis revealed several significant differences between the $1,25(OH)_2D_3$ administration (VD) and control groups regarding trabecular bone volume density (BV/TV; 1 week: 19.22 ± 0.81 % in the VD group vs. 38.71 ± 1.30 % in the control, $p < 0.01$, 2 weeks: 25.02 ± 1.09 % in the VD group vs.33.38 ± 0.68 % in the control, $p < 0.01$, 4 week: 19.07 ± 0.71 % in the VD group vs.33.10 ± 1.12 % in the control, $p < 0.01$, Fig. 2g).

Tartrate-resistant acid phosphatase (TRAP) staining and Cathepsin K (CK) immunolocalization

Compared with control group, the number of TRAP-positive osteoclasts was significantly increased on the surface
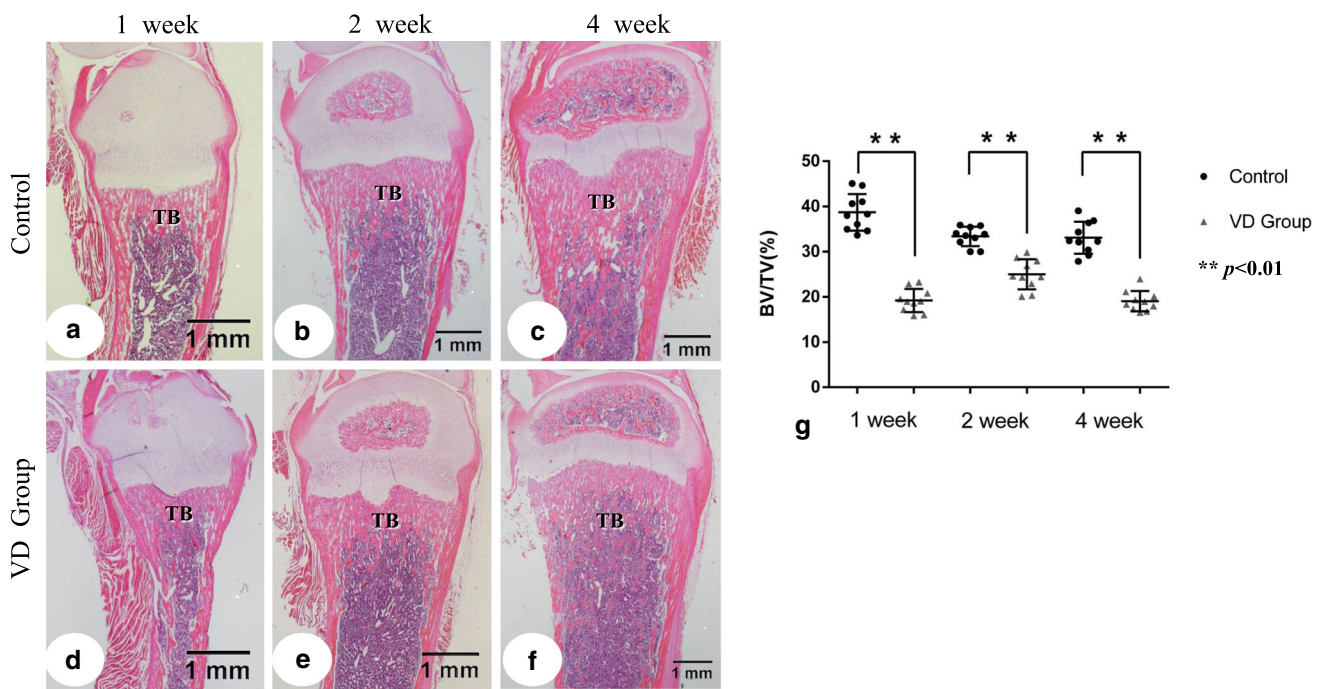
Fig. 2 Histological alterations and statistical analysis. Hematoxylin-eosin staining of tibiae shows the differences between the two groups. **a–c** low magnification of tibiae of control group (**a** 1 week, **b** 2 weeks, **c** 4 weeks); **d–f** low magnification of tibiae of 1,25(OH)$_2$D$_3$-administered group (**d** 1 week, **e** 2 weeks, **f** 4 weeks). **g** The mean values of BV/TV were assessed for both groups by using statistics (n = 10; **$p < 0.01$). At 1, 2, 4 weeks after 1,25(OH)$_2$D$_3$ administration for 3 weeks, tibiae showed obvious histological manifestation of decreased trabecular bone volume. All corresponding numerical information is found in the results section. *Error bars* indicate ±SD. *TB* trabecular bone, *BV/TV* trabecular bone volume/tissue volume. **a–f** ×40

