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ORIGINAL ARTICLE

The effect of boron on alveolar bone loss in osteoporotic rats

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KEYWORDS
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Abstract *Background/purpose:* The aim of this study is to investigate the effects of systemically administered boric acid on osteoporosis-related bone alterations, alveolar bone loss, receptor activator of nuclear factor kappa-b ligand (RANKL) expressions, and mandibular bone density in experimental periodontitis model in osteoporotic rats.
Materials and methods: Thirty-six male Wistar rats were separated into five study groups: non-ligated control (C, $n = 6$) group; periodontitis (P, $n = 6$) group; osteoporosis (O, $n = 8$) group; osteoporosis + periodontitis (O+P, $n = 8$) group, and osteoporosis + periodontitis with 50 mg/kg/d boric acid (BA50, $n = 8$) group for 15 days. Osteoporosis was created with intraperitoneal injection of 80 mg/kg retinoic acid for 15 days. Silk ligatures (4/0) were placed around the mandibular right first molar teeth to induce experimental periodontitis. After induction of osteoporosis and periodontitis, rats were sacrificed at Day 15. Alveolar bone loss was evaluated with a stereomicroscope by measuring the distance from the cement-enamel junction to the alveolar crest. Density measurements were performed on radiographs. RANKL and tartrate-resistant acid phosphatase (TRAP) staining were performed on histological slides.
Results: Alveolar bone loss was significantly higher in the O+P group than those of the other groups ($P < 0.05$). Boric acid decreased bone loss ($P < 0.05$). TRAP + osteoclast numbers were highest in the P group and lowest in the control group. The differences in TRAP + osteoclast numbers among control, P, O+P, and BA50 groups were significant ($P < 0.05$). There were no significant differences in RANKL expression and mandibular bone density ($P > 0.05$).
Conclusion: Within limitations of this study, we conclude that boric acid may decrease alveolar bone loss in a rat model with periodontitis and osteoporosis.

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Introduction

Osteoporosis is defined as "a systemic skeletal disease characterized by low bone mass and micro-architectural deterioration with consequent increase in bone fragility and susceptibility to fracture."[1] Osteoporosis is not a life-threatening disorder but still affects many people by causing pain, disability, and diminished quality of life.[2] Aging, estrogen deficiency, and inflammatory diseases are the most common factors contributing to the development of osteoporosis. There are some treatment procedures for osteoporosis and these are mainly: calcium and vitamin D reinforcement, antiresorptive agents such as bisphosphonates, selective estrogen receptor modulators, parathyroid hormone, and surgery.[2]

The relationship between osteoporosis and oral health is still a complex problem and the evidences are contradictory. Some studies have suggested an association between low skeletal bone mineral density (BMD) and periodontal bone loss and tooth loss,[3,4] while others have not.[5,6] Furthermore, the mechanisms underlying any potential association between periodontal disease and osteoporosis are not fully understood but experimental studies of osteoporosis and periodontitis suggested a strong relationship.[7,8] In both diseases, there is an increased production of cytokines that stimulate osteoclastic activity.[8] Kobayashi et al[7] showed that alveolar bone mass was reduced by ovariectomy and that estrogen deficiency significantly enhanced the loss of alveolar bone in experimental periodontitis in mice.

One of the most preferred treatment protocols for osteoporosis is antiresorptive agents such as bisphosphonates. These drugs are very effective in preventing bone resorption in long bones and vertebra but have a serious side effect on jaws. Osteonecrosis caused by bisphosphonates makes any surgical dental procedure impossible and it is very hard to treat.[9]

Being directly linked to bone metabolism,[10] boron might help reverse the effects of osteoporosis. Boron is the fifth element in the periodic table and has the characteristics of both metals and nonmetals. Boron interacts with calcium, vitamin D, and magnesium. Boron is not found alone in nature and is abundant in nature as boric acid (BA) and borate. Boron can be obtained in the diet through the consumption of fruits, vegetables (potato and avocado), legumes, nuts, eggs, milk, wine, and dried foods.[11] Many of the foods that contain boron are likely to have beneficial effects on bone.[12] The daily requirement of boron has yet to be defined, but daily multivitamin and mineral supplements contain between 3 mg and 9 mg.[12]

