

Osteoprotegerin/receptor activator of nuclear factor- κ B ligand are involved in periodontitis-promoted vascular calcification

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Abstract. The present study explored the potential role of osteoprotegerin (OPG)/receptor activator of nuclear factor- κ B (RANK)/receptor activator of nuclear factor- κ B ligand (RANKL) in promoting vascular calcification by periodontitis. Thirty-six male Wistar rats were randomly assigned to four groups to establish animal models as follows: the sham group (group C), vascular calcification group (group VDN), periodontitis group (group CP), and test group (group CP+VDN). After eight weeks, all the rats were sacrificed. The periodontal and vascular calcification indices were detected. Quantitative polymerase chain reaction (qPCR), immunohistochemistry, western blot analysis, and enzyme-linked immunosorbent assay (ELISA) were used to quantify OPG/RANK/RANKL expression in vascular tissue and serum. Protein expression analyses revealed the expression of OPG and RANKL in the vascular tissues of the four groups. The expression of OPG in group C was the highest, which was similar to group CP+VDN, and the expression of OPG in groups CP and VDN were lower. However, the expression of RANKL was inversely correlated with OPG, and the ratio of RANKL/OPG was also higher in groups CP and VDN than that in groups C and CP+VDN. In conclusion, OPG/RANK/RANKL may play an essential role in the promotion of vascular calcification by periodontitis. However, the expression levels of OPG and RANKL were not simply superimposed when periodontitis and vascular calcification co-existed.

Introduction

Periodontal disease causes chronic inflammation of the supporting tissues around teeth. Some reports (1-3) have demonstrated that periodontal disease and cardiovascular disease (CVD) are closely related. However, the mechanism of their occurrence is still unclear. Periodontal disease is associated with the development of early atherosclerotic carotid lesions (4). In addition, low-grade chronic inflammation plays an important role as a pathogenetic determinant of atherosclerosis (AS) in the development of CVD (5). Vascular calcification is a universal clinical sign of AS and is also a special expression of soft tissue calcification induced by chronic inflammatory factors (6). We believe that periodontitis can promote vascular calcification. Previous animal experiments have demonstrated that periodontitis promotes vascular calcification (7); however, the mechanism is still unclear.

Osteoprotegerin (OPG)/receptor activator of nuclear factor- κ B (RANK)/receptor activator of nuclear factor- κ B ligand (RANKL) are new members of the tumor necrosis factor (TNF) family, serving as crucial regulators of osteoclastogenesis and bone resorption, with a key role in periodontitis (8-11). They also play an essential role in vascular calcification (12-15). Compared to periodontal health, RANKL is upregulated, while OPG is downregulated in periodontitis, increasing the RANKL/OPG ratio (16). Recent research has also shown that OPG/RANK/RANKL are key molecules in the vascular system that inhibit vascular calcification and protect vascular endothelial cells (15). In addition, increased plasma levels of OPG in atherosclerotic subjects indicated that OPG might be involved in the development of AS (14). However, the potential role of OPG/RANK/RANKL in periodontitis-associated vascular calcification remains unknown.

The rat is an ideal choice for the preparation of a vascular calcification model, because the anatomical structure of alveolar bone and periodontal membrane are similar to that of human. Rats were selected as animal models in this study (17,18). In the present study, periodontitis and vascular calcification animal models were established to explore the expression of the OPG/RANK/RANKL system in vascular tissues of rats, and to identify the potential role of this system in the effect of periodontitis on vascular calcification.

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Abbreviations: OPG, osteoprotegerin; RANK, receptor activator of nuclear factor- κ B; RANKL, receptor activator of nuclear factor- κ B ligand; qPCR, quantitative polymerase chain reaction

Key words: osteoprotegerin, RANK, RANKL, animal models, vascular calcification, periodontitis