of trabecular bone in rats administered with physiologically-high-dose 1,25(OH)$_2$D$_3$. Osteoclasts in the control group were flat and slender with 2–3 nuclei, whereas osteoclasts in the VD group were round or polygonal with many nuclei (Fig. 3a–f). Statistical analysis found a significant difference in the numbers of osteoclasts between the VD and control groups (1 week: 40.00 ± 2.58 in the VD group vs. 26.02 ± 1.32 in the control, $p < 0.01$, 2 weeks: 75.03 ± 3.61 in the VD group vs. 41.05 ± 2.48 in the control, $p < 0.01$, 4 weeks: 85.01 ± 3.21 in the VD group vs. 54.04 ± 3.71 in the control, $p < 0.01$, Fig. 3g). CK staining showed stronger CK expression in the VD group compared with the control (Fig. 4a–f). Statistical analysis revealed significant differences in the optical densities of CK between the VD and control groups (1 week: 0.358 ± 0.005 in the VD group vs. 0.148 ± 0.004 in the control, $p < 0.01$, 2 weeks: 0.283 ± 0.004 in the VD group vs. 0.156 ± 0.003 in control, $p < 0.01$, 4 weeks: 0.260 ± 0.005 in the VD group vs. 0.112 ± 0.003 in the control, $p < 0.01$, Fig. 4g).

Alkaline phosphatase (ALP), RANKL and osteoprotegerin (OPG) immunolocalization

In the VD group, trabecular bone of the metaphysis showed stronger RANKL expression and weaker ALP and OPG expression than the control at all time points (Figs. 5a–f, 6a–l). Statistical analysis revealed significant differences in ALP-, RANKL-, OPG-immunoreactivity between the VD and control groups (ALP: 1 week: 0.025 ± 0.002 in the VD group vs. 0.056 ± 0.003 in the control, $p < 0.01$, 2 weeks: 0.010 ± 0.002 in the VD group vs. 0.099 ± 0.004 in the control, $p < 0.01$, 4 weeks: 0.027 ± 0.002 in the VD group vs. 0.121 ± 0.003 in the control, $p < 0.01$, Fig. 5g; RANKL: 1 week: 0.149 ± 0.005 in the VD group vs. 0.066 ± 0.003 in the control, $p < 0.01$, 2 weeks: 0.106 ± 0.002 in the VD group vs. 0.049 ± 0.003 in the control, $p < 0.01$, 4 weeks: 0.088 ± 0.002 in the VD group vs. 0.051 ± 0.003 in the control, $p < 0.01$, Fig. 6m; OPG: 1 week: 0.052 ± 0.002 in the VD group vs. 0.110 ± 0.004 in the control, $p < 0.01$, 2 weeks: 0.027 ± 0.002 in the VD group vs. 0.099 ± 0.003 in the control, $p < 0.01$, 4 weeks: 0.029 ± 0.002 in the VD group vs. 0.081 ± 0.003 in the control, $p < 0.01$, Fig. 6n).

