Therapeutic Effects of Systemic Vitamin K2 and Vitamin D3 on Gingival Inflammation and Alveolar Bone in Rats With Experimentally Induced Periodontitis

Kübra Aral,* Banu Arzu Alkan,† Recep Saraymen,‡ Arzu Yay,§ Ahmet Şen,‖ and Gözde Özge Önder§

Background: The synergistic effects of vitamin D3 and vitamin K2 on bone loss prevention have been reported. This study evaluates the effects of vitamin D3 and vitamin K2 supplementation in conjunction with conventional periodontal therapy (scaling and root planing [SRP]) on gingival interleukin (IL)-1β and IL-10, serum bone alkaline phosphatase (B-ALP) and tartrate-resistant acid phosphatase 5b (TRAP-5b), and calcium and alveolar bone levels in rats with experimentally induced periodontitis.

Methods: Seventy-two rats were divided into the following groups: 1) healthy; 2) periodontitis; 3) SRP; 4) SRP + vitamin D3; 5) SRP + vitamin K2; and 6) SRP + vitamins K2 and D3. Periodontitis was induced by ligature placement for 7 days, and vitamin K2 (30 mg/kg) and/or vitamin D3 (2 μg/kg) were administered for 10 days in the SRP + vitamin D3, SRP + vitamin K2, and SRP + vitamins K2 and D3 groups by oral gavage. On day 18, the animals were sacrificed, serum B-ALP, TRAP-5b, and calcium levels were measured, gingiva specimens were extracted for IL-1β and IL-10 analysis, and distances between the cemento-enamel junction and alveolar bone crest were evaluated.

Results: Alveolar bone levels in the periodontitis group were significantly greater than those in the other five groups. No significant differences were found in gingival IL-1β and IL-10, serum B-ALP and TRAP-5b, and calcium and alveolar bone levels between the groups receiving SRP and vitamins and the group receiving SRP alone.

Conclusion: Within the limitations of this study, vitamin D3 and K2 alone or in combination did not affect gingival IL-1β and IL-10, serum B-ALP and TRAP-5b levels, or alveolar bone compared with conventional periodontal therapy alone. *J Periodontol 2015;86:666-673.*

KEY WORDS

Biological markers; cholecalciferol; cytokines; periodontitis; vitamin K 2.

* Department of Periodontology, Faculty of Dentistry, Sifa University, İzmir, Turkey.
† Department of Periodontology, Faculty of Dentistry, Erciyes University, Kayseri, Turkey.
‡ Department of Medical Biochemistry, Faculty of Medicine, Erciyes University.
§ Department of Histology and Embryology, Faculty of Medicine, Erciyes University.
‖ Yüksekova State Hospital, Hakkari, Turkey.

Periodontitis is a disease characterized by infectious and inflammatory bone resorption.[1] It has been proposed that increased levels of cytokines in periodontally diseased tissues can act systemically, and initial periodontal treatment may affect tissue and serum cytokine levels.[2] Interleukin (IL)-1β, which is a basic marker of the inflammatory response,[3] has been reported to increase in gingival tissue in periodontal disease.[4] IL-10 plays an important role in the suppression of immune and inflammatory responses.[5] It inhibits the synthesis of proinflammatory cytokines, such as IL-1, IL-2, and IL-6, and stimulates the production of protective antibodies.[6,7]

Biochemical markers are indicative of complex bone remodeling processes and have important roles in the diagnosis and treatment of metabolic bone diseases.[8] Bone alkaline phosphatase (B-ALP) is a bone-specific isoenzyme of ALP. It is localized in the membrane of osteoblasts and released into the circulation during activation of osteoblasts.[9] It has been reported that ligature-induced periodontitis reduced the serum levels of B-ALP.[10] Tartrate-resistant acid phosphatase 5b (TRAP-5b) is a specific and sensitive marker of osteoclasts. Its serum concentration is related with bone resorption.[11,12]

doi: 10.1902/jop.2015.140467

It has been shown that, in rats with cyclosporine-induced alveolar bone loss, serum TRAP-5b levels were higher than those of healthy controls.[13]

Conventional periodontal treatment is insufficient for two main reasons: 1) microbial factors cannot be completely eliminated, and 2) conventional periodontal treatment does not have a direct effect on the host response. Therefore, to ensure the long-term success of treatment of chronic periodontitis (CP), a therapeutic approach that regulates the host response responsible for periodontal tissue breakdown has emerged, in addition to microbial elimination.[14] In conjunction with these treatment approaches, vitamin D supplementation has attracted attention because of its anti-inflammatory and immunomodulating properties.[15]

Vitamin D has regulatory effects on osteocalcin and osteopontin, which belong to the bone matrix protein family of osteoblasts, and vitamin D3 acts as an immune modulator.[16] Vitamin K plays a key role in the clotting mechanism.[17] Osteocalcin and matrix carboxyglutamate, which are both involved in bone metabolism, require vitamin K to become activated.[18] On the basis of an in vitro study, Koshihara and Hoshi[19] stated that combined administration of vitamins K2 and D3 resulted in a dramatic increase in bone calcification and that vitamin K2 was essential for high serum levels of vitamin D3.