Materials and methods

Establishment of the animal models. Male Wistar rats, aged 8 weeks and weighing 200±20 g, were purchased from the Animal Center of Affiliated Hospital of Qingdao University (Qingdao, China) [license number: SCXK (lu)20090007]. All the animals were given humane care, conforming to the Animal Management Rule of the Ministry of Health (Beijing, China). After one week of adaptation, 36 male Wistar rats were randomly assigned to four groups (n=9): the sham group (group C), vascular calcification group (group VDN), periodontitis group (group CP), and test group (group CP+VDN). The rats in each group underwent treatments similar to previous studies to establish the animal models (7). The rats in group VDN received vitamin D3 plus nicotine as follows (19). The rats were given vitamin D3 [300,000 IU/kg in peanut oil (i.m.) and nicotine (25 mg/kg in 5 ml of peanut oil, p.o.)] (Sigma-Adrich; Merck KGaA) at 9:00 on the first day. The nicotine administration was repeated at 18:00 on the same day. Rats were placed in a supine position and anesthetized by intraperitoneal injection of 10% chloral hydrate at a dose of 300 mg/kg. Group CP was treated with silk ligation and local inoculation of periodontal mixed pathogen. Subgingival plaque of periodontitis patients was scraped and placed in a centrifuge tube containing PBS solution. The plaque was dispersed by whirlpool oscillator and cultured in BHI liquid medium for 5 days. The left and right maxillary second molar were ligated with 4-0 silk thread and placed in gingival sulcus as far as possible. Meanwhile, periodontal pathogens were inoculated locally (17,18,20). The treatment in the VDN+CP group consisted of silk-ligating and simultaneous treatment with vitamin D3 (300,000 IU/kg, i.m.) plus nicotine (25 mg/kg in 5 ml, p.o.). Group C was administered an intramuscular injection of normal saline solution and gavages of pure peanut oil to replace the vitamin D3 and nicotine treatments, respectively.

After 3 months, the periodontal tissue and vascular tissue of rats in each group were detected. The probing depth and gingival sulcus bleeding index of maxillary second molars were recorded.

Before euthanasia, all rats were fasted for 12 h and anesthetized by intraperitoneal injection of 10% chloral hydrate (300 mg/kg). No peritonitis was observed in the rats. Finally, the rats were sacrificed by cervical dislocation.

Vascular calcification examination

Hematoxylin and eosin (H&E) staining, calcium deposition in the aortas, and alkaline phosphatase (ALP) activity assay. The aorta was observed by H&E staining. Image Pro Plus 6.0 medical image analysis software (Media Cybernetics, Inc.) was used for semi-quantitative analysis of the immunohistochemical results. All slides were analyzed at the same magnification (x40) and light intensity. Measurement index: Average optical density (AOD)=IG225-iga, where A represents the measured gray value. A calcium assay kit (Nanjing Jiancheng Bioengineering Institute) was used to detect calcium in the cardiovascular tissues. The supernatants were assayed for ALP activity according to the manufacturer's instructions (21).

Reverse transcription-qPCR. Total RNA was extracted from rat aortic tissue by using Trizol reagent, and reversely transcribed into cDNA using the PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd.) as previously

described (7). Gene primers were ordered from Sangon Biotech Co., Ltd.. The forward and reverse PCR primers (rat) included: GAPDH forward, 5'-ACCACCAACTGCTTA GCCCC-3' and reverse, 5'-CATGGACTGTGGTCATGA GCC-3'; OPG forward, 5'-AAGTGGCTGTGCTGTGCA CTC-3' and reverse, 5'-CGGTTTCTGGGTCATAATGC AA-3'; and RANKL forward, 5'-AGCCTTTCAAGGGGC CGTGC-3' and reverse, 5'-GGGCCACATCGAGCCACG AA-3'. The PCR cycling conditions were as follows: one cycle at 95°C for 5 min, 39 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, and final extension at 72°C for 6 min. SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd.) was used to obtain the RT-qPCR product according to the manufacturer's instructions. Lightcycler 480 (Roche, Switzerland) was detected by qPCR using the 2^{-ΔΔC_q} method (22). Gene expression results were normalized by internal control GAPDH. Each sample was tested in triplicate and then averaged for the final analysis.