Boron also has been shown to increase bone strength measures in rats and found to be effective on early bone regeneration in rabbits after expansion of midpalatal suture.[13] Hakkı et al[10] showed that boron can induce osteogenesis by regulating RunX2, bone sialoprotein (mRNA expression level), and bone morphogenetic protein-4, -6, and -7 (protein level) in osteoblastic cells *in vitro*. Also, Demirer et al[14] reported that systemically administered BA diminishes alveolar bone loss, decreases inflammatory cell infiltrate, and increases osteoblastic activity in experimental periodontitis in rats. In a previous study, we also demonstrated that 30 mg/kg and 50 mg/kg boric acid decreased osteoclastic activity in diabetic rats.[15]

In an attempt to find an alternative treatment for osteoporosis and based on these favorable aspects of BA, we hypothesized that boron might be a potent suppressor of bone loss in osteoporosis. Therefore, the aim of this study is to investigate the effect of BA on alveolar bone loss and mandibular bone density in osteoporotic rats with periodontitis.

Materials and methods

Thirty-six male Wistar rats, with an average weight of 270—320 g, were used in this study. They were housed in specially designed wire cages and maintained on a 12 hour/12 hour light/dark cycle with a constant room temperature of 23°C. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Cumhuriyet University, Sivas, Turkey. Adequate measures were taken to minimize pain or discomfort in animals. The animals were randomly divided into five groups as follows: nonligated control group (C, $n = 6$); ligated periodontitis group (P, $n = 6$); osteoporosis only group (O, $n = 8$); ligated periodontitis and osteoporosis group (O+P, $n = 8$); and ligated osteoporosis with 50 mg/kg/d BA group (BA50, $n = 8$).

Induction of osteoporosis

Twenty four rats were administered retinoic acid (80 mg/kg). The other rats were administered sham injections. Retinoic acid was mixed with olive oil and 0.5 mL of this mixture was given to the rats via intraperitoneal injection for 15 days.

Induction of experimental periodontitis

After osteoporosis was achieved in the groups, rats in the P, O+P, and BA50 groups received ligature placement performed under general anesthesia using ketamine (40 mg/kg, Eczacibasi Ilac Sanayi, Istanbul, Turkey). A 4-0 silk suture (Dogsan Ilac Sanayi, Istanbul, Turkey) was submarginally placed around both the right and left first molars of mandibular quadrants. The sutures were checked after application, and lost or loose sutures were replaced. All ligatures were placed by the same operator (H.B.Y.). The animals were kept in individual cages and received water and food *ad libitum*. BA was prepared as 50 mg/kg for 0.5-mL distilled water and systemically administered by gastric feeding at a rate of 0.5 mL daily for 15 days. The other rats were administered saline solution. On Day 15, the animals were sacrificed and the tissues were prepared for morphometrical and histopathological analyses.

Measurement of alveolar bone loss

The mandibles were stained with aqueous methylene blue (Merck & Co., Inc., Whitehouse Station, NJ, USA; 1%) to identify the cemento-enamel junction. The alveolar bone height was measured under a stereomicroscope (×25 magnification; Stemi DV4, Carl Zeiss, Jena, Germany) by recording the distance from the cemento-enamel junction to the alveolar bone crest. Measurements were taken at

three points on both the buccal and lingual sides to quantify the alveolar bone level. A mean value for each tooth was calculated. The morphometric measurement of alveolar bone loss was performed by a single examiner (H.T.) who was unaware of the identity of samples.

BMD measurement

After the removal of the soft tissue around the left mandible, digitalized X-ray investigations and BMD measurements were performed. All radiographs were taken by the same researcher (H.O.) with a digitalized intraoral imaging system in the same conditions. Briefly, a radiation tube was placed 10 cm far from a stabilized table. A certain distance was settled between the mandible and the tube. A 5-mm metallic bar was placed onto a pink wax plate, which had the same dimensions as the radiographic film. Standard radiographs were taken with the same setting of the system and then all images were transferred to an image analysis program (ImageJ, National Institutes of Health, Bethesda, MA, USA). BMD measurements were performed in a standard-sized toothless area below the apices of the first molar in all images.

