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Original Article

Role of 1,25-dihydroxyvitamin D in alleviating alveolar bone loss and gingival inflammation in ligature-induced periodontitis

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Abstract: Objectives: The goal of this study was to assess if endogenous $1,25(OH)_2D$ deficiency enhanced, whereas exogenous $1,25(OH)_2D_3$ supplementation alleviated alveolar bone loss and gingival inflammation induced by ligature-induced periodontitis. Methods: A model of ligature-induced experimental periodontitis was generated in wild-type (WT) and Cyp27b1-knockout (KO) mice on a rescue diet (RD), and un-ligated genotype-matched littermates as control, or in WT mice on a normal diet (ND) with vehicle treatment or $1,25(OH)_2D_3$ treatment, and un-ligated WT littermates as control. Alveolar bone mass and turnover, T cell infiltration and inflammatory cytokines in gingival tissues were examined. Results: In WT mice, ligature-induced alveolar bone loss occurred by inhibiting alveolar bone formation. This was characterized by reduction of osteoblast numbers, alkaline phosphatase activity and type I collagen synthesis, as well as by augmentation of osteoclastic alveolar bone resorption and gingival inflammation, including increases of osteoclast numbers, inflammatory positive cells and up-regulation of mRNA expression levels of inflammatory cytokines. Alveolar bone destruction and gingival inflammation were more severe in diet-matched Cyp27b1-KO mice than in WT littermates on RD. Supplementation of exogenous $1,25(OH)_2D_3$ alleviated alveolar bone loss and gingival inflammation in ligated WT mice on ND, but those parameters did not reach levels observed in un-ligated WT ones. Conclusions: Endogenous $1,25(OH)_2D$ deficiency enhanced, whereas exogenous $1,25(OH)_2D_3$ supplementation alleviated alveolar bone loss and gingival inflammation induced by ligature-induced periodontitis.

Keywords: 1,25-dihydroxyvitamin D, periodontitis, alveolar bone loss, inflammation

Introduction

Periodontitis, a chronic infectious disease, is characterized by the dynamic balance between bacterial invasion and host defense. The inflammatory reaction caused by local bacteria and plaque results in periodontal pocket formation, loosening of tooth attachment and alveolar bone resorption, eventually causing tooth loss [1, 2]. It may even become an infectious focus and endanger human systemic health [3]. There is mounting evidence regarding the relationship between periodontitis and many chronic diseases, such as diabetes, cardiovascular and metabolic diseases. Therefore, exploring effective methods to prevent and treat periodontal disease is of great significance to promote human oral health and improve general health and the quality of life.

Vitamin D is a fat-soluble vitamin. The native animal form is cholecalciferol (also known as vitamin D_3). Vitamin D_3 needs to be hydroxylated twice to become activated. Firstly, it is transformed into 25 hydroxyvitamin D by the action of 25 hydroxylase (Cyp27a1) in liver, and then into 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) by the 1α-hydroxylase (Cyp27b1) in kidney. $1,25(OH)_2D_3$ then combines with the vitamin D receptor (VDR) in target tissues to exert biological functions [4, 5].

VDR is not only located in target organs related to calcium and phosphorus regulation, such as

intestine, bone, kidney and parathyroid gland, but is also widely expressed in almost all organs of the whole body [6]. In addition, Cyp27b1 is found not only in kidney, but also in more than ten organs outside the kidney [7]. Therefore, activated vitamin D exerts a role beyond the promotion of calcium and phosphorus homeostasis and bone health. Thus, it has been found that active vitamin D has a regulatory role in differentiation, proliferation and activation of immune and inflammatory cells [8]. In addition, recent evidence has been presented the association of aging-related systemic inflammation and inflammation-related diseases with vitamin D [8, 9]. Epidemiological studies have also found that certain VDR gene polymorphisms are associated with periodontal disease [10]. According to these observations, it is possible that vitamin D was potentially involved in periodontal homeostasis.

Evidence regarding the relationship between vitamin D and periodontitis has been provided by studies showing that patients with periodontitis have lower levels of serum 25(OH)D, and periodontitis patients [11-14] treated with vitamin D have less markers of inflammation. Vitamin D treatment has been reported to inhibit periodontal destruction in animal models of experimental periodontitis [15-17]. However, other studies in animals [18] found that vitamin D combined with conventional periodontal treatment did not have a positive effect on inflammation of the gingiva, or on alveolar bone when compared with traditional periodontal treatment alone. Furthermore, most interventional studies with vitamin D in human inflammatory diseases have proven to be inconclusive [8]. The actions and mechanisms of vitamin D in periodontal disease therefore remain elusive and need to be further explored.

To assess the action of the active form of vitamin D, i.e. $1,25(OH)_2D$, we and others developed a mouse model with absence of $1,25(OH)_2D$ by genetic deletion or "knockout" (KO) of the Cyp27b1 (1α-hydroxylase) [19, 20]. We have previously found that $1,25(OH)_2D$ possesses anti-osteoporotic properties through alleviating oxidative stress and cell senescence [21, 22]. Cyp27b1-KO mice also develop erosive osteoarthritis, defects in mandibular bone mineralization, and degradation in periodontal tissues [23, 24]. However, the action of the $1,25(OH)_2D$ on periodontitis and its related mechanisms were not fully clarified. Therefore, the current study further assessed the function of $1,25(OH)_2D$ on periodontal destruction and inflammatory markers using an experimental periodontitis mouse model in the absence or presence of the Cyp27b1 [i.e., in Cyp27b1-KO mice and in wild-type (WT) littermates, respectively] and before or after treatment with exogenous $1,25(OH)_2D_3$. Mouse phenotypes were analyzed by histology, histopathology, and molecular biology methods. We further measured the inflammatory indicators such as CD3 positive T cells, nuclear factor-κB p65, interleukins, tumor necrosis factor and matrix metalloproteinases because the above biomarkers were related with the severity of periodontitis [25].

Materials and methods

Animals

Experimental periodontitis was induced by inserting 5-0 silk thread ligatures around the maxillary second molars of the mice as previously described [26]. After 2 weeks of ligature, all mice were humanely sacrificed, and the phenotypes of their maxillae were analyzed. The animal use protocol has been reviewed and approved by the Animal Ethical and Welfare Committee (IACUC-2002016).

The generation of Cyp27b1-KO and WT mice was conducted as previously described [19]. After weaning, all mice were fed a rescue diet (RD) to normalize the serum calcium and phosphorus levels in Cyp27b1-KO mice. Ten-week-old sex-matched Cyp27b1-KO and WT littermates were used to explore whether $1,25(OH)_2D$ deficiency enhanced periodontitis. In WT mice, one group was ligated (n=5; WT+Ligature) and one group was un-ligated (n=5; WT+Sham), while in Cyp27b1-KO mice, one group was ligated (n=5; KO+Ligature) and one group was un-ligated (n=5; KO+Sham).

Ten-week-old female WT mice on a normal diet (ND) were used in the study to assess if treatment of exogenous $1,25(OH)_2D_3$ alleviated periodontitis. Animals were randomly assigned to three groups: 1) an un-ligated group injected intraperitoneally with vehicle (normal saline containing 1% ethanol) as a control group (n=5; Sham+Vehicle); 2) a ligation group injected intraperitoneally with vehicle (n=5; Ligature+Vehicle); 3) a ligation group supplemented with

$1,25(OH)_2D_3$ injected intraperitoneally at a dose of 0.3 μg/kg/d of $1,25(OH)_2D_3$ (Sigma, St. Louis, MO, USA) [27, 28] (n=5; Ligature+ $1,25(OH)_2D_3$). $1,25(OH)_2D_3$ was dissolved in ethanol and further diluted with 300 μL PBS to a final 0.9% concentration and injected daily from the first day after the ligation.

