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# The effect of alendronate on the expression of osteopontin and osteoprotegerin in calcified aortic tissue of the rat

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ABSTRACT

Vascular calcification is a pathobiological process which leads to high morbidity and mortality in cardiovascular disease. The association between vascular calcification and osteoporosis has been reported widely, and there are close relationships among vascular calcification, related cardiovascular disease and osteoporosis, but the biochemical mechanism of vascular calcification is presently unclear. For exploring the possible mechanism of artery calcification we established aorta calcification in an animal model with vitamin $D_3$ and warfarin and tested the effect of alendronate on the expression of osteopontin and osteoprotegerin in calcified aorta tissue of the rat through measuring gene and protein expression of osteopontin and osteoprotegerin respectively. The results indicated compared with control group, the aortic calcium content of calcification group was obviously increased, osteopontin mRNA and osteoprotegerin mRNA were significantly reduced, and osteoprotegerin and osteopontin protein expressions were reduced. Compared with calcification group, the aortic calcium content of alendronate group was obviously reduced, osteopontin mRNA and osteoprotegerin mRNA were significantly increased, and osteopontin and osteoprotegerin protein expression were increased. We conclude that artery calcification may reduce the expression of osteopontin and osteoprotegerin. Alendronate may inhibit rat aorta calcification by up-regulating osteopontin and osteoprotegerin expression.



## 1. Introduction

Vascular calcification is a pathobiological process which leads to high morbidity and mortality in cardiovascular disease. Four types of vascular calcification have been identified with at least some distinct properties: intimal atherosclerotic plaque calcification, tunica media calcification, cardiac valve calcification, and vascular calciphylaxis. Tunica media calcification, also referred to as Mönckeberg's sclerosis, is associated with aging, diabetes, and chronic kidney disease (Davies and Hruska, 2001). Vascular calcification had previously been thought of as a passive, degenerative process, but now there is strong evidence to support the concept that arterial calcification is, at least in part, an active process that is associated with the key factors, including transcription factors (Msx2, Runx2, Osterix) (Towler et al., 1998), bone morphogenetic proteins, osteopontin, matrix γ-carboxyglutamic acid protein and osteoprotegerin (Collin-Osdoby, 2004). Alendronate is one of bisphosphonates which are used for the treatment of bone resorption, hypercalcemia, osteoporosis and Paget's disease for many years. It is worth noting that alendronate is not only used for treating the aforesaid diseases but may also be used for treating vascular calcification (Price et al., 2001). Price et al. (2001) have reported that alendronate inhibited artery calcification at doses comparable to those that inhibit bone resorption. Mori et al. (1998) reported that alendronate dose-dependently inhibited bovine vascular smooth muscle cells calcification. Their experimental results indicate that there are at least in part some relationships between artery calcification and osteoporosis, but concrete mechanism is unclear. Osteopontin, which is abundant at sites of medial calcification (Ahmed et al., 2001; Shanahan et al., 1999), inhibits hydroxyapatite crystallization in vitro and calcification in cultured vascular smooth muscle cells (Jono et al., 2000; Wada et al., 1999). Bennett et al. (2006) recently showed that osteoprotegerin inactivation accelerates vascular calcification in apoE$^{(-/-)}$ mice. Furthermore, the osteoprotegerin-null mouse develops medial calcification of the aorta and renal arteries (Bucay et al., 1998). However, little evidence was obtained from experiments focusing on the effect of alendronate on the expression of osteopontin and osteoprotegerin in the course of ameliorate vascular calcification. So, we created a rat calcification model during the short experimental period to study the effect of alendronate on expression of osteopontin and osteoprotegerin in medial calcification of the rat aorta, and explore possible mechanism of inhibiting artery calcification by alendronate.

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## 2. Materials and methods

### 2.1. Animals

Male Sprague–Dawley (SD) rats weighing 120–150 g were supplied by Animal Center, Tongji Medical College, Huazhong University of Science and Technology. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and is approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology.