EphrinB2 and EphB4 immunolocalization

In the VD group, trabecular bone of the metaphysis showed weaker EphrinB2 expression in osteoclasts and EphB4 in osteoblasts at all time points compared with the control group (Fig. 7a–l). Statistical analysis revealed significant
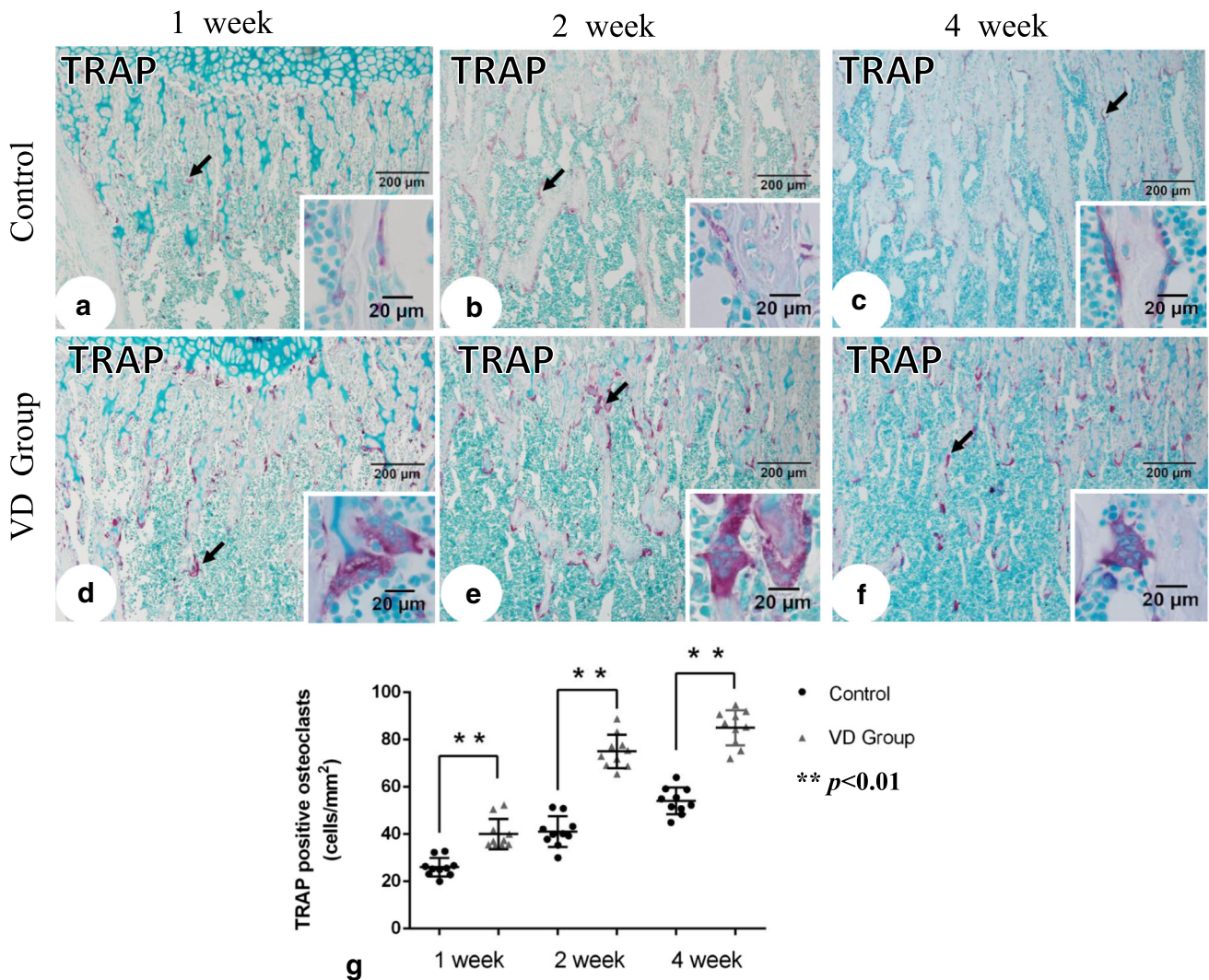
Fig. 3 TRAP staining for osteoclasts in the trabecular bone and statistical analysis. *Upper line* (**a** 1 week, **b** 2 weeks, **c** 4 weeks) control group; *lower line* (**d** 1 week, **e** 2 weeks, **f** 4 weeks) 1,25(OH)$_2$D$_3$ administration group. The osteoclasts in control group were flat and slender with 2–3 nuclei (**a**–**c**); Whereas in experimental groups the osteoclasts were round and polygon with many nuclei (**d**–**f**). **g** TRAP-positive osteoclasts were counted in the metaphysis of tibiae for both groups (n = 10; **p < 0.01). All corresponding numerical information is found in the results section. *Error bars* indicate ±SD. *SD* standard deviation; **a**–**f** ×100; cells ×400

differences in EphrinB2- and EphB4-immunoreactivity between the VD and control groups (EphrinB2, 1 week: 0.071 ± 0.002 in the VD group vs. 0.117 ± 0.004 in the control, $p < 0.01$, 2 weeks: 0.038 ± 0.002 in the VD group vs. 0.098 ± 0.003 in the control, $p < 0.01$, 4 weeks: 0.040 ± 0.003 in the VD group vs. 0.126 ± 0.003 in the control, $p < 0.01$, Fig. 7m; EphB4, 1 week: 0.078 ± 0.003 in the VD group vs. 0.104 ± 0.003 in control, $p < 0.01$, 2 weeks: 0.062 ± 0.002 in the VD group vs. 0.091 ± 0.004 in the control, $p < 0.01$, 4 weeks: 0.052 ± 0.002 in the VD group vs. 0.110 ± 0.003 in the control, $p < 0.01$, Fig. 7n).

Discussion

In this study, 3-week-continuous administration of high-dose 1,25(OH)$_2$D$_3$ stimulated osteoclastogenesis and inhibited the activity of osteoblasts, which resulted in decreased trabecular bone volume (Fig. 8). In addition, these negative effects could continuously affect bone metabolism after drug withdrawal.