The aim of this study is to evaluate the effects of vitamin K2 and/or vitamin D3 administration in addition to conventional periodontal treatment (scaling and root planing [SRP]) on gingival levels of IL-1β and IL-10, serum levels of B-ALP and TRAP-5b, and alveolar bone loss in rats with experimentally induced periodontitis.

MATERIALS AND METHODS

Animals

Seventy-two male Wistar rats weighing 270 to 330 g were randomly assigned to six equal groups, and periodontitis was experimentally induced in all groups except group S (the healthy control group). The remaining treatment groups were included the following: 1) group P (periodontitis only); 2) group PT (SRP was performed); 3) group D (SRP was performed and vitamin D3 was administered); 4) group K (SRP was performed and vitamin K2 was administered); and 5) group DK (SRP was performed and vitamins D3 and K2 were administered). The experimental protocol was approved by the Animal Experiment Ethics Committee of Erciyes University (approval no. 12.06.2013.TS.06.KN.13/87). During the experimental procedure, the animals were maintained in a quiet room with a controlled temperature (21°C ± 1°C) and a 12-hour light/dark cycle. The rats were fed standard chow and had access to tap water *ad libitum*.

Experimental Periodontal Disease

Animals were examined before placement of ligatures using a dental loupe. No inflammation was observed. The animals were randomly selected, weighed, and anesthetized with a combination of ketamine¶ (1 mL/kg intraperitoneally) and xylazine# (0.1 mL/kg intraperitoneally). The ligature model of periodontitis was implemented by tying 4/0 cotton ligatures in the subgingival region around the right and left maxillary first molars. After 7 days, the ligatures were removed in groups P, PT, D, K, and DK, and conventional periodontal treatment (SRP) was only performed in the treatment groups (groups PT, D, K, and DK).[20]

Periodontal Therapy Procedure

The animals were anesthetized for ligature removal and SRP procedure. Immediately after ligature removal, periodontal therapy was performed with curets** in groups PT, D, K, and DK as described previously.[21] A curette was used in the buccal and lingual surfaces in a disto-mesial direction and in furcation and interproximal areas in an apico-coronal direction 10 times with a pull stroke.[21] Periodontal pockets were irrigated with 1 mL saline solution after SRP.[22]

Vitamin Treatments

Vitamin supplementation was initiated when the animals woke from anesthesia after the SRP procedure. Calcitriol†† was prepared daily in corn oil (vehicle)‡‡ at a concentration of 2 μg/mL.[13] Animals in groups D and DK were given 2 μg/kg vitamin D3 by oral gavage once a day for 10 days. Vitamin K2 (menatetrenone)§§ was converted into a mixture at 30 mg/5 mL in the corn oil vehicle and administered orally once a day for 10 days at 30 mg/kg to groups K and DK.[23] The vitamins in the combined vitamin group (DK) were administered 30 minutes apart. The animals were sacrificed 24 hours after the last day of vitamin administration.

Histologic Analysis

Each rat's maxilla, including molar teeth, was resected as a block and divided into two sections from the midline. Right sections were used for histologic analysis, and left sections were used for biochemical analysis. Right fragments were dissected, fixed in 10% formaldehyde for 48 hours, decalcified in 10% EDTA for ≈2 weeks, washed, dehydrated in graded alcohol concentrations, cleared in xylene, and embedded in paraffin. The paraffin blocks were serially cut in a mesio-distal direction along the long axis of the teeth to generate 7-μm-thick longitudinal

¶ Ketalar, Pfizer, Istanbul, Turkey.
# Rompun, Bayer, Istanbul, Turkey.
** 1/2 Mini Five Gracey, Hu-Friedy, Chicago, IL.
†† Santa Cruz Biotechnology, Santa Cruz, CA.
‡‡ Sigma-Aldrich, St. Louis, MO.
§§ Santa Cruz Biotechnology.

sections. Sections were transferred to polylysine slides, stained with Masson trichrome, and examined under a light microscope.‖‖ Digital images were captured and analyzed with software.¶¶ Alveolar height measurements were performed, taking into consideration an imaginary line uniting the cemento-enamel junction of the maxillary first and second molars and the alveolar bone crest (CEJ–ABC distance). The histologic sections were clustered in groups of 10 sections by an examiner (AY) unaware of study design.[24] Intra-examiner reproducibility was checked before and during the histometric analysis using seven sections from each group, which were selected at random and analyzed twice with a 1-week interval between analyses.[25]

Serum Analysis of B-ALP, TRAP-5b, and Calcium Levels

After being fasted overnight, on day 18, the animals were anesthetized, and 8 mL blood was drawn directly from the heart of each animal, immediately before they were sacrificed. Blood samples were transferred to gel tubes and then placed in the centrifuge. After centrifugation, dissociated serum samples were transferred to 1.5-mL tubes and stored at –70°C before analysis. B-ALP and TRAP-5b levels were analyzed using enzyme-linked immunosorbent assay (ELISA) kits## in accordance with the instructions of the manufacturer. Calcium levels were analyzed using a chemical analyzer.***

Analysis of Gingival IL-1β and IL-10 Levels

The tissues harvested around the first molar teeth were dissected and placed into sterile tubes containing 400 μL phosphate-buffered saline (PBS) and 0.05% polysorbate 20.††† Tissues were weighed, sliced into small pieces with scissors, and solubilized in PBS to a final concentration of 100 mg tissue/mL. After extraction with a vortex mixer for 10 minutes, each gingival sample was centrifuged at 370 × g for 5 minutes. The supernatants were collected and stored at –70°C before analysis. To avoid protease activity, the entire procedure was performed at 4°C.[26] IL-10 and IL-1β levels were analyzed using ELISA kits‡‡‡ in accordance with the instructions of the manufacturer.