### 2.2. Preparation of rat vascular calcification model

Rat vascular calcification model were induced by treatment with vitamin $D_3$ and warfarin (Johnson et al., 2006; Price et al., 2000). One hundred twenty rats were randomly divided into control group ($n=20$), calcification group ($n=20$) and 4 alendronate groups (each group consists of 20 rats). From the first day to the eighth day, alendronate group was treated with daily subcutaneously injection of alendronate (Sigma, USA); the doses of the 4 groups were 0.01 mg/kg/d, 0.05 mg/kg/d, 0.1 mg/kg/d and 0.5 mg/kg/d respectively. On the fifth day calcification group and alendronate group were subcutaneously injected with warfarin (15 mg/100 g per 12 hours for 4 days; Sigma, USA) and 1,25-dihydroxyvitamin $D_3$ (vitamin $D_3$, 300,000 U/kg per day for 3 days; Sigma, USA). The control group was given daily subcutaneous injection of normal saline. All injections were administered subcutaneously into the backs of the animals. All rats received doses of 1.5 mg vitamin $K_1$ (Sigma, USA) per 100 g of body weight for preventing bleeding at 24 and 48 hours before the first warfarin dose. On the ninth day all rats were anesthetized intraperitoneally with sodium pentobarbital (45 mg/kg), and the aortas (from the arch to the renal arteries) were removed in a sterile manner and frozen at −80 °C for subsequent studies.

### 2.3. von Kossa stain of aortic calcification

The selected tissue samples were placed in formalin and processed for dehydrating paraffin embedding. Five-micrometer thick sections were cut in transverse sections. Mineral deposition was assessed by von Kossa staining (6% silver nitrate), as previously described (Qi et al., 2003).

### 2.4. Real-time quantitative PCR

Total RNA was isolated from the aortas using TRIzol reagent according to the manufacturer's instructions (Invitrogen, USA) and reverse transcribed into cDNA with a Toyoba reverse transcription kit (Toyoba, Japan). The real-time quantitative PCR was carried out with ABI PRISM 7900 sequence detector system (Applied Biosystems, Foster City, Canada) according to the manufacturer's instructions. GAPDH was used as endogenous control. PCR reaction mixture contained the SYBR GREEN I (Takara, Japan), cDNA, and the primers. The sequences of primers for real-time quantitative PCR were used: osteopontin (5′-CCAGCACACAAGCAGACGTT-3′ and 5′-TCAGTCCATAAGCCAAGCTATCAC-3′), osteoprotegerin (5′-TCACTGGGCTGTTTCTTCAG-3′ and 5′-TCCTCTTTCTCAGGGTGCTT-3′), and GAPDH (5′-GAAGGGCTCATGACCACAGT-3′ and 5′-GGATGCAGGGATGATGTTCT-3′). Relative gene expression level (the amount of target, normalized to endogenous control gene) was calculated using the comparative Ct method formula 2-ΔΔCt.

### 2.5. Western blotting

Aorta tissue protein was electrophoresed on a 10% SDS-polyacrylamide gel and then transblotted onto nitrocellulose membranes. Membranes were treated overnight with TBS-Tween/5% dry milk and incubated with goal anti-rat osteopontin antibody at 1:300 dilution (Santa Cruz, USA) and goal anti-rat osteoprotegerin antibody at 1:250 dilution (Santa Cruz, USA), after washing the blot was incubated with horseradish peroxidase-conjugated rabbit anti-goal secondary antibodies for 2 hours at room temperature. Chemiluminescent detection was performed using ECL (Amersham Biosciences) followed by autoradiography.

### 2.6. Measurement of calcium content in rat aorta

The aorta tissues were dissolved in $HNO_3$ and then dried in an oven and redissolved with blank solution (27 nmol/L KCl, 27 μmol/L $LaCl_3$ in deionized water). Calcium content was measured using an atomic absorption spectrophotometer (SpectrAA-240FS, USA) at 422.7 nm (Wu et al., 2003).

### 2.7. Statistical analysis

The results are shown as mean ± S.E.M. The significance of differences was estimated by ANOVA followed by Student–Newmann–Keuls multiple comparison tests. $P<0.05$ was considered statistically significant. All statistical analyses were performed using SPSS software (version 11.0, SPSS Inc., Chicago, IL).

## 3. Results

### 3.1. von Kossa stain of aortic calcification

Representative photographs of von Kossa staining were shown in Fig. 1. von Kossa staining showed no calcification can be detected in untreated control animals (A), and there were strongly positively-stained black areas in the animals treated with warfarin and vitamin $D_3$ (B, black arrowhead). There was less calcification in the animals treated with warfarin and vitamin $D_3$ plus 0.1 mg/kg/d alendronate (C, green arrow head) (original magnification 400×). In the calcified aorta, calcification was mainly located in the elastic fibers of the media layer.