Mice were maintained in the pathogen-free situation. All experimental steps involved in this study were carried out in strict accordance with the guidelines of the Institute of experimental animals of Nanjing Medical University (approval number: IACUC-1802007).

Serum biochemical analyses

Serum calcium, phosphorus and $1,25(OH)_2D_3$ levels of mice were measured as previously described [29].

Microtomography (μCT)

Maxillae were isolated and analyzed using μCT based on previous methods [30]. For maxilla reconstruction, the ROI (region of interest) was in the region under the furcation roof and the root apex of the maxillary second molar. The parameter of BMD (bone mineral density) was measured in the ROI based on a previously described method [31].

Tissue samples and histology

The right maxillae were fixed and decalcified as previously described [32]. The specimen was then embedded in paraffin wax and 5-μm sections were harvested in the mesio-distal plane. Samples were routinely stained with H&E (hematoxylin and eosin), or total collagen, or ALP (alkaline phosphatase) or TRAP (tartrate-resistant acid phosphatase) as previously described [10]. Alveolar bone height loss (ABHL), which was measured between the roof of furcation and the crest of alveolar bone in the area of the second molar, was evaluated based on H&E staining. Alveolar bone volume, which was measured in the ROI, was evaluated based on total collagen staining. The gingivae of the left maxillary molars were dissected for RNA extraction.

Immunohistochemistry

Type I collagen (Col-I), CD3 and NF-κB p65 (nuclear factor-κB p65) were examined by immunohistochemistry as described previously [30]. Briefly, immuno-histochemical staining was carried out based on the avidin-biotin-peroxidase complex technique with affinity-purified rabbit anti-mouse Col-I, CD3 and NF-κB p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and with secondary antibody (biotinylated goat anti-mouse IgG, Sigma). Sections were incubated using the Vectastain Elite ABC reagent (Vector Laboratories).

Real-time RT-PCR

RNA was isolated from the gingivae of maxillary second molars with TRIzol reagent. RT-PCRs were performed based on a previous method [33], and the sequences of PCR primers were used as our previous study [30]. Briefly, reverse transcription reactions were conducted using the SuperScript First-Strand Synthesis System (Invitrogen). The specificity of primers was tested by conventional PCR, and then all primers were used for real-time RT-PCR quantitative analysis. After normalization of β-actin mRNA, results of the relative amount of mRNA were measured using SDS 7300 software.

Statistical analysis

Data are demonstrated as the mean ± SEM, and comparisons of differences among groups were conducted through a one-way ANOVA followed by Bonferroni's test. *P* values <0.05 were believed statistically significant.

Results

Increased alveolar bone loss in Cyp27b1-KO mice with ligature-induced periodontitis

The levels of serum calcium and phosphorus were normal, but the serum $1,25(OH)_2D$ level was undetectable in 12-week-old sham and ligated Cyp27b1-KO mice (**Figure 1A-C**). After 2 weeks of the sham ligation or ligature, maxillae from WT+Sham, WT+Ligature, KO-sham and KO-ligature mice were analyzed using μCT and histology. ABHL, BMD and total collagen area relative to tissue area were analyzed. The results showed that ABHL were significantly increased in both WT+ and KO+Ligature mice relative to genotype-matched sham mice and were increased more dramatically in KO+Ligature mice relative to WT+Ligature ones (**Figure 1F** and **1H**). Significant differences of ABHL in root furcation area were detected between

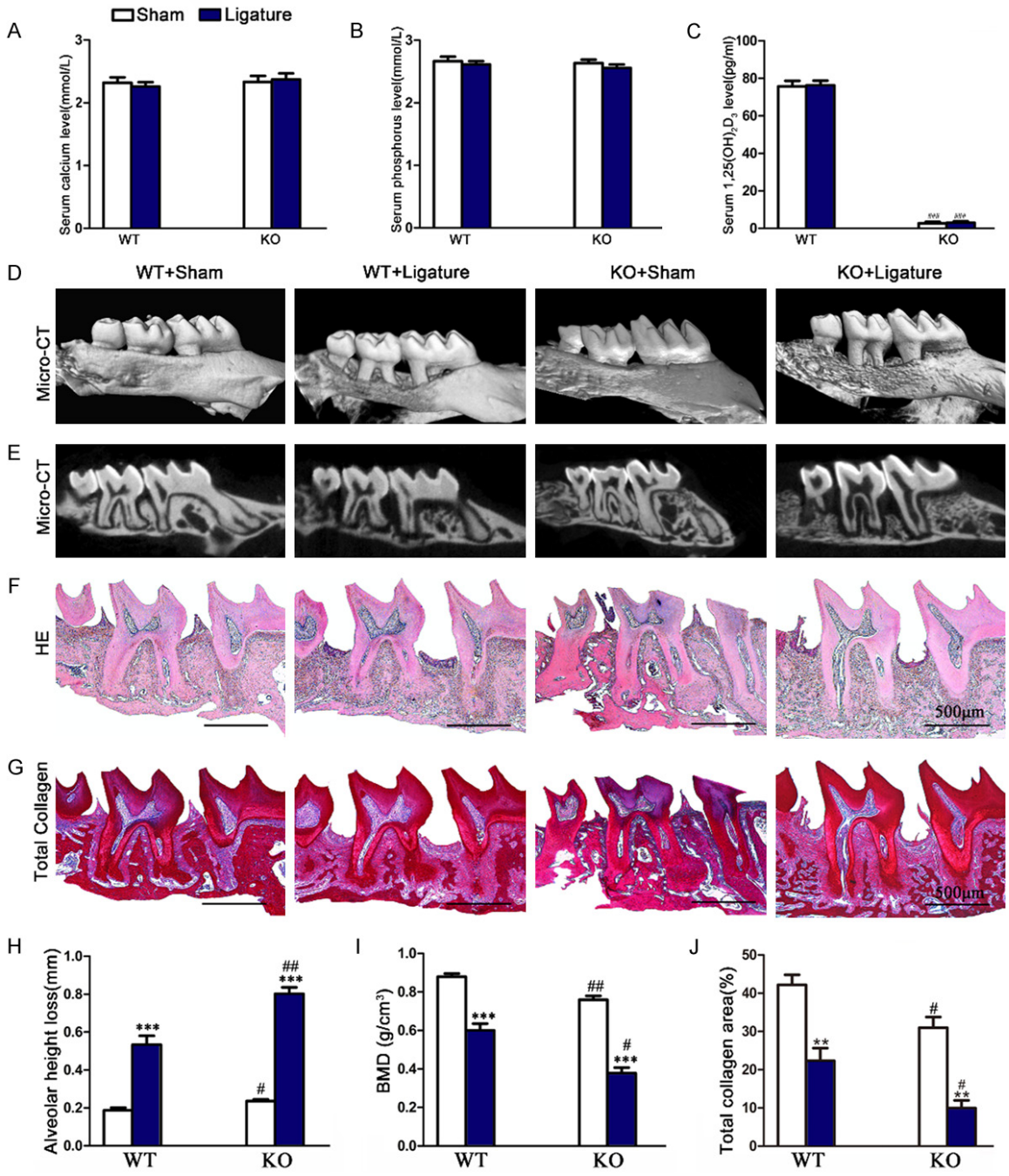

Figure 1. Increased alveolar bone resorption in ligature-induced Cyp27b1-KO mice. (A) Calcium, (B) phosphorus and (C) 1,25(OH)$_2$D$_3$ levels in serum. (D) 3D and (E) 2D Micro-CT reconstructed maxillae from WT-sham, WT-ligature, KO-sham and KO-ligature ones. (F) Images of maxillae stained with H&E. (G) Images of maxillae stained with total collagen. (H) Alveolar bone height loss (ABHL) which was measured as the distance between the roof of furcation and the crest of alveolar bone in the area of the second molar (M2) based on the H&E image. (I) BMD of ROI. (J) The percent collagen positive area in the alveolar bone of the M2. Values are mean ± s. e. m. **: P<0.01, ***: P<0.001, compared with genotype-matched sham mice; #: P<0.05, ##: P<0.01, compared with WT-sham or WT-ligature ones, respectively.