Statistical Analysis

Data are expressed as mean ± SD. Statistical analysis was performed using a program package.§§§ The groups were compared via one-way analysis of variance (ANOVA). Intergroup multiple comparisons (post hoc) were performed using Tukey test, and *P* values <0.05 were deemed to indicate statistical significance in all tests.

RESULTS

Serum B-ALP, TRAP-5b, and Calcium Levels

There were statistically significant differences in serum B-ALP levels among the groups ($P < 0.05$) (Fig. 1). Compared with group S, groups PT ($P < 0.05$), K ($P < 0.001$), and DK ($P < 0.05$) had higher levels of serum B-ALP. Comparisons among the vitamin groups revealed that B-ALP levels in group D were statistically lower than those in group K ($P < 0.05$). There were no significant differences between group PT and any of the vitamin groups ($P > 0.05$). Serum B-ALP levels in group P were significantly lower than those in group K ($P < 0.001$).

There were significant differences in serum TRAP-5b levels among the groups ($P < 0.05$) (Fig. 1). Group S had significantly lower levels than group K ($P < 0.05$). Among the vitamin groups, serum TRAP-5b levels in group K were significantly higher than those in group DK ($P < 0.05$). There were no significant differences between group PT and any of the vitamin treatment groups or between group P and any of the other the groups ($P > 0.05$).

No significant differences in serum calcium levels were observed among all groups ($P > 0.05$) or between the specific vitamin groups ($P > 0.05$). Furthermore, significant differences were not found between group PT and any of the vitamin groups ($P > 0.05$) (Fig. 1).

Gingival IL-10 and IL-1β Levels

There were no significant differences in gingival IL-10 levels when the experimental groups were compared with the healthy group ($P > 0.05$) (Fig. 2). There were also no significant differences in IL-10 among the vitamin groups (D, K, and DK) or between the vitamin groups and group PT ($P > 0.05$). Gingival IL-10 levels in group P were significantly lower than those in groups D and K ($P < 0.05$).

Gingival IL-1β levels were significantly lower in group S than in group P ($P < 0.05$). There were no significant differences among the vitamin groups or between the vitamin groups and group PT ($P > 0.05$). Gingival IL-1β levels in group P were significantly higher than those in group PT ($P < 0.05$) (Fig. 2).

Histologic Analysis

Repeated measurements of the CEJ–ABC distance on histologic sections revealed that there was no significant difference between the first and second measurements of the examiner ($P > 0.05$). Of all the experimental groups, only group P had significantly higher CEJ–ABC distance values than group S (healthy control group) ($P < 0.001$) (Fig. 3). There were no significant differences among the vitamin groups D, K, and DK ($P > 0.05$). Moreover, no significant differences were observed between the

‖‖ Olympus BX50, Olympus, Tokyo, Japan.
¶¶ NIH ImageJ, National Institutes of Health, Bethesda, MD.
Sunred Biological Technology, Shanghai, China.
*** cobas 8000, Roche Diagnostics, Basel, Switzerland.
††† Tween 20, Thermo Fisher Scientific, Waltham, MA.
‡‡‡ eBioscience, San Diego, CA.
§§§ SPSS, IBM, Chicago, IL.

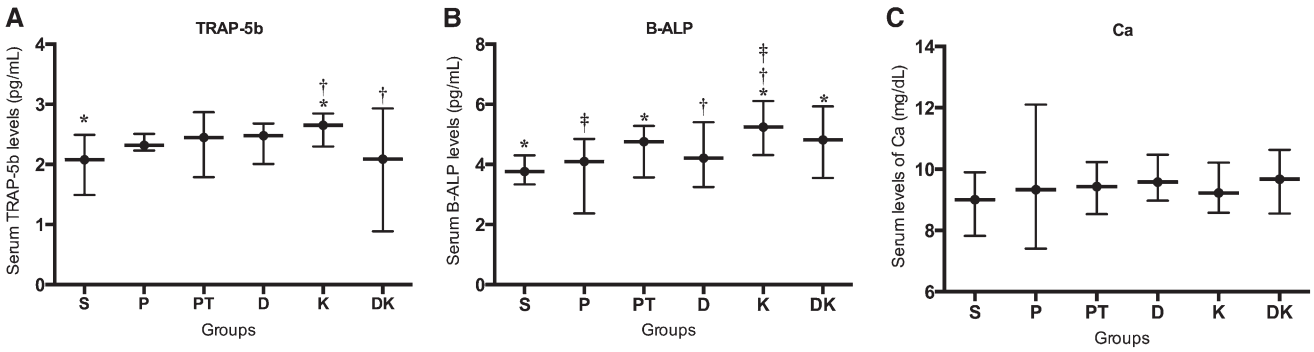

Figure 1.