### 3.2. Osteopontin and osteoprotegerin expression in rat aorta

Osteopontin and osteoprotegerin mRNA expression were quantitatively analyzed by real-time quantitative PCR, and osteopontin and osteoprotegerin protein expression were quantitatively measured by western blotting. As shown in Fig. 2, Osteopontin mRNA expression was consistent with fgl2 protein expression. Osteopontin expression in calcification group was significantly reduced as compared with those in control group ($^*P<0.05$), compared with calcification group, osteopontin exhibited a higher expression level in all alendronate groups ($\#P<0.05$). Furthermore, alendronate up-regulated osteopontin expression in a dose-dependent manner ($\S P<0.05$). There was a significant decrease in osteoprotegerin expression of calcification group when compared with control group ($^*P<0.05$). There was a significant increase in osteoprotegerin expression of all alendronate groups when compared with calcification group ($\#P<0.05$). In addition, osteoprotegerin expression was elevated by alendronate in a dose-dependent manner ($\S P<0.05$) (Fig. 3).

### 3.3. Calcium content in rat aorta

As illustrated in Fig. 4, calcium content in rat aorta was significantly increased in calcification group as compared with control group ($^*P<0.05$), and significantly decreased in all alendronate groups as compared with calcification group ($\#P<0.05$), and alendronate reduced calcium content in rat aorta in a dose-dependent manner ($\S P<0.05$).

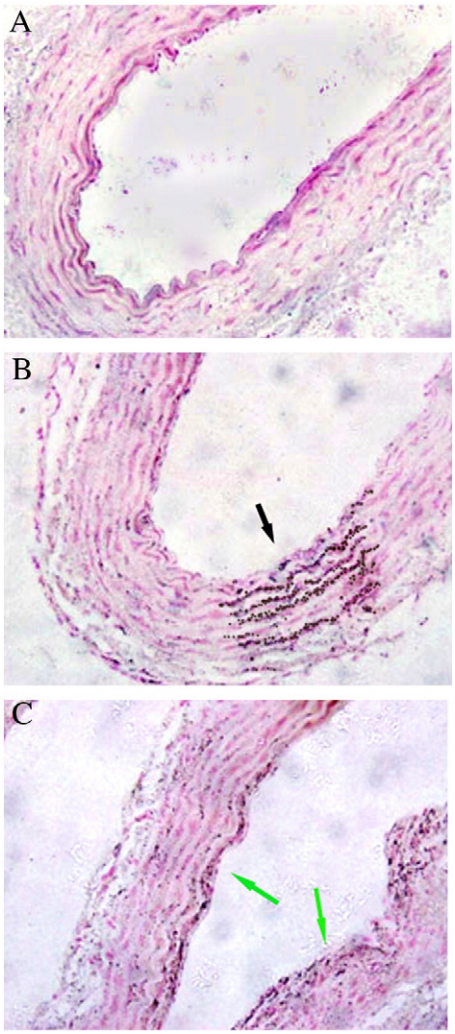


**Fig.1.** von Kossa staining for the aorta of the rat.Representative photographs of von Kossa staining of control animals (A), calcification animals (B black arrowhead) and animals treated with alendronate (0.1 mg/kg/d) (C, green arrowhead) (original magnification 400×).