WT+ and KO+Sham mice. BMD and total collagen area were significantly reduced in both WT+ and KO+Ligature mice relative to genotype-matched sham mice and were reduced more dramatically in KO+Sham and KO+Ligature mice relative to WT+Sham and WT+Liga-

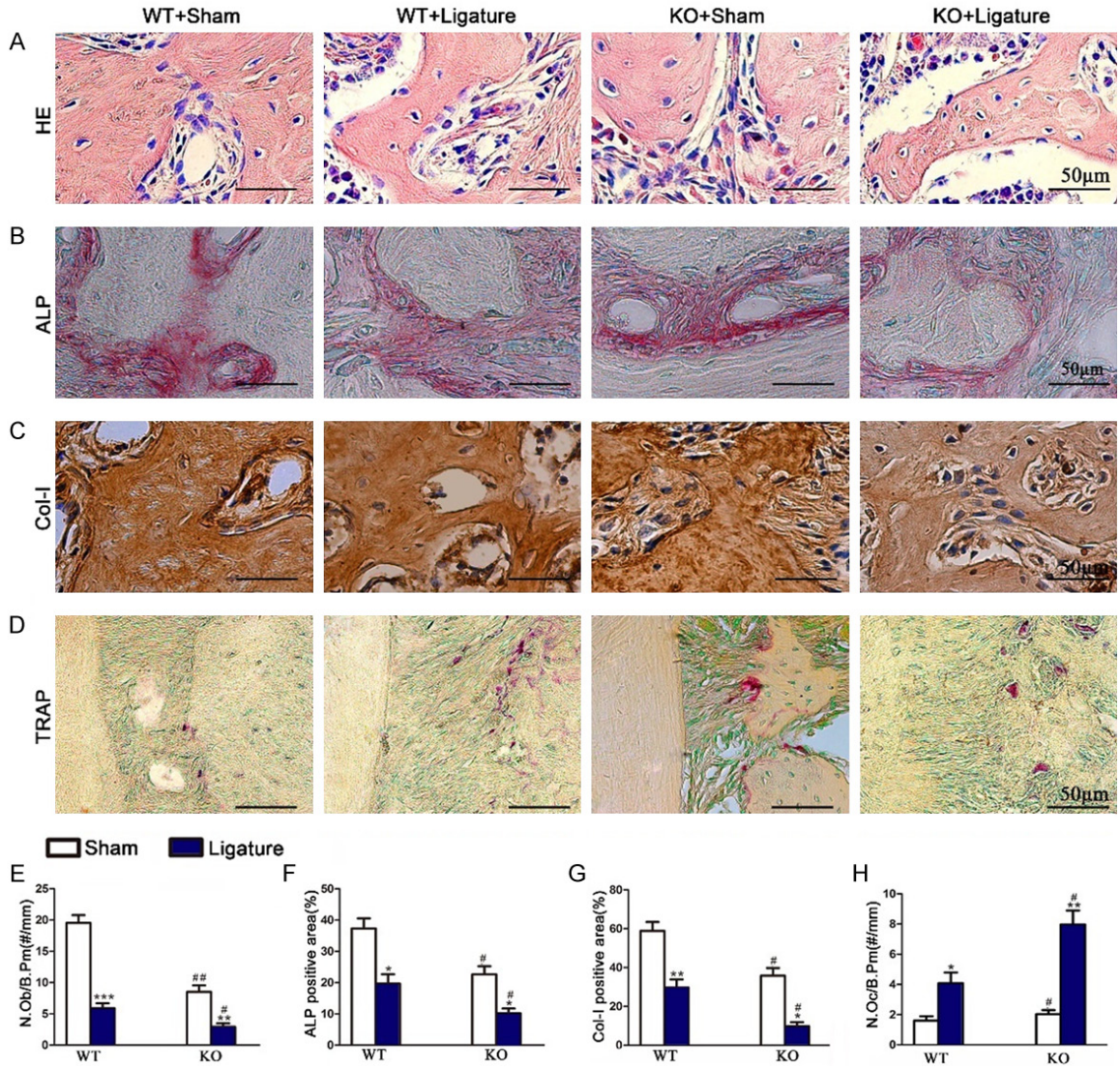

Figure 2. Changes of alveolar bone turnover in ligature-induced Cyp27b1-KO mice. A. Images of maxillae stained with H&E. B. Images of maxillae stained with ALP. C. Images of maxillae stained immunohistochemically with Col-I. D. Images of maxillae stained histochemically for TRAP. E. The osteoblast numbers per mm bone perimeter (N.Ob/B.Pm, #/mm) was measured based on H&E-stained images. F. ALP positive area. G. Col-I positive area. H. The number of TRAP-positive osteoclasts per mm bone perimeter (N.Oc/B.Pm, #/mm). Values are mean ± s. e. m. *: P<0.05, **: P<0.01, ***: P<0.001, compared with genotype-matched sham mice; #: P<0.05, ##: P<0.01, compared with WT-sham or WT-ligature mice, respectively.

ture mice, respectively (**Figure 1D**, **1E**, **1G**, **1I** and **1J**). These results demonstrated that ligature resulted in alveolar bone loss in both WT and KO mice, however, absence of 1,25(OH)$_2$D aggravated ligature-induced alveolar bone resorption.

Changes of alveolar bone turnover in Cyp27b1-KO mice with ligature-induced periodontitis

There were significant reductions of osteoblast numbers, ALP and type I collagen areas in both WT+ and KO+Ligature mice relative to genotype-matched sham mice, and in both KO+Sham and KO+Ligature mice relative to WT+Sham and WT+Ligature mice, respectively (**Figure 2A-C**, **2E-G**). In contrast, TRAP positive osteoclast numbers were significantly greater in both WT+ and KO+Ligature mice relative to genotype-matched sham mice, and also were significantly more in KO+Sham and KO+Ligature mice relative to WT+Sham and WT+Ligature mice, respectively (**Figure 2D** and **2H**). These results reveal that both ligature and