Histopathological evaluations

Right mandibles were fixed in 10% neutral buffered formalin. Tissues were then decalcified in a fixative-added decalcification solution containing EDTA with a change twice a week for 8 weeks until decalcification was completed. The specimens were then dehydrated through an ethanol series and embedded in paraffin. Each sample was sliced into 5-μm continuous sections and prepared for hematoxylin and eosin, histochemical staining for tartrate-resistant acid phosphatase (TRAP), and immunohistochemistry staining for inducible receptor activator of nuclear factor kappa-B ligand (RANKL). The periodontal tissues around the mandibular first molar teeth were examined. Inflammatory cell infiltration (ICI) was determined and ICI scoring was based on the inflammatory cell accumulation around the first molars. ICI was determined with a semiquantitative scoring as no visible ICI (0), slightly visible ICI (1), moderately visible ICI (2), and the dense ICI (3). Osteoblast cells, i.e., forming surfaces, by the visibility of active bone formation surfaces that were bordered by the osteoid and cuboidal osteoblasts in the examined area were counted.

TRAP histochemistry

Deparaffinized sections were subjected to TRAP staining, to identify osteoclasts. TRAP staining was performed according to the manufacturer's protocol using the TRAP staining kit (Sigma-Aldrich, St Louis, MO, USA). Bright red staining of the TRAP+ osteoclasts was closely monitored under the microscope. Stained sections were washed in deionized water and sections were counterstained with Gill's hematoxylin and analyzed using light microscopy (Nikon Eclipse, E 600; Nikon, Tokyo, Japan). Multinucleated giant cells with ruffled border and resorption lacunae were considered to be osteoclasts and TRAP+ osteoclast cells neighboring periodontal ligament surrounding the tooth were counted.

RANKL immunohistochemistry

After deparaffinization and dehydration of the sections, antigen retrieval was performed using 10mM sodium citrate buffer (pH 6.0) for 2 hours at 70°C. The sections were then treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. After incubation with normal rabbit serum for 30 minutes, samples were incubated with primary antibodies overnight. The antibodies and conditions used were as follows: goat polyclonal anti-*inducible nitric oxide synthase* (Santa Cruz Biotechnology, Inc., Dallas, Texas, U.S.A.; 1:100). After washing several times with phosphate-buffered saline, the sections were incubated with biotinylated immunoglobulin G for 30 minutes, washed several times with phosphate-buffered saline, and reacted with streptavidin—horseradish peroxidase conjugated reagent for 30 minutes. Following 5-minute washes (3 times) with phosphate-buffered saline, samples were incubated with 3,3′-diaminobenzidine chromogen to visualize the immunoreactivity. Sections were counterstained with hematoxylin and analyzed using light microscopy (Nikon Eclipse, E 600; Nikon). Alveolar bone areas surrounding roots of the first molars were examined and RANKL evaluation was made by measuring the RANKL+ areas of the bone surrounding teeth. The percentage of RANKL+ area to the examined area was calculated. RANKL presence $< 25\%$ of the areas surrounding teeth were scored as 1, 25—50% were scored as 2, 50—75% were scored as 3, and $> 75\%$ were scored as 4.

Statistical analysis

Data were presented as mean ± standard deviation or percentage as appropriate. Osteoclast and osteoblast numbers, alveolar bone loss, RANKL, and mandibular bone density were analyzed with analysis of variance followed by Tukey test for pair-wise comparisons. A P value < 0.05 was considered statistically significant.

Results

Experimental procedures were performed successfully and there were no complications.

The presence of the silk ligature around the first molar induced an inflammatory reaction in the periodontal tissue. Measurement of alveolar bone loss in the mandibular molar tooth revealed significantly higher bone loss values in the O+P group compared with the other groups ($P < 0.05$; Figure 1). Administration of BA decreased the negative effects of osteoporosis on periodontal destruction. The lowest alveolar bone loss was 0.57 mm in the C group. Also, there was no significant difference in alveolar bone loss between the C and O groups ($P > 0.05$).

Histological sections from the C group showed normal architecture in both the periodontal ligament and the alveolar bone tissues. Sections from the O groups revealed thinning of the bony trabeculae with multiple resorption foci along the bone surface. The TRAP-positive osteoclast cell numbers of the study groups are shown in Figure 2. The

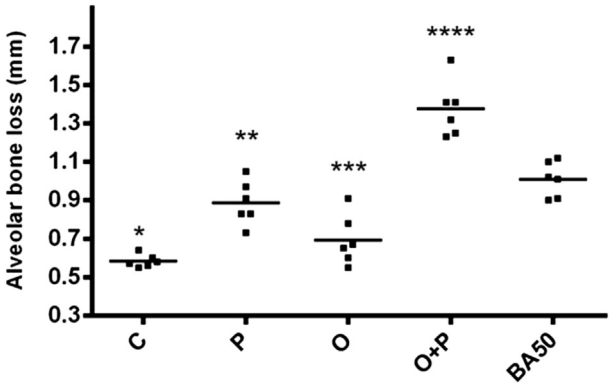

Figure 1 Alveolar bone loss in the study groups. * $P < 0.05$ versus P, O+P, and BA50 groups. ** $P < 0.05$ versus O+P group. *** $P < 0.05$ versus O+P and BA50 groups. **** $P < 0.05$ versus BA50 group. BA50 = boric acid 50 mg/kg/d group; C = control group; O = osteoporosis group; O+P = osteoporosis and periodontitis group.