Immunohistochemistry. Immunohistochemistry of tissue slides was performed as described previously (23,24). The EnVision™ immunohistochemistry system was used to detect the expression of OPG and RANKL in aortic tissues. After conventional dewaxing, EDTA antigen retrieval was performed for 2 min, followed by adding 3% H₂O₂ to each slice for 10 min. After blocking, the slides were incubated with a primary anti-OPG antibody (dilution 1:100; cat. no. orb247239; Biorbyt) or a primary anti-RANKL antibody (dilution 1:100; cat. no. orb11190; Biorbyt) at 37°C for 1 h. Then the slices were rinsed with PBS three times for 5 min. The goat anti-rabbit secondary antibody (cat. no. DS-0004; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) was added to slices at 37°C for 30 min. Finally, the visualization signal was developed with a diaminobenzidine-(DAB)-peroxidase substrate for 1 min. The sections were counterstained with hematoxylin, dehydrated, made transparent, and finally sealed with neutral gum.

Western blot analysis. Aortic tissue samples were homogenized on ice using lysis buffer and centrifuged at 13,000 x g for 15 min at 4°C. The BCA assay was applied to determine protein concentrations. All the samples were prepared to the same concentration with loading buffer (5X) and, boiled for 5 min. Protein (30 μg) was separated by 12% SDS-PAGE and transferred to PVDF membranes. Next, the membranes were incubated with primary antibodies of OPG and RANKL (cat. nos. orb247239 and orb11190; Biorbyt) diluted 1:300 in 5% skimmed milk in TBST overnight at 4°C. Horseradish peroxidase-conjugated anti-rabbit IgG (cat. no. ZB-2306) was used to detect the bound antibody. After the membranes were rinsed, a Bio-Rad image analysis system (Quantity One, Bio-Rad Laboratories, Inc.) was used to quantify the band intensities by densitometry. The results were normalized against the β-actin protein purchased from Santa Cruz Biotechnology, Inc. Each experiment was repeated three times with each animal.

Measurement of serum OPG and RANKL concentration. Sandwich enzyme-linked immune-sorbent assay (ELISA) (DuoSet ELISA, R&D Systems, Inc.) was used to determine serum concentrations of OPG and RANKL in the collected serum samples. All the samples were tested in duplicate, and