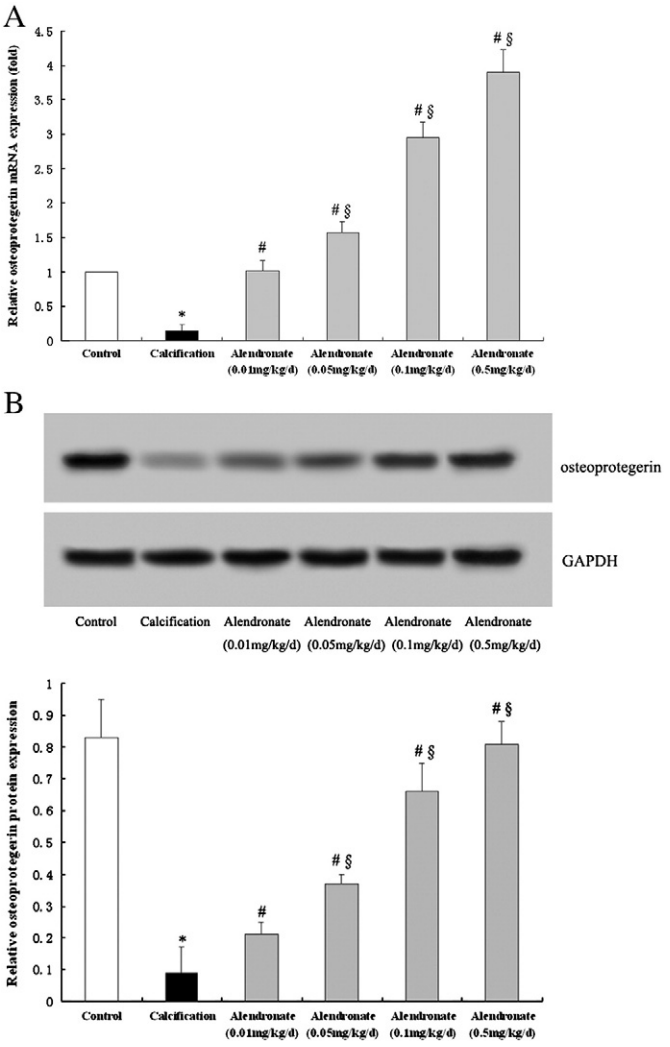


**Fig.2.** Osteopontin expression in rat aorta.Osteopontin mRNA and protein expression were analyzed respectively by real-time quantitative PCR and western blotting. Osteopontin expression in calcification group was significantly decreased as compared with control group; alendronate up-regulated osteopontin expression as compared with calcification group. (A) Real-time quantitative PCR. (B) Western blotting. Data are expressed as mean ± S.E.M. *$P<0.05$ compared with control; #$P<0.05$ compared with calcification; §$P<0.05$ compared with alendronate (0.01 mg/kg/d).

## 4. Discussion

This is the first report that alendronate inhibits media calcification through up-regulating expression of osteopontin and osteoprotegerin. The probable process of vascular calcification is that diabetes and end-stage renal disease rapidly induce adventitial inflammation, oxidative stress, and macrophage and T cell infiltration; and it up-regulates expression of TNF-α, bone morphogenetic protein (BMP) 2, Msx2, and gene expression. BMP2 and Msx2 expression occurs in adventitial pericytic myofibroblasts (Shao et al., 2006), medial vascular and smooth muscle cells and enhances adventitial myofibroblast (osteoprogenitor) migration, proliferation, medial thickening, and matrix metalloproteinase (MMP)-dependent matrix turnover (Vattikuti and Towler, 2004).

Clinical and experimental studies have consistently established an association between vascular calcification and bone resorption (Kiel et al., 2001). Alendronate is a drug widely applied to the treatment of bone resorption through the specific biochemistry process which interferes with the analepsia, differentiates function of osteoclast and causes to its apoptosis at last. Recently, some evidence indicates that vascular calcification and osteoporosis frequently occur together and share many of the same risk factors (e.g. aging, inflammatory disease, glucocorticoid use, chronic renal failure, or estrogen deficiency) (Hofbauer and Schoppet, 2004). However, Price et al. (2000, 2001) showed that alendronate inhibited arterial calcification and cardiac valve calcification after 2 and 4 weeks in a warfarin-induced ectopic calcification model. Therefore, a hypothesis is proposed that vascular calcification is linked to osteoporosis. This hypothesis is supported by the fact that both artery calcification and osteoporosis increase dramatically in women after menopause; a prospective study on 2662 postmenopausal women from Denmark demonstrated that advanced aortic calcification was significantly associated with lower bone mineral density and rapid bone loss of the proximal femur (Bagger et al., 2006). Similar finding was obtained from Bucay et al. (1998), which showed that there was an association between early-onset osteoporosis and medial artery calcification in the osteoprotegerin-deficient mouse.