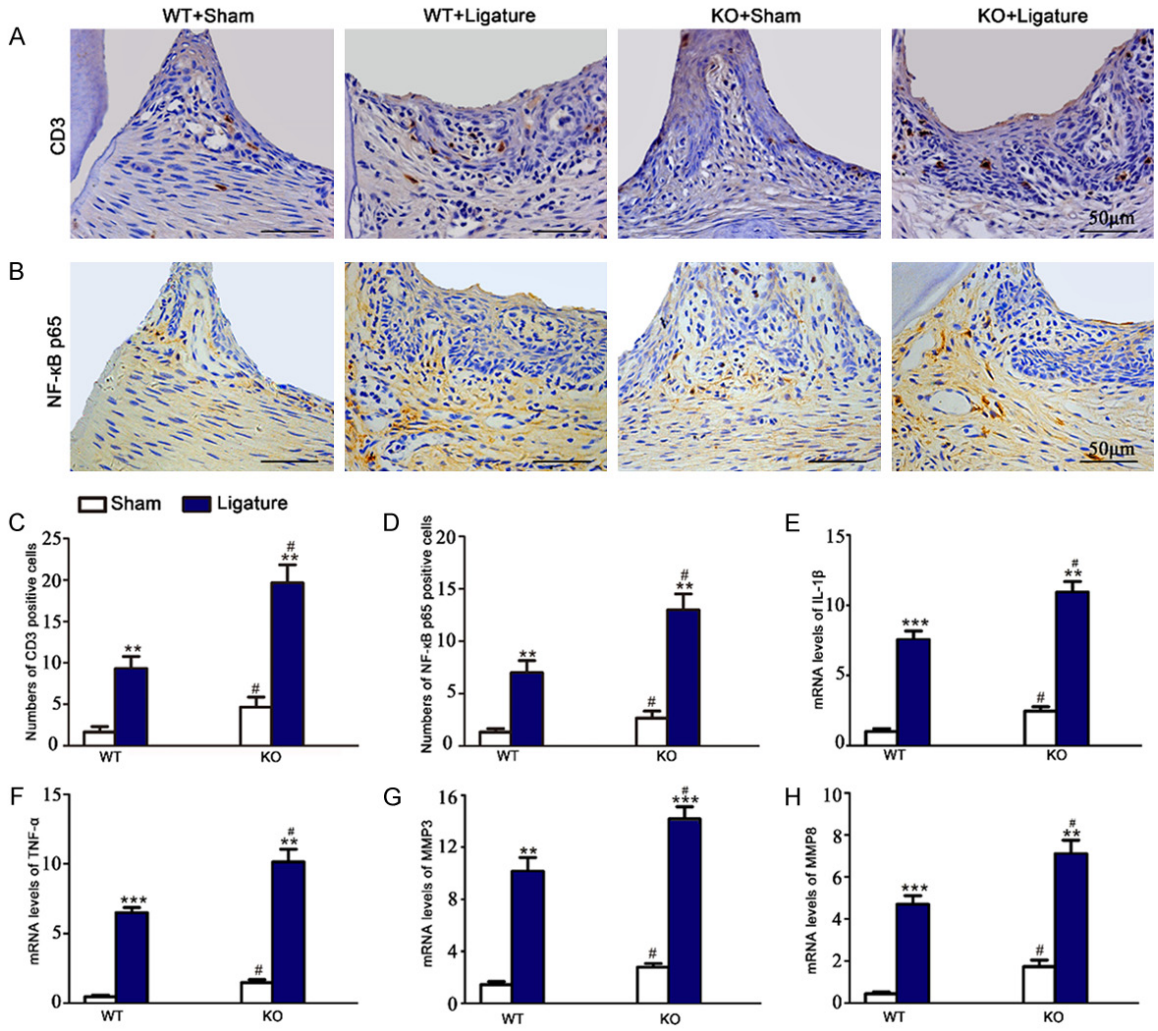

Figure 3. Enhanced T cell infiltration and proinflammatory cytokine-related genes in ligature-induced Cyp27b1-KO mice. A. Images of maxillary tissues stained immunohistochemically with CD3. B. Images of maxillary tissues stained immunohistochemically for NF-κB p65. C. The numbers of CD3 positive cells. D. The numbers of NF-κB p65 positive cells. E-H. Real-time RT-PCR was conducted on extracts of gingivae for the gene expression of *IL-1β*, *TNF-α*, *MMP3* and *MMP8*. Values are mean ± s. e. m. of 5 determinations per group. **: P<0.01, ***: P<0.001, compared with genotype-matched sham ones; #: P<0.05, compared with WT-sham or WT-ligature ones, respectively.

absence of 1,25(OH)$_2$D aggravated periodontal bone loss, which was related with inhibition of osteoblastic formation and augmentation of osteoclastic resorption.

Enhanced T cell infiltration and proinflammatory cytokine-related genes in Cyp27b1-KO mice with ligature-induced periodontitis

In order to explore the effect of 1,25(OH)$_2$D on periodontal inflammation, some proinflammatory cytokines in gingiva were measured. We found that the numbers of CD3$^+$ and NF-κB p65$^+$ cells, and mRNA expression levels of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), matrix metalloproteinases-3 and -8 (MMP3 and MMP8), were significantly higher in both WT+ and KO+Ligature mice relative to genotype-matched sham mice and were also significantly greater in KO+Sham and KO+Ligature mice relative to WT+Sham and WT+Ligature mice, respectively (**Figure 3**). These results indicate that alveolar bone destruction induced by ligature and aggravated by absence of 1,25(OH)$_2$D were associated with increased T cell infiltration and proinflammatory cytokine production.