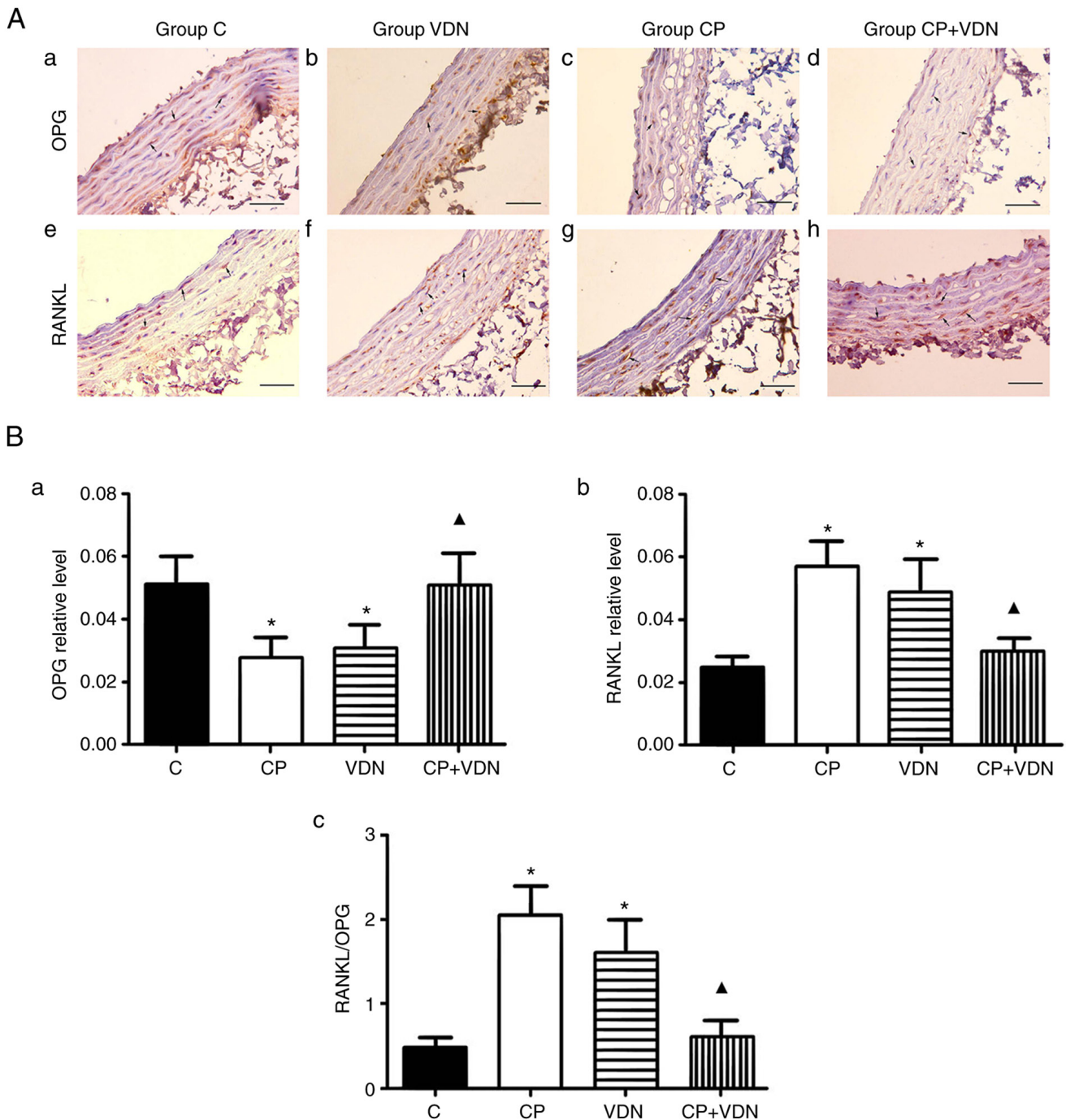


Figure 1. (A) (a-h) Representative vascular immunohistochemistry. OPG expression was higher in group C than in any other group. RANKL expression was higher in the other three groups, while the CP+VDN group exhibited the highest expression (black arrows indicate positive cells; scale bar, 50 μ m.). (B) (a) IOD/area value of OPG was calculated with Image-Pro Plus software. (b) IOD/area value of RANKL was calculated with Image-Pro Plus software. (c) Representative RANKL/OPG value. * $P < 0.05$, compared with groups CP and VDN; $\blacktriangle P > 0.05$, compared with group C (non-significant difference). Groups: the sham group (group C), vascular calcification group (group VDN), periodontitis group (group CP), and test group (group CP+VDN). OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor- κ B ligand.

then average values were calculated. The detection limit of OPG was 0.07 pmol/l. The intra-assay and inter-assay precision were ≤ 3 and $\leq 5\%$, respectively. The limit of detection for RANKL was 0.01 pmol/l. The intra-assay and inter-assay precision were ≤ 5 and $\leq 3\%$, respectively.

Statistical analysis. The data conformed to normal and the variance was uniform. The data are expressed as means \pm standard deviation (mean \pm SD). All the statistical analyses were

carried out using SPSS 19.0 (IBM Corp.). One-way analysis of variance (ANOVA) and Tukey's multiple comparison method were used to compare the groups. Statistical significance was defined at $P < 0.05$ for all tests.

Results

Periodontal and vascular tissues. H&E staining, calcium assay and ALP assay results were examined in our previous