A) *Effects of vitamin D and vitamin K supplementation on serum TRAP-5b levels.* *******Comparison with group S: serum TRAP-5b levels were significantly lower in group S than in group K (P <0.05).* ***†****Among the vitamin groups, serum TRAP-5b levels were significantly higher in group K than in group DK (P <0.05). One-way ANOVA was used for statistical analysis.* ***B)*** *Effects of vitamin D and vitamin K supplementation on serum B-ALP levels.* *******Comparison with group S: serum B-ALP levels were lower in group S compared with groups PT, K, and DK (P <0.05).* ***†****Among the vitamin groups, serum B-ALP levels were significantly higher in group K than in group D (P <0.05).* ***‡****Comparison with group P: serum B-ALP levels were significantly lower in group P than in group K (P <0.001). One-way ANOVA was used for statistical analysis.* ***C)*** *Effects of vitamin D and vitamin K supplementation on serum calcium (Ca) levels. No significant differences were found between the groups (P >0.05). The Kruskal-Wallis test was used for statistical analysis.*

vitamin groups (D, K, and DK) and group PT ($P>0.05$). The CEJ–ABC distance values in groups PT ($P = 0.001$), D ($P<0.001$), K ($P= 0.001$), and DK ($P<0.001$) were significantly lower than those in group P (Fig. 3). Histologic sections from the study groups are shown in Figure 4. Histologic analysis of group S confirmed that the periodontal and gingival structures were healthy.

DISCUSSION

Studies on the use of antiresorptive agents for anti-inflammatory and anti-infective periodontal treatment have become popular in recent years. It is expected that efficient treatment procedures targeting control of bone resorption, in addition to conventional mechanical periodontal therapy aimed at eliminating inflammation, will soon become available.[15] Vitamins may be considered candidates in this sense, because they play roles in the regulation of host responses, the enhancement of innate immune responses, and the reduction of alveolar bone resorption.[15,27] Many studies have confirmed that vitamin D deficiency is associated with bone loss.[28] In addition to promoting bone homeostasis, vitamin D also has anti-inflammatory and immunomodulating properties.[29]

In this study, conventional periodontal therapy alone or in combination with the administration of vitamin D3, vitamin K2, and vitamins D3 and K2 together affects the CEJ–ABC distance favorably. However, the differences in alveolar bone loss between the vitamin groups and group PT were not significant. Thus, these findings did not support the notion that vitamin D has additional positive effects on alveolar bone.

There are previous reports of human studies examining the relationship between periodontal status and vitamin D deficiency.[30,31] Jönsson et al.[30] examined the effects of female sex hormones and vitamin D on periodontal pathology. The authors reported that the antagonistic effects of estrogen and progesterone on periodontitis depend on the presence of high levels of vitamin D and suggested that this situation may originate from the anti-inflammatory effects of these hormones and vitamin D via a reduction of cytokines, such as IL-1β and IL-6. Boggess et al.[32] investigated the relationship between periodontal status and vitamin D levels in pregnant females. Their findings showed that vitamin D levels in females with moderate and severe periodontal disease were lower than those of periodontally healthy females. The authors suggested that vitamin D may be a potential therapeutic strategy to enhance maternal oral health.[32] Teles et al.[33] investigated the relationships between serum adipokines, vitamin D, and clinical/microbiologic periodontal parameters in patients with CP. Their study showed that the patients who had the highest levels of serum vitamin D exhibited less bleeding on probing, better probing depth, less clinical attachment loss (AL), fewer missing teeth, and lower levels of pathogenic bacteria.[33] Dietrich et al.[34] examined the relationship between serum vitamin D levels and AL in a large population and suggested that lower serum vitamin D levels were associated with increased AL. In another study,[31] the same investigator reported that vitamin D may reduce sensitivity to gingival inflammation by way of its anti-inflammatory effects. The present study shows that conventional periodontal therapy alone was not effective in increasing the levels of the gingival anti-inflammatory cytokine IL-10 or the serum bone formation marker B-ALP and that vitamin D3 and/or K2 supplementation was required to achieve this. Of all