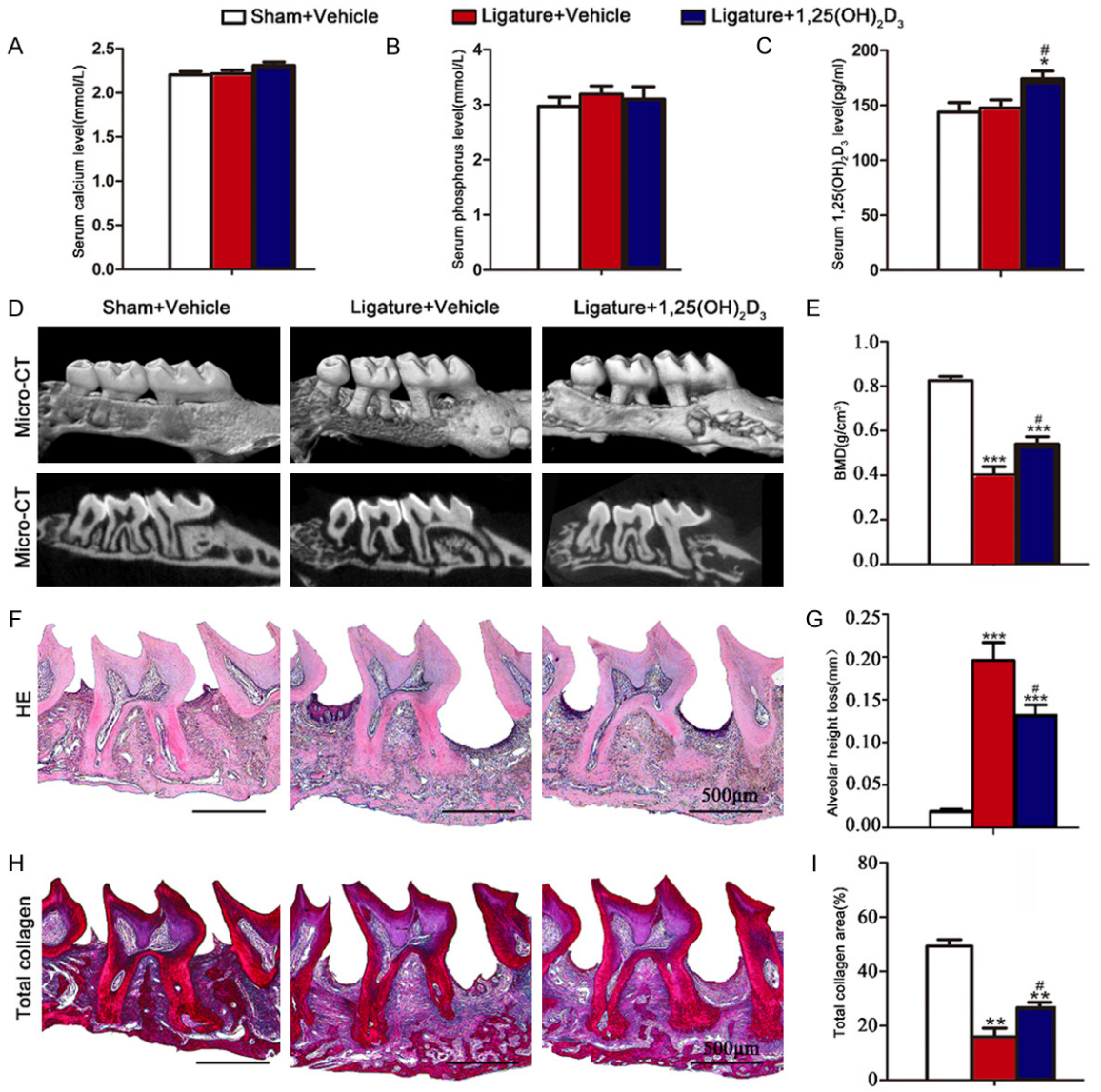

Figure 4. Alleviated alveolar bone resorption in ligature-induced mice treated with $1,25(OH)_2D_3$. A. Calcium levels in serum. B. Phosphorus levels in serum. C. $1,25(OH)_2D_3$ levels in serum. D. 3D and 2D Micro-CT reconstructed maxillae from Sham+Vehicle, Ligature+Vehicle and Ligature+$1,25(OH)_2D_3$ mice. E. BMD of ROI. F. Images of maxillae stained with H&E. G. Alveolar bone height loss (ABHL) which was defined as the distance between the roof of furcation and the crest of alveolar bone in the area of M2 based on H&E image. H. Images of maxillae stained with total collagen. I. Percent of collagen$^+$ area in the alveolar bone of the M2. Values are mean ± s. e. m. *: P<0.05, **: P<0.01, compared with Sham+Vehicle group; #: P<0.05, ##: P<0.01, compared with Ligature+Vehicle group.

Alleviated alveolar bone loss in the ligature-induced mice treated with $1,25(OH)_2D_3$

To assess the action of $1,25(OH)_2D_3$ supplementation on periodontal bone in mice with experimental periodontitis, 10-week-old WT mice on ND were un-ligated as sham control (Sham+Vehicle) or ligated with vehicle treatment (Ligature+Vehicle) or with $1,25(OH)_2D_3$ treatment (Ligature+$1,25(OH)_2D_3$). After 2 weeks, the levels of serum calcium, phosphorus and $1,25(OH)_2D$, ABHL, BMD and total collagen$^+$ area relative to tissue area were analyzed. We found that serum calcium and phosphorus were not altered, whereas the serum $1,25(OH)_2D$ level was increased significantly in 12-week-old ligated WT mice with $1,25(OH)_2D$ treatment when compared with sham or ligated WT mice with vehicle treatment (**Figure 4A-C**). ABHL was significantly enhanced in

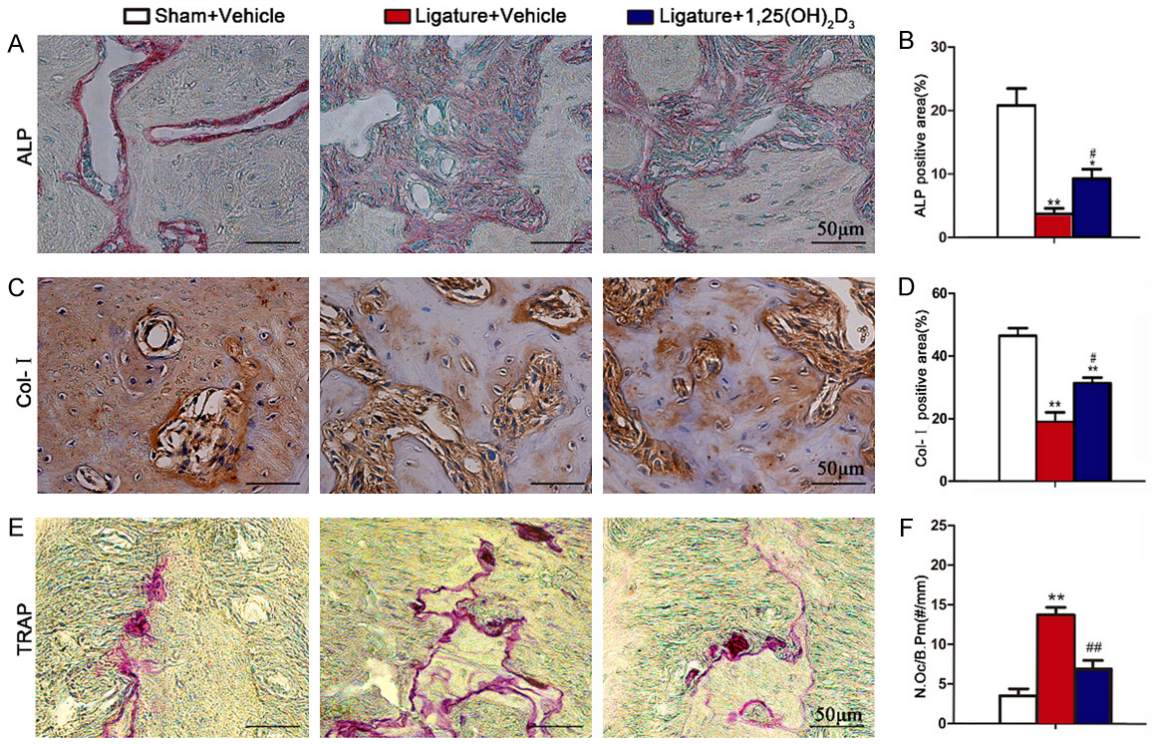

Figure 5. Changes of alveolar bone turnover in ligature-induced mice treated with $1,25(OH)_2D_3$. A. Images of maxillae stained with ALP. B. ALP positive area. C. Images of maxillae stained with Col-I. D. Col-I positive area. E. Images of maxillae stained with TRAP. F. The number of $TRAP^+$ osteoclasts per mm bone perimeter (N.Oc/B.Pm, #/mm). Values are mean ± s. e. m. *: P<0.05, **: P<0.01, compared with Sham+Vehicle group; #: P<0.05, ##: P<0.01, compared with Ligature+Vehicle group.

Ligature+Vehicle mice relative to Sham+Vehicle mice, but was significantly alleviated in Ligature+$1,25(OH)_2D_3$ mice relative to Ligature+Vehicle (**Figure 4F** and **4G**). BMD and total $collagen^+$ areas were significantly lower in Ligature-Vehicle mice relative to Sham+Vehicle mice, but were significantly higher in Ligature-$1,25(OH)_2D_3$ mice relative to Ligature+Vehicle (**Figure 4D**, **4E**, **4H** and **4I**). However, all parameters related to alveolar bone mass in Ligature+$1,25(OH)_2D_3$ mice did not reach the levels of Sham+Vehicle mice (**Figure 4D-I**). These results proved that $1,25(OH)_2D_3$ supplementation could alleviate alveolar bone degradation caused by ligature-induced periodontitis.

Changes of alveolar bone turnover in the ligature-induced mice treated with $1,25(OH)_2D_3$

We found that ALP and type I $collagen^+$ areas were significantly less in Ligature+Vehicle mice relative to Sham+Vehicle mice, but were significantly more in Ligature+$1,25(OH)_2D_3$ mice compared with Ligature+Vehicle mice (**Figure 5A-D**). In contrast, $TRAP^+$ osteoclast numbers were significantly greater in Ligature+Vehicle mice relative to Sham+Vehicle mice, but were significantly fewer in Ligature+$1,25(OH)_2D_3$ mice compared with Ligature+Vehicle mice (**Figure 5E** and **5F**). The parameters related to alveolar bone turnover in Ligature+$1,25(OH)_2D_3$ mice did not however reach the levels observed in Sham+Vehicle mice (**Figure 5**). Nevertheless, these results indicate that $1,25(OH)_2D_3$ might play a positive role in alveolar bone turnover.