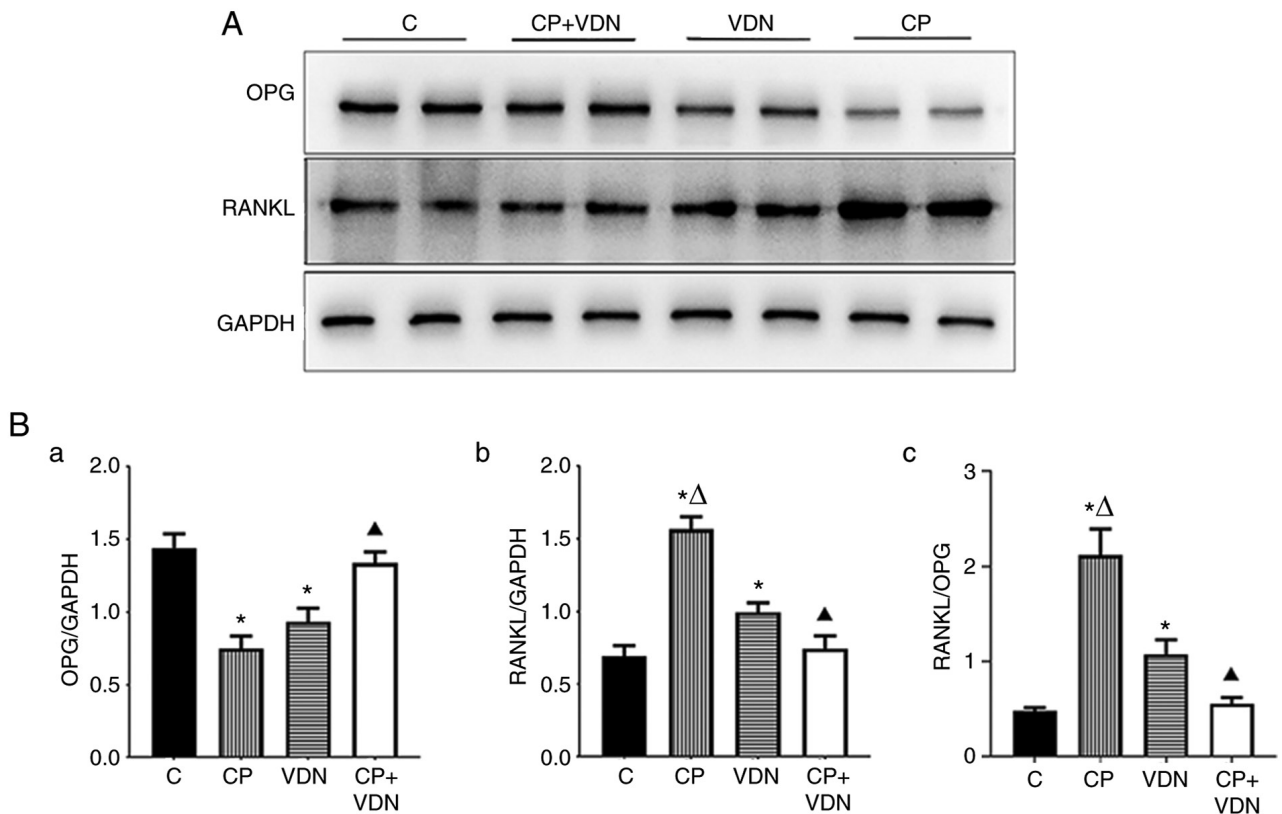


Figure 2. (A) Quantitative analysis of OPG and RANKL, using western blot analysis of the vascular tissue. (B) The relative levels of (a) OPG (b) RANKL and (c) RANKL/OPG value by western blot analysis. * $P < 0.05$, compared with groups C and CP+VDN; ▲ $P > 0.05$, compared with group C (non-significant difference); Δ $P < 0.05$, compared with group CP. Groups: the sham group (group C), vascular calcification group (group VDN), periodontitis group (group CP), and test group (group CP+VDN). OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor- κ B ligand.