1,25(OH)$_2$D$_3$ has been shown to affect certain bone cells, targeting each of the major bone cell types either directly or indirectly to influence the anabolic and catabolic processes of bone remodeling. Calcitriol, the activated
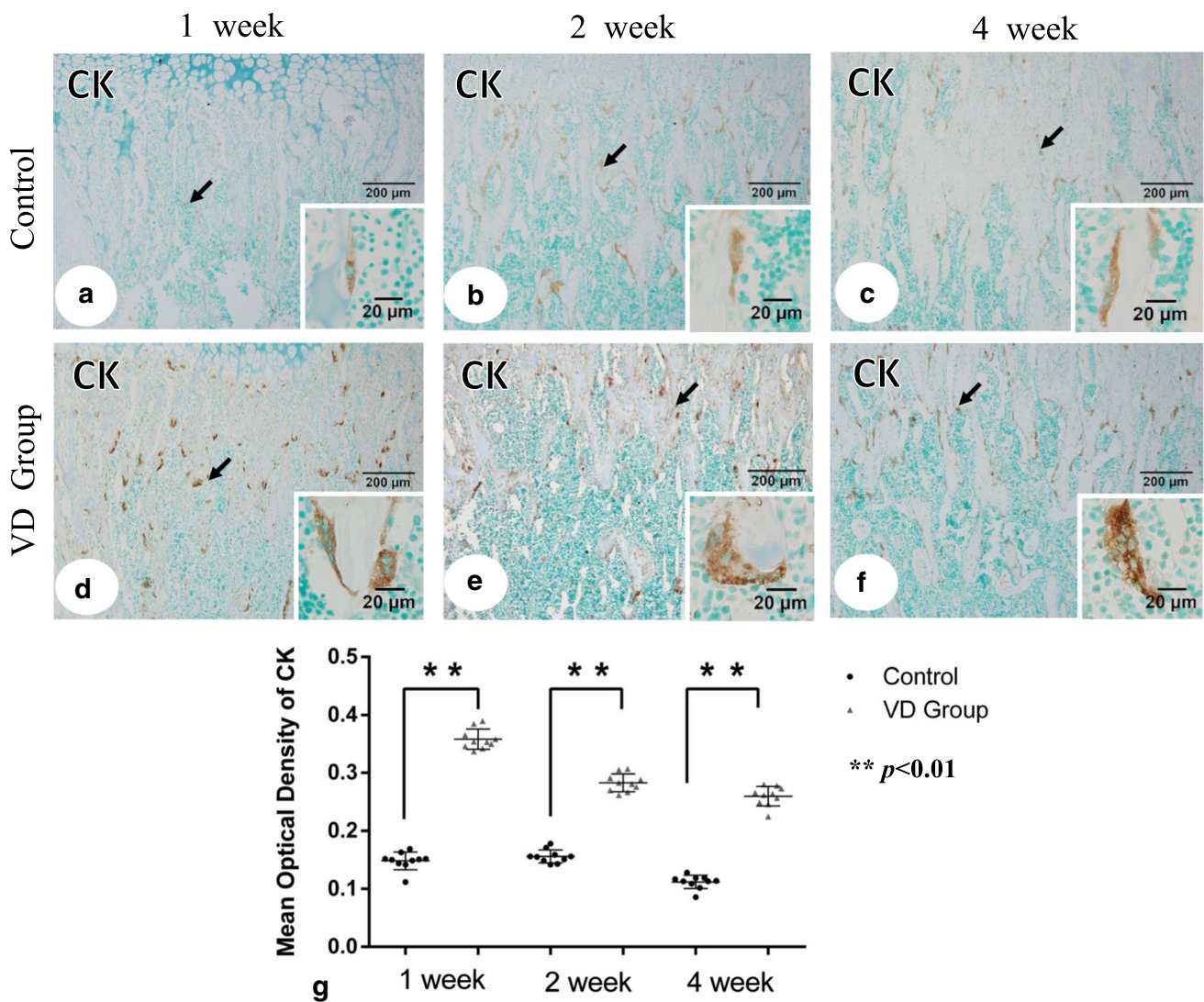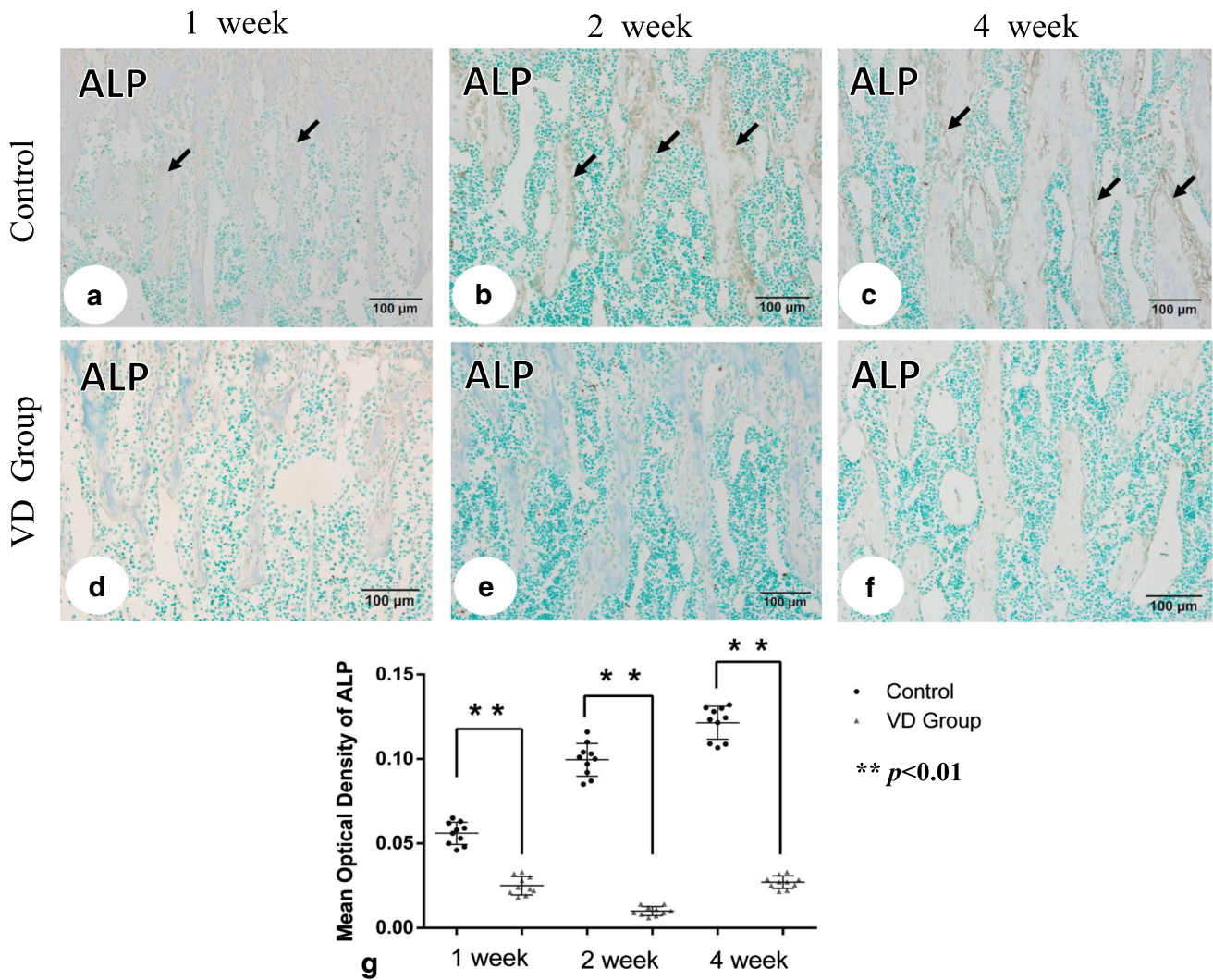
Fig. 4 Immunolocalization of CK in the trabecular bone and statistical analysis. *Upper line* (**a** 1 week, **b** 2 weeks, **c** 4 weeks) control group; *lower line* (**d** 1 week, **e** 2 weeks, **f** 4 weeks) 1,25(OH)$_2$D$_3$ administration group. Weak expression of CK in the control group with flat and slender osteoclasts (**a** 1 week, **b** 2 weeks; **c** 4 weeks), while intense expression of CK in the 1,25(OH)$_2$D$_3$ administration group with round and polygon osteoclasts (**d** 1 week, **e** 2 weeks; **f** 4 weeks). **g** Mean optical density of CK immunoreactivity were assessed in the trabecular bone for both groups (n = 10; **p < 0.01). All corresponding numerical information is found in the results section. *Error bars* indicate ±SD. *SD* standard deviation; **a**–**f** ×100; cells ×400