Reduced T cell infiltration and proinflammatory cytokine-related genes in the ligature-induced mice treated with $1,25(OH)_2D_3$

We found that the numbers of CD3 and NF-κB p65 positive cells, mRNA levels, such as IL-1β, TNF-α, MMP3 and MMP8, were significantly greater in Ligature+Vehicle group relative to Sham+Vehicle group, however, they were significantly less in Ligature+$1,25(OH)_2D_3$ group relative to Ligature+Vehicle group. Nevertheless, they did not reach the levels observed in

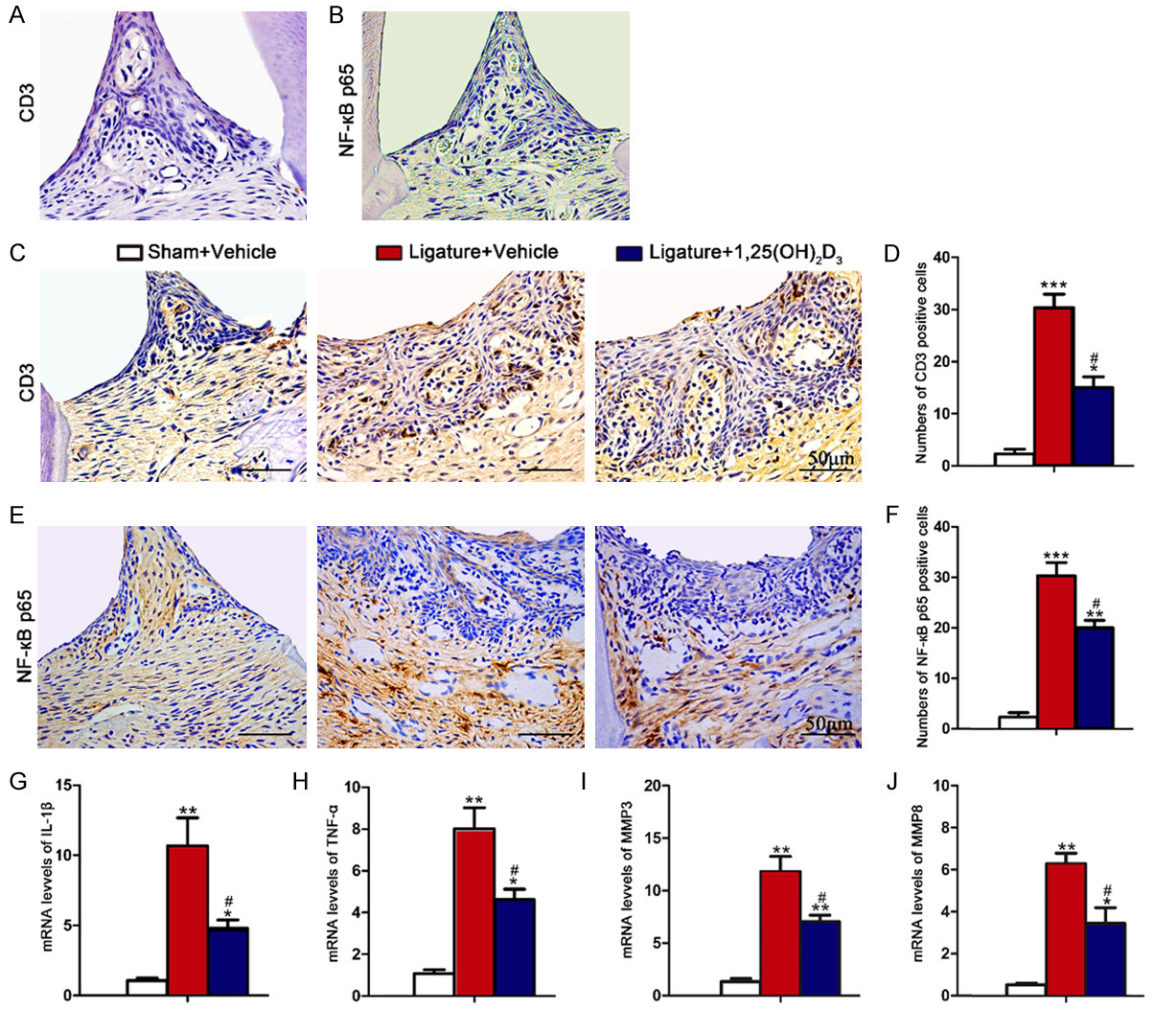

Figure 6. Reduced T cell infiltration and proinflammatory cytokine-related genes in ligature-induced mice treated with $1,25(OH)_2D_3$. A. A negative isotype control image of CD3. B. A negative isotype control image of NF-κB p65. C. Images of maxillary tissues stained with CD3. D. The numbers of CD3 positive cells. E. Images of maxillary tissues stained with NF-κB p65. F. The numbers of NF-κB p65 positive cells. G-J. Real-time RT-PCR was conducted on extracts of gingivae for the gene expression of *IL-1β*, *TNF-α*, *MMP3* and *MMP8*. Values are mean ± s. e. m. of 5 determinations per group. *: P<0.05, **: P<0.01, ***: P<0.001, compared with Sham+Vehicle group; #: P<0.05, ##: P<0.01, compared with Ligature+Vehicle group.

Sham+Vehicle group (**Figure 6**). The above results indicate that treatment with $1,25(OH)_2D_3$ could mitigate ligature-induced alveolar bone resorption by inhibiting inflammation.

Discussion

We recently reported that absence of $1,25(OH)_2D$ in mice could lead to periodontal bone resorption and gingival inflammation [30]. However, it is unclear whether loss of $1,25(OH)_2D$ function aggravates inflammatory bone loss in periodontitis. Therefore, we generated a model with ligure-induced periodontitis in WT and Cyp27b1-KO mice on RD and compared their maxillary phenotypes with genotyping-matched sham control ones. To exclude the effects of calcium and phosphorus, sham and ligated WT and Cyp27b1-KO mice were fed RD, and our results demonstrated that levels of calcium and phosphorus in serum were normal in sham or ligated WT and Cyp27b1-KO mice, whereas levels of $1,25(OH)_2D$ in serum were undetectable in both sham and ligated Cyp27b1-KO mice. The above results are in accordance with our other findings [22] and indicate that such mice can be used to investigate the deletion of $1,25(OH)_2D$ per se on

inflammatory bone loss in experimental periodontitis.

The mouse ligature-induced periodontitis model is thought to mimic human periodontitis [26]. In this study, we employed this model and demonstrated that in WT mice, bone loss after ligation was the result of inhibiting bone formation and enhancing bone resorption and inflammation in periodontium. Furthermore, inflammatory bone degradation was more severe in ligated mice deficient in $1,25(OH)_2D$. In view of our current studies demonstrating a pathogenetic role of vitamin D deficiency in ligature-induced periodontitis, even in the presence of normal serum calcium and phosphate, vitamin D deficiency might also be considered an independent risk factor for the development of periodontal disease [34]. This is supported by a systematic review and meta-analysis which revealed lower levels of 25(OH)D in serum in patients with chronic periodontitis compared with healthy controls [12].