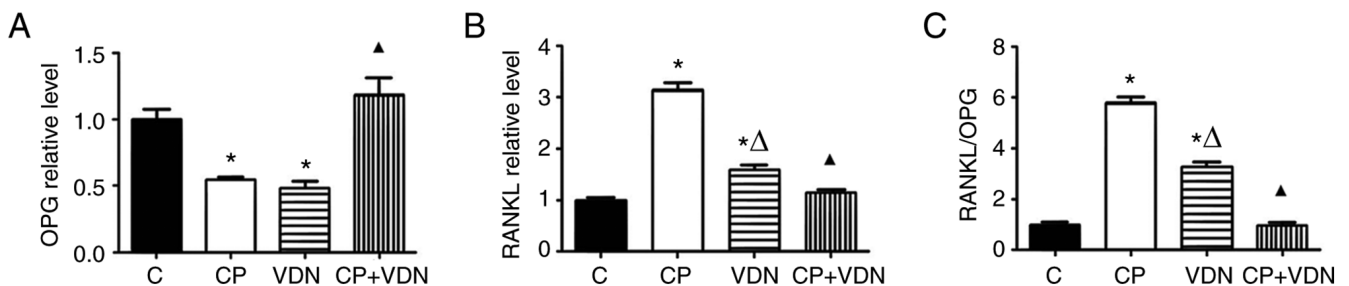


Figure 3. Expression of (A) OPG, (B) RANKL and (C) the ratio of RANKL/OPG by real-time qPCR. * $P < 0.05$, compared with groups C and CP+VDN; ▲ $P > 0.05$, compared with group C (non-significant difference); Δ $P < 0.05$, compared with group CP. Groups: the sham group (group C), vascular calcification group (group VDN), periodontitis group (group CP), and test group (group CP+VDN). OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor- κ B ligand.

study (7). Calcium content in group VDN and group CP+VDN were significantly higher than that in group C and group CP ($P < 0.05$), and the calcium content in group CP+VDN was higher than that in group VDN, and the difference was statistically significant ($P < 0.05$). The alkaline phosphatase activity of group CP, group VDN and group CP+VDN were higher than that of group C; the difference was statistically significant ($P < 0.05$), and the difference was statistically significant ($P < 0.05$) between CP+VDN group and CP and VDN group.

Expression of OPG/RANKL. The expression of OPG/RANKL in vascular tissues was detected by real-time qPCR,

immunohistochemistry, and western blot analysis, from mRNA to protein, from quantification to location.

Immunohistochemistry. OPG and RANKL were both expressed in the vascular tissues of the four groups with different degrees. OPG expression was stronger in group C than in groups CP and VDN; however, it was similar to that in group CP+VDN (Fig. 1Aa-d). Instead, RANKL expression was different from OPG, which was stronger in groups VDN and CP than in groups C and CP+VDN (Fig. 1Aa-h). IOD/area value of OPG and RANKL were calculated with Image-Pro Plus software (Media Cybernetics, Inc.; Fig. 1Ba and b); the OPG/RANKL value is presented in Fig. 1Bc. The value of

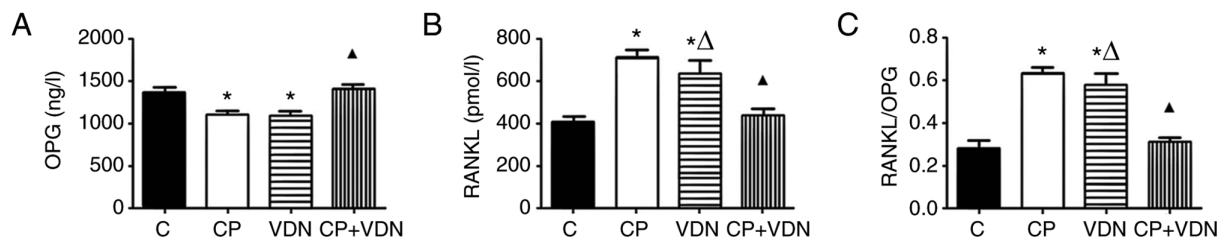


Figure 4. Expression of (A) OPG, (B) RANKL and (C) the RANKL/OPG ratio by ELISA. * $P < 0.05$, compared with groups C and CP+VDN; ▲ $P > 0.05$, compared with group C (non-significant difference); Δ $P < 0.05$, compared with group CP. Groups: the sham group (group C), vascular calcification group (group VDN), periodontitis group (group CP), and test group (group CP+VDN). OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor- κ B ligand.

RANKL/OPG in the CP and VDN groups was higher than that in the C and CP+VDN groups, indicating that both periodontitis and vascular calcification could affect OPG/RANK/RANKL expression; however, when periodontitis was tested with vascular calcification, the effect was not simply superimposed.

Western blot analysis. OPG protein gray values in the C and CP+VDN groups were higher than that in the other groups (Fig. 2A and Ba). RANKL protein gray value and the value of RANKL/OPG (Fig. 2A and Bb and c) showed the contrast tendency compared to OPG. Comparisons between the four groups showed statistically significant differences.

Reverse transcription-qPCR. OPG expression in the C and CP+VDN groups was higher than that in the CP and VDN groups. However, OPG expression in the CP+VDN group was similar to that in the C group (Fig. 3A). The quantitative analysis revealed a significant decrease in RANKL expression levels in the aorta of groups C and CP+VDN compared with groups CP and VDN, with the highest expression in the CP group (Fig. 3B). The RANKL/OPG ratio was the highest in the CP group, with no significant difference between the C and CP+VDN groups ($P > 0.05$) (Fig. 3C).