form of vitamin D3, has opposing effects on osteoblast differentiation (Hicok et al. 1998; Lohmann et al. 2000; Li et al. 2008). Furthermore, supraphysiological doses of 1,25(OH)$_2$D$_3$ inhibit cancellous bone formation, exerting a negative influence on bone quality, beginning from 1-week post-administration and intensifying after 1 month of exposure (Idelevich et al. 2011). In the present study, we noticed that even at a physiologically-high dose, 1,25(OH)$_2$D$_3$ inhibited osteogenesis, inhibited the activity of osteoblasts and reduced bone formation in vivo.

TRAP, a histochemical and biochemical marker for osteoclasts, allows for the examination of osteoclastic differentiation and function (Cole and Walters 1987). Regarding osteoclastogenesis, our results revealing that high-dose calcitriol increased the number of TRAP-positive osteoclasts are consistent with some previous studies, in which calcitriol strongly stimulated the formation of osteoclasts and induced the expression of RANKL and macrophage colony-stimulating factor (M-CSF), both in vitro and in vivo, leading to bone resorption (Suda et al. 1999; Sato et al. 2007). However, other studies have shown that calcitriol inhibited osteoclast formation. Specifically, calcitriol was reported to inhibit the induction of c-fos and nuclear factor of activated T cells c1 (NFATc1) and
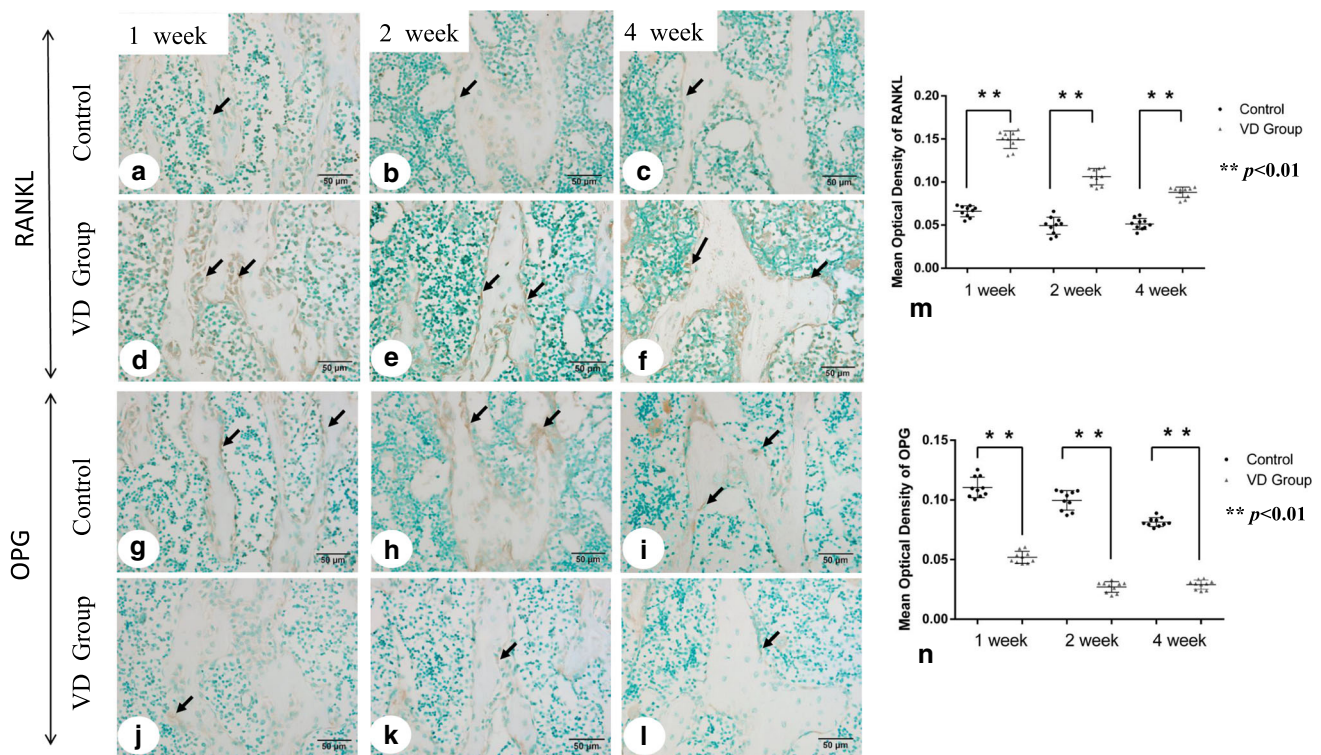
Fig. 5 Immunolocalization of ALP in the trabecular bone and statistical analysis. *Upper line* (**a** 1 week, **b** 2 weeks, **c** 4 weeks) control group; *lower line* (**d** 1 week, **e** 2 weeks, **f** 4 weeks) 1,25(OH)$_2$D$_3$ administration group. Comparing to the control group (**a** 1 week, **b** 2 weeks, **c** 4 weeks), weaker expressions of ALP were showed in the 1,25(OH)$_2$D$_3$ administration group (**d** 1 week, **e** 2 weeks, **f** 4 weeks). **g** Mean optical density of ALP immunoreactivity were assessed in the trabecular bone for both groups (n = 10; **p* < 0.01). All corresponding numerical information is found in the results section. *Error bars* indicate ±SD. *SD* standard deviation; **a–f** ×200

upregulated the expression of interferon-beta (IFN-beta), which are key regulators of osteoclast formation (Takasu et al. 2006; Sakai et al. 2009). In addition, our previous studies reported that local administration of calcitriol promoted osteogenesis and mineralization, which could be promising for the restoration of mandibular bone defects (Feng et al. 2015; Liu et al. 2015). More interestingly, in thyroparathyroidectomized rats infused with parathyroid hormone (PTH), systemically administered calcitriol inhibited PTH-induced bone resorption at a physiological dose and stimulated bone resorption at a toxic dose (Ueno et al. 2003). Overall, these results indicate that calcitriol may function by stimulating or inhibiting bone resorption, depending on the dose and route of administration.