The possibility of alendronate inhibiting artery calcification is that the inhibiting effect of alendronate on bone resorption lowers the concentration of calcium and/or phosphate in blood and thereby reduces the tendency of mineral nuclei to form and grow in the artery wall. Besides, another possibility is the hypothesis we proposed in this experiment, that alendronate could activate osteopontin and osteoprotegerin which

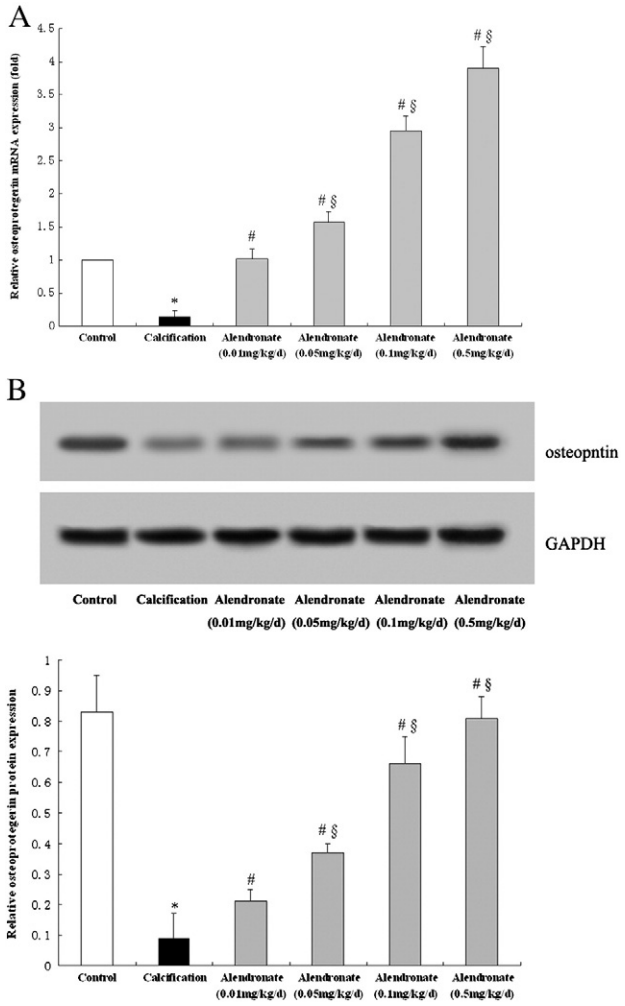


**Fig.3.** Osteoprotegerin expression in rat aorta.Osteoprotegerin mRNA and protein expression was measured respectively by real-time quantitative PCR and western blotting. There was a significant decrease in osteoprotegerin expression of calcification group when compared with control group. There was a significant increase in osteoprotegerin expression of all alendronate groups when compared with calcification group. (A) Real-time quantitative PCR. (B) Western blotting. Data are expressed as mean ± S.E.M. $^{*}P<0.05$ compared with control; $\#P<0.05$ compared with calcification; $\S P<0.05$ compared with alendronate (0.01 mg/kg/d).

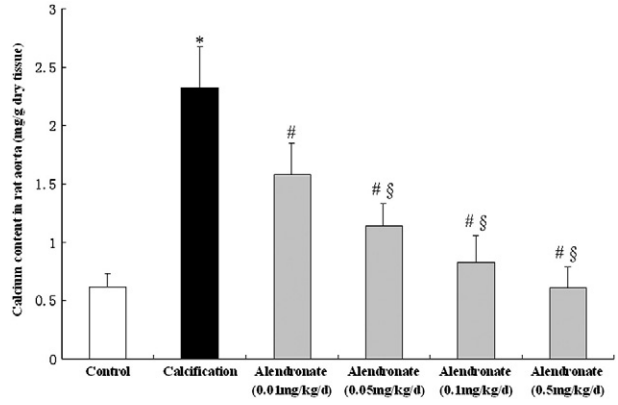


**Fig.4.** Calcium content in rat aorta.Calcium content in rat aorta was significantly increased in calcification group as compared with control group ($^{*}P<0.05$), and significantly decreased in all alendronate groups as compared with calcification group. Data are expressed as mean ± S.E.M. $^{*}P<0.05$ compared with control; $\#P<0.05$ compared with calcification; $\S P<0.05$ compared with alendronate (0.01 mg/kg/d).

may be potential inhibitors of vascular calcification. So, we designed the experiment.