It has been reported that in an experimental periodontitis mouse model, supplementation of 25(OH)D or $1,25(OH)_2D_3$ ameliorated periodontitis [15-17]. Short-term treatment of vitamin D_3 contributed to raised 25(OH)D levels in serum and improved periodontal attachment loss in patients with periodontitis who received nonsurgical periodontal therapy [35]. However, a recent report concluded that the action of vitamin D treatment as an adjunct to nonsurgical periodontal therapy is unclear because of the lack of available studies [12]. In the current study, we found that supplementation of $1,25(OH)_2D_3$ alleviated alveolar bone loss by stimulating periodontal bone formation and inhibiting osteoclastic alveolar bone resorption in ligated WT mice on ND. Furthermore, treatment of $1,25(OH)_2D_3$ mitigated gingival inflammation in ligature-induced periodontitis. We recently reported that exogenous $1,25(OH)_2D_3$ could improve osteoporosis due to natural aging or absence of $1,25(OH)_2D$ [21]. A previous study reported that $1,25(OH)_2D_3$ influenced proliferation and homing of T cell [36]. The expression intensity of NF-κB appears to increase with the severity of the lesion in gingivitis and periodontitis [37]. Vitamin D analogues can down-regulate NF-κB p65 nuclear translocation, and significantly mitigate the expression of pro-inflammatory chemokines, so as to inhibit the inflammatory response [38]. Human gingival epithelial cells could converse vitamin D to $25(OH)D_3$ and subsequently to $1,25(OH)_2D_3$, and topical use of both 25(OH)D or $1,25(OH)_2D_3$ resulted in inhibition of pro-inflammatory cytokine expression in mice [39]. Other studies also showed that $1,25(OH)_2D_3$ decreased NF-κB p65 phosphorylation, and expression levels of IL-1β protein in gingival epithelium [15]. Results from our current study suggest that $1,25(OH)_2D_3$ alleviated alveolar bone loss and gingival inflammation in part by inactivating NF-κB signaling pathway, supporting the anti-inflammatory role of $1,25(OH)_2D_3$.

To date, however, investigations concerning inflammatory and immune diseases treated with vitamin D were inconclusive in humans. Results from our ongoing studies using Cyp27b1 KO mice have demonstrated that although serum 25(OH)D level is not decreased in Cyp27b1 KO mice relative to WT ones [19], they display rickets, female infertility, male infertility, hypertension, premature aging, high incidence of multiple tumors and osteoporosis [19, 21, 22, 29, 33, 40-45]. These results suggest that sufficient $1,25(OH)_2D$ level rather than 25(OH)D level is necessary for the prevention of vitamin D deficiency-induced diseases. In human studies, as a result of declining renal function with age, a decrease in $1,25(OH)_2D$ production by approximately 50% has been reported [46]. In our recent study, we examined the protein expression levels of 1α-hydroxylase in kidney, intestine and bone of 3-, 9- and 18-month-old WT mice using Western blots and found that they were progressively down-regulated [41]. These results support the view that supplementation of vitamin D in the elderly cannot prevent the occurrence and development of aging related diseases, mainly because, at least renal 1α-hydroxylase expression is too low to synthesize sufficient $1,25(OH)_2D$. The prevalence of periodontitis is even higher in the elderly adult population. It has been approximated that roughly two-thirds of Americans 65 years of age and older have moderate to severe periodontitis [47]. Therefore, we suggest that $1,25(OH)_2D_3$ rather than vitamin D_3 should be supplemented in the population with periodontitis.

In conclusion, $1,25(OH)_2D$ deficiency can aggravated, whereas exogenous $1,25(OH)_2D_3$ supplementation alleviated bone loss and gingival inflammation caused by ligature-induced

periodontitis, supporting that absence of $1,25(OH)_2D$ might be believed a risk factor for the etiology of periodontal disease and $1,25(OH)_2D_3$ supplementation could be used as an adjunct of periodontal therapy.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

[1] Listgarten MA. Nature of periodontal diseases: pathogenic mechanisms. J Periodontal Res 1987; 22: 172-178.

[2] Page RC. The role of inflammatory mediators in the pathogenesis of periodontal disease. J Periodontal Res 1991; 26: 230-242.

[3] Genco RJ and Sanz M. Clinical and public health implications of periodontal and systemic diseases: an overview. Periodontol 2000 2020; 83: 7-13.

[4] Liao J, Ozono K, Sone T, McDonnell DP and Pike JW. Vitamin D receptor interaction with specific DNA requires a nuclear protein and 1,25-dihydroxyvitamin D3. Proc Natl Acad Sci U S A 1990; 87: 9751-9755.

[5] Dusso AS, Brown AJ and Slatopolsky E. Vitamin D. Am J Physiol Renal Physiol 2005; 289: F8-28.

[6] Norman AW. From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health. Am J Clin Nutr 2008; 88: 491S-499S.

[7] Townsend K, Evans KN, Campbell MJ, Colston KW, Adams JS and Hewison M. Biological actions of extra-renal 25-hydroxyvitamin D-1alpha-hydroxylase and implications for chemoprevention and treatment. J Steroid Biochem Mol Biol 2005; 97: 103-109.

[8] Colotta F, Jansson B and Bonelli F. Modulation of inflammatory and immune responses by vitamin D. J Autoimmun 2017; 85: 78-97.

[9] Goncalves de Carvalho CM and Ribeiro SM. Aging, low-grade systemic inflammation and vitamin D: a mini-review. Eur J Clin Nutr 2017; 71: 434-440.

[10] Wan QS, Li L, Yang SK, Liu ZL and Song N. Role of vitamin D receptor gene polymorphisms on the susceptibility to periodontitis: a meta-analysis of a controversial issue. Genet Test Mol Biomarkers 2019; 23: 618-633.

[11] Antonoglou GN, Knuuttila M, Niemela O, Raunio T, Karttunen R, Vainio O, Hedberg P, Ylostalo P and Tervonen T. Low serum level of 1,25(OH)2 D is associated with chronic periodontitis. J Periodontal Res 2015; 50: 274-280.

[12] Machado V, Lobo S, Proenca L, Mendes JJ and Botelho J. Vitamin D and periodontitis: a systematic review and meta-analysis. Nutrients 2020; 12: 2177.

[13] Khammissa RAG, Ballyram R, Jadwat Y, Fourie J, Lemmer J and Feller L. Vitamin D deficiency as it relates to oral immunity and chronic periodontitis. Int J Dent 2018; 2018: 7315797.

[14] Meghil MM, Hutchens L, Raed A, Multani NA, Rajendran M, Zhu H, Looney S, Elashiry M, Arce RM, Peacock ME, Dong Y and Cutler CW. The influence of vitamin D supplementation on local and systemic inflammatory markers in periodontitis patients: a pilot study. Oral Dis 2019; 25: 1403-1413.

[15] Li H, Zhong X, Li W and Wang Q. Effects of 1,25-dihydroxyvitamin D3 on experimental periodontitis and AhR/NF-kappaB/NLRP3 inflammasome pathway in a mouse model. J Appl Oral Sci 2019; 27: e20180713.

[16] Li H, Xie H, Fu M, Li W, Guo B, Ding Y and Wang Q. 25-hydroxyvitamin D3 ameliorates periodontitis by modulating the expression of inflammation-associated factors in diabetic mice. Steroids 2013; 78: 115-120.

[17] Wang Q, Li H, Xie H, Fu M, Guo B, Ding Y, Li W and Yu H. 25-Hydroxyvitamin D3 attenuates experimental periodontitis through downregulation of TLR4 and JAK1/STAT3 signaling in diabetic mice. J Steroid Biochem Mol Biol 2013; 135: 43-50.

[18] Aral K, Alkan BA, Saraymen R, Yay A, Sen A and Onder GO. Therapeutic effects of systemic vitamin k2 and vitamin d3 on gingival inflammation and alveolar bone in rats with experimentally induced periodontitis. J Periodontol 2015; 86: 666-673.