ELISA. OPG expression in the C and CP+VDN groups was higher than that in CP and VDN groups, while its expression in the CP+VDN group was similar to that in group C (Fig. 4A). However, compared to the other two groups, RANKL expression level in groups C and CP+VDN was significantly lower, with the highest level in group CP (Fig. 4B). The RANKL/OPG ratio had a tendency similar to the expression of RANKL (Fig. 4C).

Discussion

Periodontal disease and cardiovascular disease (CVD) are closely related, but the mechanism is still unclear. Many studies have demonstrated that vascular calcification, a common occurrence in CVD, results in increased cardiovascular mortality (25). We previously found in animal experiments that periodontitis promotes vascular calcification (7). However, the mechanism is still unclear.

The present study investigated the relationship and mechanism between periodontitis and vascular calcification by establishing rat models of periodontitis and vascular calcification. There are different types of experimental periodontitis models. One of the most commonly used models is the rat

model of periodontitis induced by ligation, which results in continuous accumulation of calculus and plaque at the ligation site (26,27). Periodontal probing depth (PD), sulcus bleeding index (SBI), and hematoxylin and eosin (H&E) staining of periodontal tissue serve as indicators of periodontal disease. Periodontitis models were successfully established. Vitamin D3 combined with nicotine-induced vascular calcification in rats has the advantages of simple operation, high survival rate, and good reproducibility (19,28). The calcium assay and alkaline phosphatase (ALP) can serve as indicators of vascular calcification. The rat vascular calcification models were successfully established by the higher levels of calcium and ALP. As for the group CP+VDN, the symptoms of both periodontitis and vascular calcification were found which were more severe than any other group, indicating an interaction between periodontitis and vascular calcification.

Osteoprotegerin (OPG), a member of the tumor necrosis factor receptor family, is a soluble decoy receptor of receptor activator of nuclear factor- κ B ligand (RANKL), which is usually expressed by osteoblasts and inhibits bone resorption by binding to RANKL, thereby preventing RANKL from binding to RANK (29). The bone tissue destruction that occurs in periodontitis is regulated by the balance between OPG and RANKL levels (30). The expression of RANKL increases significantly in patients with chronic periodontitis compared to healthy individuals (31,32). There are also reports that OPG and RANKL have been detected in mice, consistent with the present study that RANKL-positive cells are present in healthy gingival tissue samples (33). These all indicate that RANKL and OPG play a key role in periodontitis.

The RANKL/RANK/OPG system has been extensively explored in vascular biology, which is an essential factor in balancing bone metabolism, in regulating the immune system, and in participating in vascular calcification (34). RANKL and OPG perform vital functions in osteogenic modulation of the vascular system. OPG can be produced by various tissues, including cardiovascular tissue, and is found in high concentrations in renal and aortic arteries. In contrast to OPG, RANKL is not usually detectable in the normal vascular system (14). By immunohistochemical staining, Kaden *et al* (35) found that the expression of OPG in the local calcified area was decreased, while the expression of RANKL was increased in the vascular tissue of atherosclerosis and calcified aortic valve stenosis. Bucay *et al* (36) found significant calcification in the renal artery and aortic media in OPG-knockout mice, indicating that OPG plays an important role in protecting the media of large vessels from calcification. Similar to the former research, OPG

protein and RNA were strongly expressed in the normal vasculature and weakly in vascular calcification. As for RANKL, which is also found at a low level in the normal vasculature, it was upregulated in vascular calcification. This also indirectly showed that OPG had a protective effect on blood vessels and an inhibitory effect on arterial calcification. In the vascular system of the periodontitis group, similar results were achieved as that in the vascular calcification group, suggesting that perhaps periodontitis affects the vascular system by OPG/RANK/RANKL, which might be the same as vascular calcification.