Vitamin D is a moderator of osteoclastic activity and not just an inducer of osteoclastogenesis. The effects of vitamin D on osteoclastic activity has largely been established to be mediated via 1,25(OH)$_2$D$_3$ activity on osteoblasts, which regulates the secretion of the key osteoclastogenesis factor, RANKL. RANKL/OPG ratio is an index of osteoclastogenic stimulation (Tanaka et al. 2011) and plays a key role in the regulation of bone metabolism (Martin 2013). In this study, we found that high-dose 1,25(OH)$_2$D$_3$ obviously increased the expression of RANKL and decreased the expression of OPG. This change in RANKL/OPG ratio resulted in augmented osteoclast numbers and activities, accelerating trabecular bone remodeling. Such metabolic effects of active vitamin D were in line with a

Fig. 6 Immunolocalization of RANKL and OPG in the trabecular bone and statistical analysis. *Upper line* (**a** 1 week, **b** 2 weeks, **c** 4 weeks, **g** 1 week, **h** 2 weeks, **i** 4 weeks) control group; *lower line* (**d** 1 week, **e** 2 weeks, **f** 4 weeks, **j** 1 week, **k** 2 weeks, **l** 4 weeks) 1,25(OH)$_2$D$_3$ administration group. Weak expression of RANKL in the control group (**a** 1 week, **b** 2 weeks, **c** 4 weeks), while stronger expression of RANKL were showed in the 1,25(OH)$_2$D$_3$ administration group (**d** 1 week, **e** 2 weeks, **f** 4 weeks). Comparing to the control group (**g** 1 week, **h** 2 weeks, **i** 4 weeks), weaker expression of OPG were observed in the 1,25(OH)$_2$D$_3$ administration group (**j** 1 week, **k** 2 weeks, **l** 4 weeks). M and N: mean optical density of RANKL and OPG immunoreactivity were assessed in the trabecular bone for both groups, respectively (n = 10; **$p < 0.01$). All corresponding numerical information is found in the results section. *Error bars* indicate ±SD. *SD* standard deviation. **a–l** ×200

previous report that 1,25(OH)$_2$D$_3$ promoted the expression of RANKL in osteoblasts in vitro (Takahashi et al. 2014).

Recent studies revealed the importance of EphB4 and EphrinB2 during the "coupling" of osteoblast and osteoclast function in bone remodeling. Activation of EphB receptors by the ephrinB ligands is referred to as "forward signaling," whereas activation of the ephrinB ligands by the EphB receptors is designated "reverse signaling." (Zhao et al. 2006; Allan et al. 2008; Irie et al. 2009). The osteogenic potential of osteoblasts was enhanced by forward signaling via EphB4, while the differentiation and function of osteoclasts was inhibited by reverse signaling through EphrinB2 (Zhao et al. 2006). In the present study, we demonstrated that the expressions of both EphrinB2 and EphB4 were downregulated in tibia of the VD group, with decreased bone mass and increased osteoclastic activities compared with the control group. Supportive studies demonstrated that inhibition of EphrinB2/EphB4 signaling using a blocking peptide (TNYL) or a decoy soluble form

of EphB4 (sEphB4) caused the inhibition of mineralization of human mesenchymal stem cells (Arthur et al. 2011) and mouse bone marrow stromal cells (Allan et al. 2008; Martin et al. 2010) in vitro. Moreover, increased expressions of EphB4 and EphrinB2 by genistein, an isoflavone that can bind to and activate estrogen receptors, improved glucocorticoid-induced osteoporosis (Cheng et al. 2015). Taken together, EphrinB2/EphB4 signaling may play an important role in the reduction of bone mass associated with high dose of vitamin D administration.