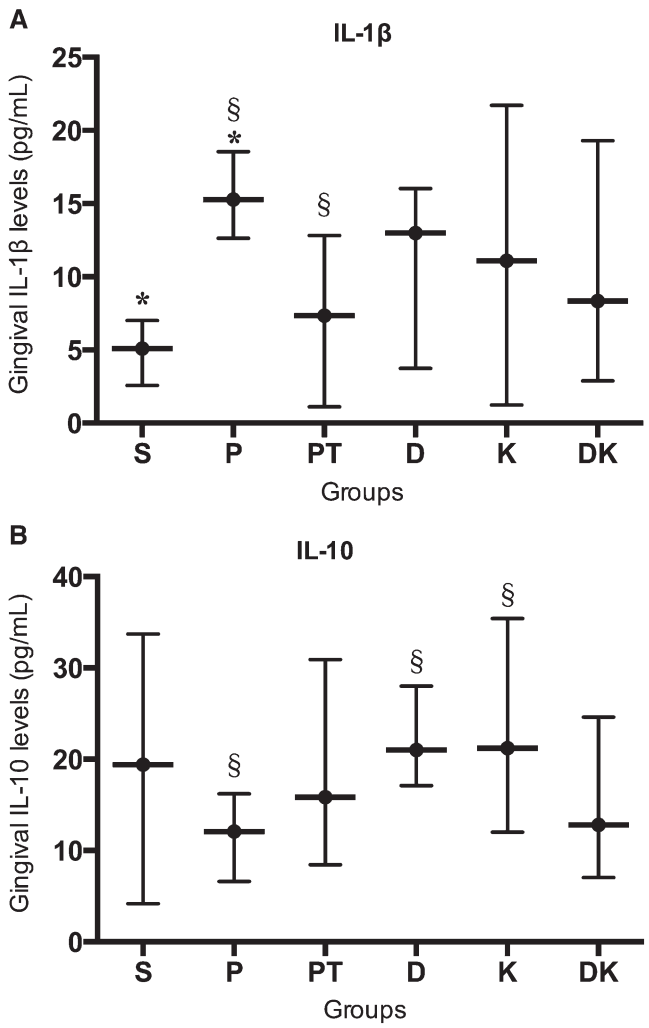

Figure 2.
A) *Effects of vitamin D and vitamin K supplementation on gingival IL-1β levels.* ******* *Comparison with group S: gingival IL-1β levels were significantly lower in group S than in group P (P <0.05).* ***§*** *Comparison with group P: gingival IL-1β levels were significantly higher in group P than in group PT (P <0.05). One-way ANOVA was used for statistical analysis.* ***B)*** *Effects of vitamin D and vitamin K supplementation on gingival IL-10 levels.* ***§*** *Comparison with group P: gingival IL-10 levels were significantly lower in group P than in groups D and K (P <0.05). One-way ANOVA was used for statistical analysis.*

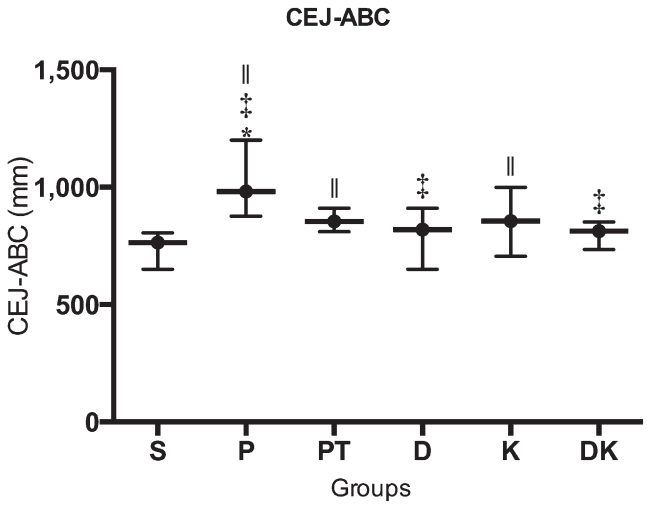

Figure 3.
Effects of vitamin D and vitamin K supplementation on the CEJ–ABC distance. ******* *Comparison with group S: The CEJ–ABC distance was significantly higher in group P than in group S (P <0.05).* ***‡*** *Comparison with group P: the CEJ–ABC distance was significantly higher in group P than in groups D and DK (P <0.001).* ***‖*** *Comparison with group P: the CEJ–ABC distance was significantly higher in group P than in groups PT and K (P = 0.001). One-way ANOVA was used for statistical analysis.*

the treatment groups, including group PT, only group K demonstrated significantly higher B-ALP levels compared with group P (untreated).

A few studies have examined the effects of vitamin D supplementation on periodontal status in humans.[34-38] Krall[35] reported that higher vitamin D intake was associated with reduced AL and a lower risk of tooth loss. Miley et al.[36] suggested that patients receiving maintenance periodontal therapy and vitamin D and calcium supplementation had better periodontal health.

A limited number of animal studies on the effects of vitamin administration on periodontal parameters and alveolar bone resorption are available.[13,37,38] Hidekazu et al.[37] investigated the effects of vitamin K2 on periodontitis. The authors suggested that administration of vitamin K2 may reduce the number of osteoclasts and the amount of bone resorption in rapidly progressive experimental periodontitis.[37] In the present study, B-ALP levels are only significantly higher than those of group P (untreated) in group K. This finding was also concordant with histologic findings. Although the findings of this study show positive effects of vitamin K2 administration on alveolar bone, as reported by Hidekazu et al.,[37] it is not appropriate to compare the findings of the two studies. The major difference between the two studies is the timing of vitamin K administration in relation to the initiation of experimental periodontitis. The other difference is that conventional periodontal therapy (SRP) was used in this study.

Spolidorio et al.[13] investigated the effects of calcitonin and vitamin D administration on serum parameters and alveolar bone in rats with cyclosporine-induced alveolar bone loss. It was reported that vitamin D and calcitonin reduced osteopenic changes, and the levels of TRAP-5b and inflammatory cytokines (IL-1β, tumor necrosis factor [TNF]-α, and IL-6) induced by cyclosporine. Although that study and this study both investigated the effects of vitamin D administration on alveolar bone and inflammatory cytokine levels, the methods (experimental periodontitis model versus drug-induced alveolar bone loss model) used for induction of alveolar bone loss and the administration