Osteopontin is a non-collagenous, glycosylated phosphoprotein associated with biomineralization in osseous tissues (Lomashvili et al., 2004), as well as vascular calcification. Speer et al. (2002) have demonstrated that inactivation of the osteopontin gene enhances vascular calcification of matrix Gla protein-deficient mice as an inducible inhibitor of ectopic calcification in vivo, and that elevated phosphate stimulates smooth muscle cell phenotypic transition and mineralization via the activity of a sodium-dependent phosphate cotransporter. Osteopontin, however, appears to block vascular calcification most likely by preventing calcium phosphate crystal growth and inducing cellular mineral resorption, and the administration of exogenous osteopontin could reverse calcification partly to these calcified tissue (Giachelli et al., 2005). Similar findings findings have been reported in several vivo systems; for example, osteopontin inhibits calcification in cultured vascular smooth muscle cells (Jono et al., 2000; Wada et al., 1999).

Our experimental results agree that osteopontin is an inhibitor of medial calcification. As shown in the Fig. 2 osteopontin mRNA and protein expression in calcification group are significantly reduced as compared with the control group ($^{*}P<0.05$). Alendronate increased osteopontin expression in all alendronate groups compared with calcification group ($\#P<0.05$).

Osteoprotegerin is tumor necrosis factor receptor superfamily, member 11b (Tnfrsf11b) (Cundy et al., 2002), also called osteoclastogenesis inhibitory factor (OCIF). Bucay et al. (1998) demonstrated that targeted deletion of osteoprotegerin in mice results in severe, early-onset osteoporosis, and that loss of osteoprotegerin also results in calcification of the aorta and renal arteries, sites of endogenous osteoprotegerin expression in normal animals. This finding suggested that regulation of osteoprotegerin, its signaling pathway, or its ligand(s) may play a role in the long observed association between osteoporosis and vascular calcification. Similar findings have been reported by Price et al. (2001); they have reported that osteoprotegerin can inhibit vascular calcification induced by warfarin and vitamin $D_3$ in vivo. From understanding of the molecular mechanisms, the osteoblasts express and secrete the tumor necrosis factor-like molecule, RANK-L (receptor activator of nuclear factor kB ligand) in response to various hormonal, cytokine and mechanical stimuli. RANK-L binds to its receptor RANK (receptor activator of nuclear factor kB), expressed on the surface of osteoclast precursors. The binding of RANK-L to RANK initiates the differentiation of these precursors to mature, active osteoclasts. At the same time osteoblasts also secrete a decoy receptor, osteoprotegerin, which acts as an inhibitor of bone resorption by binding to RANK-L, preventing activation of osteoclast precursors through RANK (Theoleyre et al., 2004). Animal experiments indicate that imbalances in this system can cause osteoporosis or/and arterial calcification. For instance, osteoprotegerin-deficient mice exhibit a phenotype of osteoporosis (Bucay et al., 1998), and two thirds of the animals also display medial calcifications of the aorta and the renal arteries that can be successfully treated by an osteoprotegerin transgene delivered during midgestation (Min et al., 2000).

Our experimental results just show the relationship between osteoprotegerin and arterial calcification. Osteoprotegerin mRNA and protein in calcification group were decreased as compared with control group ($^{*}P<0.05$), and alendronate as a drug of osteoporosis inhibited arterial calcification. As shown in Fig. 3, osteoprotegerin mRNA and protein in all alendronate groups were increased as compared with calcification group ($\#P<0.05$). Besides, alendronate decreased calcium content of rat aorta in all alendronate groups as compared with calcification group ($\#P<0.05$), and alendronate reduced calcium content in rat aorta in a dose-dependent manner ($\S P<0.05$).

Extrapolating these results to the clinical settings, not only alendronate may be used to prevent and treat media calcification, but

also osteoprotegerin and osteopontin may be used to antagonize media calcification in the future, just like that AMGN-0007 (a recombinant osteoprotegerin construct) is developed as a potential therapeutic agent in the treatment of bone disease (Body et al., 2003). Our findings support the potential relevance to vascular calcification in human disease but uncertain, because the experiments were very short term. Further research is required to clarify the effect of alendronate on vascular calcification in human disease. It is worth noting that it is very important that patients with diabetes, end-stage renal disease and with risk factors of vascular calcification should get prophylactic therapy in early stage of disease, because it is very difficult to dissolve hydroxyapatite and eject it out of patients after the formation of hydroxyapatite crystal.

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