As to serum OPG and RANKL levels, the results were complex and even controversial. As shown in previous studies, high levels of serum OPG are associated with age, end-stage renal disease, high cardiovascular mortality, diabetes, coronary artery disease, and acute cerebral infarction (37-39). There are many reports that serum OPG is elevated in the supportive condition of AS (14,15,40). It has been suggested that the serum concentration of RANKL is the highest in ischemic cerebrovascular disease, acute myocardial infarction, and other acute vascular syndromes (41). The RANKL/OPG ratio was positively correlated with total coronary artery calcification, while OPG was negatively correlated with total coronary artery calcification. There was no significant correlation between RANKL serum concentration and the degree of coronary artery calcification (42). However, in this research, serum OPG and RANKL levels differed from other studies, with the same tendency to be expressed in the vessel, i.e., the OPG level was lower in CP and VDN groups, while the level of RANKL was higher.

However, no studies are available on the potential role of OPG and RANKL in the effect of periodontitis on vascular calcification. In addition, the mechanism of interaction between vascular calcification and periodontitis is unclear. Immunohistochemistry, western blot analysis, quantitative real-time qPCR, and ELISA were used to analyze OPG and RANKL in vascular tissues and serum of different groups to investigate whether OPG/RANK/RANKL is involved in the effect of periodontitis on vascular calcification. The RANKL/OPG ratio has also been studied. Although OPG/RANK/RANKL plays a key role in both periodontitis and vascular calcification, periodontitis promotes vascular calcification. The expression levels of RANKL and OPG were slightly different from the previous speculation. The OPG level in the group CP+VDN was not lower than that in the groups CP and VND, but the same as the sham group. RANKL in the CP+VDN group was not more highly expressed than that in group CP or VDN, but it was the same as that in the sham group. The same tendency was observed in vascular tissues and serum by four methods, and the RANKL/OPG ratio was the same.

The results were surprising and far different from the previous speculation, which may be related to several aspects. Firstly, vascular calcification is actively regulated by a variety of networks, including negative and positive regulators, activation of modulators or temporal expression, and multiple amplification or suppression of feedback loops that coordinate function, cell recruitment, survival, differentiation, and interactions with other cells or matrix molecules (43-45). OPG/RANK/RANKL may participate in several aspects of the above processes that govern vascular calcification. Secondly, the expression of RANKL and OPG might be affected by many factors, such as inflammatory cytokines. High levels of OPG are associated with insulin resistance and HbA1c level, as well as with

high inflammatory markers, such as fibrinogen, interleukin-6, and CRP (46). Periodontitis is a chronic infection with high inflammatory cytokine levels. Finally, in the VDN+CP group, the effect of periodontitis and vascular calcification on OPG/RANKL/RANK might be contrary and considerable, leading to the expression of OPG and RANKL, which was similar to the sham group. Although OPG has attracted much attention as a connecting molecule between the vascular system and bone, there is controversy concerning the role the OPG/RANK/RANKL system plays in cardiovascular diseases, and the mechanism of OPG/RANK/RANKL in the process of vascular calcification remains unclear. There is yet no consensus in opinion unifying the dichotomy of OPG in animal models and human studies (47). In atherosclerotic lesions, the elevated OPG level might be a response to the damage of blood vessels and persistent inflammatory processes. It is believed that the increased level of OPG might be a self-defensive and compensatory response against AS progression.

OPG/RANK/RANKL has been involved in the progression of vascular calcification. However, it is only one aspect of the complicated network and is regulated by many other factors. When periodontitis is involved, the progression is more complex.

In conclusion, taken together, RANKL/RANK/OPG may have important functions in the process in which periodontitis could promote vascular calcification. However, if periodontitis and vascular calcification co-exist, the expression levels of RANKL and OPG are not simply superimposed. If OPG or RANKL is used as a marker to indicate one disease, the effect of the other disease should be considered.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MJ and PZ contributed to the design and implementation of the experiment and the writing of the paper. XY and ML contributed to the design of the experiment. PS, YQ and KP put forward valuable suggestions on the design of the experiment and the revision of the paper, and supervised the implementation of the experiment. PZ and KP contributed to the data collection and guaranteed the authenticity of the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of the Affiliated Hospital of Qingdao University (approval

no. AHQU20140425). The study is consistent with the 1964 Declaration of Helsinki and its subsequent amendments or similar ethical standards.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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