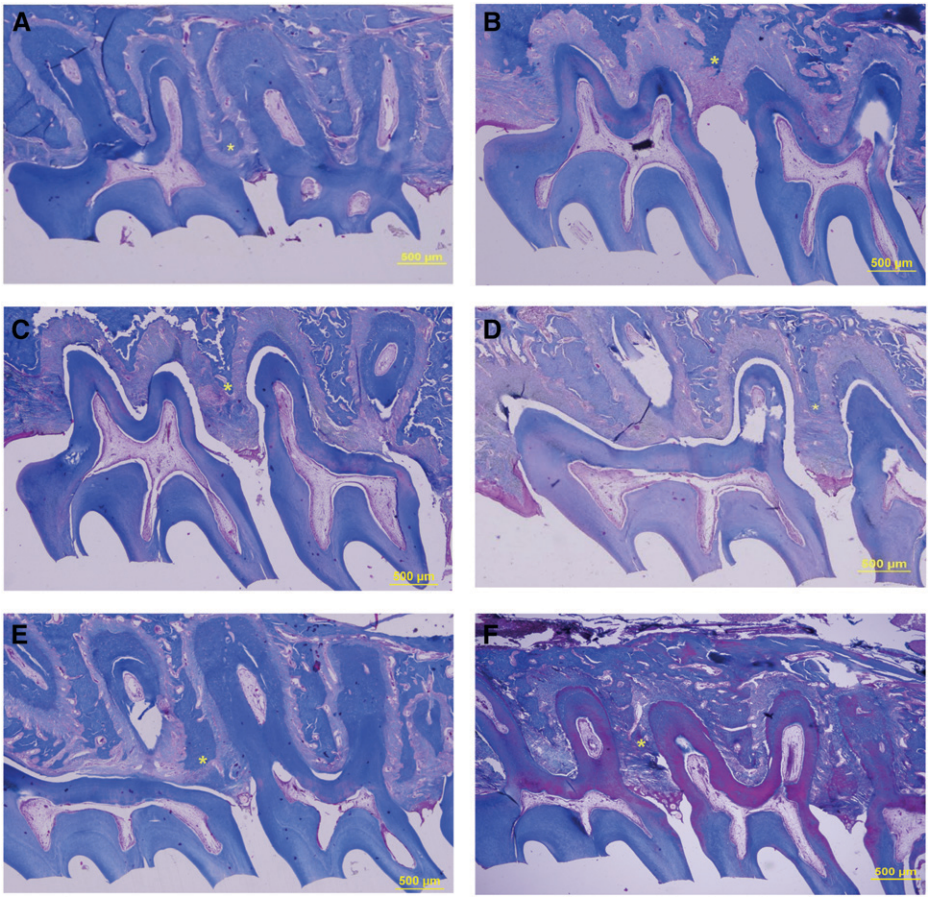

Figure 4.
Histologic sections of the study groups stained with Masson trichrome (MT). *******Defined as the ABC.*
A) *Group S.* ***B)*** *Group P.* ***C)*** *Group PT.* ***D)*** *Group D.* ***E)*** *Group K.* ***F)*** *Group DK.*

of different drug combinations (vitamins D and K versus vitamin D and calcitonin) for different durations (10 days versus 6 weeks) are some of the differences between the two studies. However, the major difference is that, regardless of the type of alveolar bone loss induced, in this study, vitamin administration in animals with established alveolar bone loss is initiated, whereas Spolidorio et al.[13] initiated drug administration simultaneously with resorptive agent administration.

In the present study, the administration of vitamins D3 and K2, alone or in combination, did not significantly enhance the gingival levels of IL-10 or the serum levels of B-ALP and did not reduce the levels of gingival IL-1β and serum TRAP-5b compared with conventional periodontal therapy alone. Serum TRAP-5b levels indicative of a systemic effect were not evident during the period of vitamin administration (10 days). Taking into consideration that no difference existed even in serum levels of TRAP-5b between non-treated periodontitis animals and the healthy group, this situation may be interpreted as periodontitis affecting local anti-inflammatory markers, rather than systemic markers, during the study period. Furthermore, the lack of significant differences in calcium levels among all groups may have been attributable to the dosage and short study duration. Additionally, using more and different biochemical markers in future studies may clarify the effects of vitamin D and vitamin K supplementation in the treatment of periodontitis.

Li et al.[38] examined the effects of vitamin D3 on alveolar bone loss and gingival inflammation in mice with and without diabetes with *Porphyromonas gingivalis*-induced experimental periodontitis. The authors reported that vitamin D3 treatment reduced serum TNF-α levels, alveolar bone loss, and fasting blood glucose levels in mice with diabetes and had a protective effect against diabetic periodontitis. Although that study was similar to this study in terms of vitamin administration after the induction of experimental periodontitis, the two studies differ in two respects. First, Li et al.[38] did not perform any conventional periodontal treatment. Second, their animals had diabetes, which is known to have adverse effects on periodontal tissues. The fact that supplementation with vitamin D3 in conjunction with conventional periodontal therapy reduced gingival IL-10 levels significantly suggests that vitamin D3 may have protective functions on gingival inflammation. A similar effect also exists for vitamin K2.

There are reports on the effects of combined vitamins D and K administration on bone loss and bone mineral density.[23,39,40] However, to the best of the authors' knowledge, no published study has investigated the relationship between periodontal status and the administration of this vitamin combination. Matsunaga et al.[41] reported that vitamins D and K combined had synergistic effects in the reduction of bone loss in ovariectomized rats.[41] However, it is not possible to compare the results of that study with the present findings because the experimental designs (experimental periodontitis versus bone loss attributable to osteoporosis) and the types of bone examined (alveolar bone versus tibia) differ between the two studies.