In conclusion, our results suggest that long-term administration of high-dose 1,25(OH)$_2$D$_3$ during bone development, even a maximal physiological dose, can increase osteoclast numbers and activities, and decrease bone formation through the RANKL/RANK/OPG and EphrinB2/EphB4 signaling pathways, subsequently impairing bone metabolism. Optimal dosages for vitamin D supplementation for its positive effects on bone metabolism, without causing side effects, need to be determined.
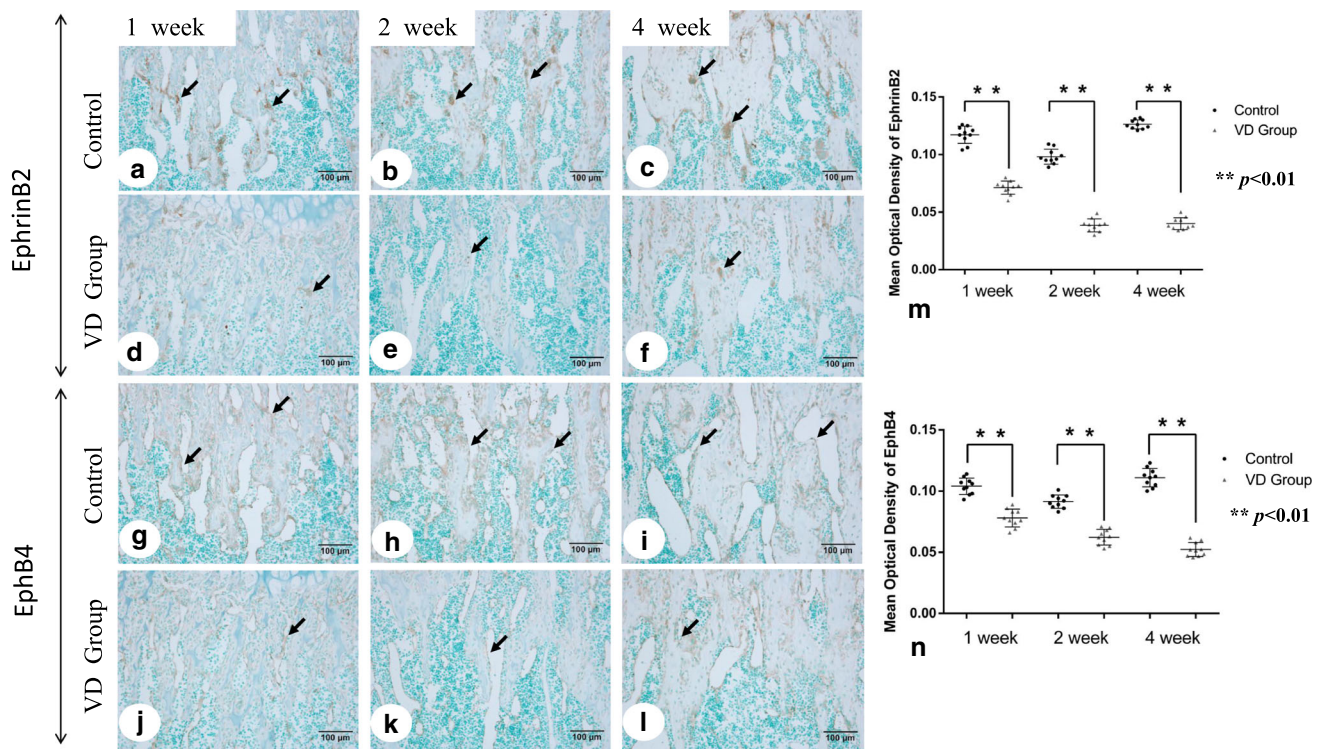
Fig. 7 Immunolocalization of EphrinB2 and EphB4 in the trabecular bone and statistical analysis. *Upper line* (**a** 1 week, **b** 2 weeks, **c** 4 weeks, **g** 1 week, **h** 2 weeks, **i** 4 weeks) control group; *lower line* (**d** 1 week, **e** 2 weeks, **f** 4 weeks, **j** 1 week, **k** 2 weeks, **l** 4 weeks) $1,25(OH)_2D_3$ administration group. Intense expression of EphrinB2 of the control group (**a** 1 week, **b** 2 weeks, **c** 4 weeks), while weak expression of EphrinB2 (**d** 1 week, **e** 2 weeks, **f** 4 weeks) in the $1,25(OH)_2D_3$ administration group. Intense expression of EphB4 of the control group (**g** 1 week, **h** 2 weeks, **i** 4 weeks), while weak expression of EphB4 (**j** 1 week, **k** 2 weeks, **l** 4 weeks) in the $1,25(OH)_2D_3$ administration group. M and N: mean optical density of EphrinB2 and EphB4 immunoreactivity were assessed in the trabecular bone for both groups, respectively (n = 10; **$p < 0.01$). All corresponding numerical information is found in the results section. *Error bars* indicate ±SD. *SD* standard deviation. **a–l** ×200
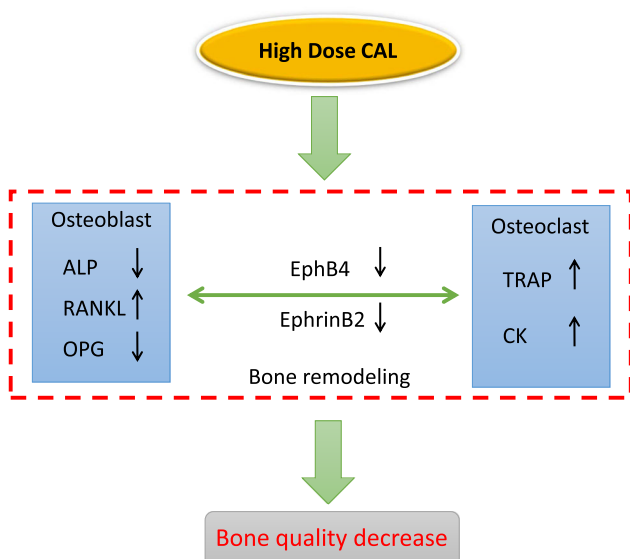
Fig. 8 Schema of high-dose $1,25(OH)_2D_3$ affects bone remodeling in young growing rats

Conflict of interest We declare that we have no conflict of interest.

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