[19] Panda DK, Miao D, Tremblay ML, Sirois J, Farookhi R, Hendy GN and Goltzman D. Targeted ablation of the 25-hydroxyvitamin D 1alpha -hydroxylase enzyme: evidence for skeletal, re-

productive, and immune dysfunction. Proc Natl Acad Sci U S A 2001; 98: 7498-7503.
[20] Dardenne O, Prud'homme J, Arabian A, Glorieux FH and St-Arnaud R. Targeted inactivation of the 25-hydroxyvitamin D(3)-1(alpha)-hydroxylase gene (CYP27B1) creates an animal model of pseudovitamin D-deficiency rickets. Endocrinology 2001; 142: 3135-3141.
[21] Yang R, Chen J, Zhang J, Qin R, Wang R, Qiu Y, Mao Z, Goltzman D and Miao D. 1,25-Dihydroxyvitamin D protects against age-related osteoporosis by a novel VDR-Ezh2-p16 signal axis. Aging Cell 2020; 19: e13095.
[22] Chen L, Yang R, Qiao W, Zhang W, Chen J, Mao L, Goltzman D and Miao D. 1,25-Dihydroxyvitamin D exerts an antiaging role by activation of Nrf2-antioxidant signaling and inactivation of p16/p53-senescence signaling. Aging Cell 2019; 18: e12951.
[23] Liu H, Guo J, Wang L, Chen N, Karaplis A, Goltzman D and Miao D. Distinctive anabolic roles of 1,25-dihydroxyvitamin D(3) and parathyroid hormone in teeth and mandible versus long bones. J Endocrinol 2009; 203: 203-213.
[24] Shen M, Luo Y, Niu Y, Chen L, Yuan X, Goltzman D, Chen N and Miao D. 1,25(OH)2D deficiency induces temporomandibular joint osteoarthritis via secretion of senescence-associated inflammatory cytokines. Bone 2013; 55: 400-409.
[25] Yang B, Pang X, Li Z, Chen Z and Wang Y. Immunomodulation in the treatment of periodontitis: progress and perspectives. Front Immunol 2021; 12: 781378.
[26] Abe T and Hajishengallis G. Optimization of the ligature-induced periodontitis model in mice. J Immunol Methods 2013; 394: 49-54.
[27] Chow EC, Quach HP, Vieth R and Pang KS. Temporal changes in tissue 1alpha,25-dihydroxyvitamin D3, vitamin D receptor target genes, and calcium and PTH levels after 1,25(OH)2D3 treatment in mice. Am J Physiol Endocrinol Metab 2013; 304: E977-989.
[28] Li F, Liu P, Zhang X, Zhang Q, Tang S, Zhu M and Qiu M. 1,25(OH)2D3-mediated amelioration of aortic injury in streptozotocin-induced diabetic rats. Inflammation 2013; 36: 1334-1343.
[29] Panda DK, Miao D, Bolivar I, Li J, Huo R, Hendy GN and Goltzman D. Inactivation of the 25-hydroxyvitamin D 1alpha-hydroxylase and vitamin D receptor demonstrates independent and interdependent effects of calcium and vitamin D on skeletal and mineral homeostasis. J Biol Chem 2004; 279: 16754-16766.
[30] Gong A, Chen J, Wu J, Li J, Wang L, Goltzman D and Miao D. 1,25-dihydroxyvitamin D deficiency accelerates alveolar bone loss independent of aging and extracellular calcium and phosphorus. J Periodontol 2018; 89: 983-994.
[31] Liu YF, Wu LA, Wang J, Wen LY and Wang XJ. Micro-computerized tomography analysis of alveolar bone loss in ligature- and nicotine-induced experimental periodontitis in rats. J Periodontal Res 2010; 45: 714-719.
[32] Miao D, Bai X, Panda D, McKee M, Karaplis A and Goltzman D. Osteomalacia in hyp mice is associated with abnormal phex expression and with altered bone matrix protein expression and deposition. Endocrinology 2001; 142: 926-939.
[33] Xue Y, Karaplis AC, Hendy GN, Goltzman D and Miao D. Exogenous 1,25-dihydroxyvitamin D3 exerts a skeletal anabolic effect and improves mineral ion homeostasis in mice that are homozygous for both the 1alpha-hydroxylase and parathyroid hormone null alleles. Endocrinology 2006; 147: 4801-4810.
[34] Hildebolt CF. Effect of vitamin D and calcium on periodontitis. J Periodontol 2005; 76: 1576-1587.
[35] Gao W, Tang H, Wang D, Zhou X, Song Y and Wang Z. Effect of short-term vitamin D supplementation after nonsurgical periodontal treatment: a randomized, double-masked, placebo-controlled clinical trial. J Periodontal Res 2020; 55: 354-362.
[36] Topilski I, Flaishon L, Naveh Y, Harmelin A, Levo Y and Shachar I. The anti-inflammatory effects of 1,25-dihydroxyvitamin D3 on Th2 cells in vivo are due in part to the control of integrin-mediated T lymphocyte homing. Eur J Immunol 2004; 34: 1068-1076.
[37] Golz L, Memmert S, Rath-Deschner B, Jager A, Appel T, Baumgarten G, Gotz W and Frede S. Hypoxia and P. gingivalis synergistically induce HIF-1 and NF-kappaB activation in PDL cells and periodontal diseases. Mediators Inflamm 2015; 2015: 438085.
[38] Giarratana N, Penna G, Amuchastegui S, Mariani R, Daniel KC and Adorini L. A vitamin D analog down-regulates proinflammatory chemokine production by pancreatic islets inhibiting T cell recruitment and type 1 diabetes development. J Immunol 2004; 173: 2280-2287.
[39] Menzel LP, Ruddick W, Chowdhury MH, Brice DC, Clance R, Porcelli E, Ryan LK, Lee J, Yilmaz O, Kirkwood KL, McMahon L, Tran A and Diamond G. Activation of vitamin D in the gingival epithelium and its role in gingival inflammation and alveolar bone loss. J Periodontal Res 2019; 54: 444-452.
[40] Chen L, Yang R, Qiao W, Yuan X, Wang S, Goltzman D and Miao D. 1,25-Dihydroxy vitamin D prevents tumorigenesis by inhibiting oxidative stress and inducing tumor cellular senescence in mice. Int J Cancer 2018; 143: 368-382.
[41] Qiao W, Yu S, Sun H, Chen L, Wang R, Wu X, Goltzman D and Miao D. 1,25-Dihydroxyvita-

min D insufficiency accelerates age-related bone loss by increasing oxidative stress and cell senescence. Am J Transl Res 2020; 12: 507-518.
[42] Sun H, Qiao W, Cui M, Yang C, Wang R, Goltzman D, Jin J and Miao D. The polycomb protein Bmi1 plays a crucial role in the prevention of 1,25(OH)2 D deficiency-induced bone loss. J Bone Miner Res 2020; 35: 583-595.
[43] Sun W, Chen L, Zhang W, Wang R, Goltzman D and Miao D. Active vitamin D deficiency mediated by extracellular calcium and phosphorus results in male infertility in young mice. Am J Physiol Endocrinol Metab 2015; 308: E51-62.
[44] Sun W, Xie H, Ji J, Zhou X, Goltzman D and Miao D. Defective female reproductive function in 1,25(OH)2D-deficient mice results from indirect effect mediated by extracellular calcium and/or phosphorus. Am J Physiol Endocrinol Metab 2010; 299: E928-935.
[45] Zhou C, Lu F, Cao K, Xu D, Goltzman D and Miao D. Calcium-independent and 1,25(OH)2D3-dependent regulation of the renin-angiotensin system in 1alpha-hydroxylase knockout mice. Kidney Int 2008; 74: 170-179.
[46] Gallagher JC. Vitamin D and aging. Endocrinol Metab Clin North Am 2013; 42: 319-332.
[47] Eke PI, Wei L, Borgnakke WS, Thornton-Evans G, Zhang X, Lu H, McGuire LC and Genco RJ. Periodontitis prevalence in adults ≥65 years of age, in the USA. Periodontol 2000 2016; 72: 76-95.