In this study, the vitamin groups did not show significant positive effects on the evaluated parameters compared with group PT. This may have been attributable to the short duration of this study (10 days), which was one of the limitations of the study. If the duration of vitamin administration is extended, vitamin supplementation in addition to conventional periodontal therapy may change the treatment outcome. In addition, the lack of significant differences between the treatment groups may have been the result of the small sample size, which was another limitation of the present study.

To the best of the authors' knowledge, this study is the first to investigate the effects of vitamin D3 and vitamin K2 administration alone and in combination, in conjunction with conventional periodontal therapy (SRP) for periodontitis. The findings of the present study could not be compared with those of other studies because of differing experimental designs and drug combinations and variations in the parameters examined.

CONCLUSION

Within the limitation of this study, vitamin D3 and K2 supplementation (either alone or in combination) after conventional periodontal treatment did not have a positive effect on gingival IL-1β and IL-10, serum B-ALP and TRAP-5b levels, or alveolar bone compared with conventional periodontal treatment alone.

ACKNOWLEDGMENTS

The authors thank Dr. Ikramuddin Aukhil, Department of Periodontology, College of Dentistry, University of Florida, Gainesville, Florida, for his assistance with this study; Dr. Ahmet Öztürk, Department of Biostatistics, Faculty of Medicine, Erciyes University, Kayseri, Turkey, for statistical analysis; and Dr. Eren Demirpolat, Faculty of Pharmacology, Erciyes University, for his assistance in preparing the vitamin solutions. This study was supported by Erciyes University Research Fund Grant TDK-2013-4709. The authors report no conflicts of interest related to this study.

REFERENCES

1. Page RC, Engel LD, Narayanan AS, Clagett JA. Chronic inflammatory gingival and periodontal disease. *JAMA* 1978;240:545-550.
2. Loos BG. Systemic effects of periodontitis. *Ann R Australas Coll Dent Surg* 2006;18:27-29.
3. Oppenheim JJRF, Faltneyk CR. Interleukins and interferons. In: Stites DP, Stobo JD, Wells JV, eds. *Basic and Clinical Immunology*, 6th ed. Connecticut: Appleton & Lange; 1987:82-87.
4. Hou LT, Liu CM, Liu BY, Lin SJ, Liao CS, Rossomando EF. Interleukin-1beta, clinical parameters and matched cellular-histopathologic changes of biopsied gingival tissue from periodontitis patients. *J Periodontal Res* 2003;38: 247-254.
5. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. *J Immunol* 1991;147:3815-3822.
6. Rousset F, Garcia E, Defrance T, et al. Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proc Natl Acad Sci USA* 1992; 89:1890-1893.
7. Scarel-Caminaga RM, Trevilatto PC, Souza AP, Brito RB, Camargo LE, Line SR. Interleukin 10 gene promoter polymorphisms are associated with chronic periodontitis. *J Clin Periodontol* 2004;31:443-448.
8. Seibel MJRS, Bilezikian JP. Biochemical markers of bone metabolism. In: Becker KL, ed. *Principles and Practice of Endocrinology and Metabolism*, 2nd ed. Philadelphia: Lippincott; 1995:498-508.
9. Colford J, Sailer D, Langman C. Five osteocalcin assays compared: Tracer specificity, fragment interference, and calibration. *Clin Chem* 1997;43:1240-1241.
10. Goes P, Melo IM, Dutra CS, Lima AP, Lima V. Effect of alendronate on bone-specific alkaline phosphatase on periodontal bone loss in rats. *Arch Oral Biol* 2012;57: 1537-1544.
11. Halleen J, Hentunen TA, Hellman J, Väänänen HK. Tartrate-resistant acid phosphatase from human bone: Purification and development of an immunoassay. *J Bone Miner Res* 1996;11:1444-1452.
12. Halleen JM, Räisänen S, Salo JJ, et al. Intracellular fragmentation of bone resorption products by reactive oxygen species generated by osteoclastic tartrate-resistant acid phosphatase. *J Biol Chem* 1999;274: 22907-22910.
13. Spolidorio LC, Herrera BS, Coimbra LS, Spolidorio DM, Muscará MN, Rossa C Jr. Intermittent therapy with 1,25 vitamin D and calcitonin prevents cyclosporin-induced alveolar bone loss in rats. *Calcif Tissue Int* 2010;87: 236-245.
14. Caton JG. Evaluation of Periostat for patient management. *Compend Contin Educ Dent* 1999;20:451-456, 458-460, 462; quiz 463.
15. Bartold PM, Cantley MD, Haynes DR. Mechanisms and control of pathologic bone loss in periodontitis. *Periodontol 2000* 2010;53:55-69.
16. Toubi E, Shoenfeld Y. The role of vitamin D in regulating immune responses. *Isr Med Assoc J* 2010; 12:174-175.
17. Shearer MJ. Vitamin K. *Lancet* 1995;345:229-234.
18. Feskanich D, Weber P, Willett WC, Rockett H, Booth SL, Colditz GA. Vitamin K intake and hip fractures in women: A prospective study. *Am J Clin Nutr* 1999;69: 74-79.
19. Koshihara Y, Hoshi K. Vitamin K2 enhances osteocalcin accumulation in the extracellular matrix of human osteoblasts in vitro. *J Bone Miner Res* 1997;12:431-438.
20. Pavone C, Perussi LR, de Oliveira GJ, et al. Effect of Er, Cr:YSGG laser application in the treatment of experimental periodontitis [published online ahead of print January 30, 2014]. *Lasers Med Sci* doi:10.1007/s10103-014-1526-3.
21. Fernandes LA, de Almeida JM, Theodoro LH, et al. Treatment of experimental periodontal disease by photodynamic therapy in immunosuppressed rats. *J Clin Periodontol* 2009;36:219-228.
22. Fernandes LA, Martins TM, Almeida JM, et al. Experimental periodontal disease treatment by subgingival irrigation with tetracycline hydrochloride in rats. *J Appl Oral Sci* 2010;18:635-640.

23. Iwamoto J, Seki A, Sato Y, Matsumoto H, Tadeda T, Yeh JK. Vitamin K2 promotes bone healing in a rat femoral osteotomy model with or without glucocorticoid treatment. *Calcif Tissue Int* 2010;86:234-241.
24. Benatti BB, Campos-Júnior JC, Silva-Filho VJ, et al. Effects of a *Mikania laevigata* extract on bone resorption and RANKL expression during experimental periodontitis in rats. *J Appl Oral Sci* 2012;20:340-346.
25. Semenoff TA, Semenoff-Segundo A, Bosco AF, Nagata MJ, Garcia VG, Biasoli ER. Histometric analysis of ligature-induced periodontitis in rats: A comparison of histological section planes. *J Appl Oral Sci* 2008;16: 251-256.
26. Pimentel SP, Barrella GE, Casarin RC, et al. Protective effect of topical *Cordia verbenacea* in a rat periodontitis model: Immune-inflammatory, antibacterial and morphometric assays. *BMC Complement Altern Med* 2012;12:224.
27. Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system: Vitamins A and D take centre stage. *Nat Rev Immunol* 2008;8:685-698.
28. Kulie T, Groff A, Redmer J, Hounshell J, Schrager S. Vitamin D: An evidence-based review. *J Am Board Fam Med* 2009;22:698-706.
29. Walters MR. Newly identified actions of the vitamin D endocrine system. *Endocr Rev* 1992;13:719-764.
30. Jönsson D, Aggarwal P, Nilsson BO, Demmer RT. Beneficial effects of hormone replacement therapy on periodontitis are vitamin D associated. *J Periodontol* 2013;84:1048-1057.
31. Dietrich T, Nunn M, Dawson-Hughes B, Bischoff-Ferrari HA. Association between serum concentrations of 25-hydroxyvitamin D and gingival inflammation. *Am J Clin Nutr* 2005;82:575-580.
32. Boggess KA, Espinola JA, Moss K, Beck J, Offenbacher S, Camargo CA Jr. Vitamin D status and periodontal disease among pregnant women. *J Periodontol* 2011;82: 195-200.
33. Teles FR, Teles RP, Martin L, Socransky SS, Haffajee AD. Relationships among interleukin-6, tumor necrosis factor-α, adipokines, vitamin D, and chronic periodontitis. *J Periodontol* 2012;83:1183-1191.
34. Dietrich T, Joshipura KJ, Dawson-Hughes B, Bischoff-Ferrari HA. Association between serum concentrations of 25-hydroxyvitamin D3 and periodontal disease in the US population. *Am J Clin Nutr* 2004;80:108-113.
35. Krall EA. The periodontal-systemic connection: Implications for treatment of patients with osteoporosis and periodontal disease. *Ann Periodontol* 2001;6:209-213.
36. Miley DD, Garcia MN, Hildebolt CF, et al. Cross-sectional study of vitamin D and calcium supplementation effects on chronic periodontitis. *J Periodontol* 2009;80:1433-1439.
37. Konishi H, Kawanami M, Sakagami R, et al. Influence of menatetrenone (vitamin K2) administration on bone resorption in rapidly progressive experimental periodontitis in rats. *Jpn J Conserv Dent* 2001;44:255-264.
38. Li H, Xie H, Fu M, et al. 25-hydroxyvitamin D3 ameliorates periodontitis by modulating the expression of inflammation-associated factors in diabetic mice. *Steroids* 2013;78:115-120.
39. Ushiroyama T, Ikeda A, Ueki M. Effect of continuous combined therapy with vitamin K(2) and vitamin D(3) on bone mineral density and coagulofibrinolysis function in postmenopausal women. *Maturitas* 2002;41: 211-221.
40. Yonemura K, Fukasawa H, Fujigaki Y, Hishida A. Protective effect of vitamins K2 and D3 on prednisolone-induced loss of bone mineral density in the lumbar spine. *Am J Kidney Dis* 2004;43:53-60.
41. Matsunaga S, Ito H, Sakou T. The effect of vitamin K and D supplementation on ovariectomy-induced bone loss. *Calcif Tissue Int* 1999;65:285-289.

Correspondence: Assistant Prof. Kübra Aral, Department of Periodontology, Faculty of Dentistry, Sifa University, 35100 Bornova, İzmir, Turkey. Fax: 90-232-308-0-308; e-mail: drkubraaral@gmail.com.

Submitted August 13, 2014; accepted for publication December 4, 2014.