osteoclast number was highest in the O+P group and lowest in the C group. The differences between the C and O+P groups were significant ($P < 0.05$). The differences among the other groups were not statistically significant ($P > 0.05$). RANKL staining scores were lowest in the C group and highest in the O+P group but the difference was not statistically significant ($P > 0.05$; Figure 3).

Osteoblast number was highest in the BA50 group and lowest in the O group. Although osteoblast number in the experimental groups was decreased after osteoporosis induction, the difference in osteoblast numbers did not reach significance between P and BA50 groups ($P > 0.05$). The osteoblast number in the O group was significantly lower than C, P, and BA50 groups ($P < 0.05$) but there were no significant differences between the O and O+P groups ($P > 0.05$; Table 1).

Mandibular density measurements were highest in the control group and lowest in the O+P group but there was no significant difference among the groups either ($P > 0.05$; Figure 4). The results are shown in Table 1.

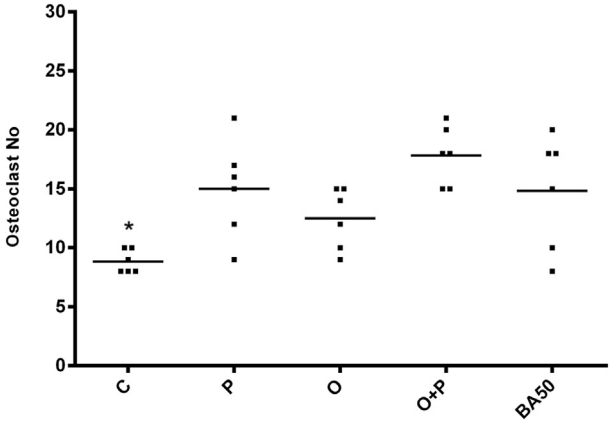

Figure 2 Osteoclast numbers in the study groups. * $P < 0.05$ versus O+P group. BA50 = boric acid 50 mg/kg/d group; C = control group; O = osteoporosis group; O+P = osteoporosis and periodontitis group.

Discussion

Osteoporosis is a systemic disorder characterized by reduced BMD throughout the skeletal system, including the jaws, and compromises bone strength that predisposes to increased risk of fracture, particularly hip fractures.[16] In a systematic review, it has been suggested that systemic bone loss could increase the risk of osteoporotic fractures affecting the mandible and increase the risk of developing periodontitis.[17] The correlation between periodontitis and systemic osteoporosis is generally determined based on radiological criteria, clinical criteria, or both. The results of the studies are controversial; some studies found no relationship between osteoporosis and periodontitis and some found a positive correlation.[17,18] There is no standardization in the studies investigating the relationship between periodontitis and osteoporosis.[17,18]

Pharmacologic options for osteoporosis prevention or treatment are bisphosphonates (alendronate, ibandronate, risedronate, and zolendronic acid), teraparatide (*parathyroid hormone* N-terminal amino acids 1–34), estrogens, the estrogen receptor modulators raloxifene or bazedoxifene, calcitonin, and the RANKL inhibitor denosumab.[19] These drugs are also used in patients with metastasizing bone disease.[20] In spite of being very effective in osteoporosis treatment and preventing osteoprotic fractures, these drugs might have serious side effects such as medication-related osteonecrosis of the jaw.[19] Bisphosphonate-related osteonecrosis of the jaw has been characterized as the main side effect of bisphosphonate therapy and it is a hard-to-treat condition.[9,21]

Recently boron was shown to promote skeletal health by targeting the pathways of osteoblast and osteoclast differentiation and survival of these cells.[22] Ying et al[22] reported that boron can increase osteogenic effects by stimulating osteogenic differentiation-related marker gene synthesis during the proliferation and differentiation phase in human *bone marrow stromal cells* and could be a promising approach for enhancing osteogenic capacity. Boron increased bone strength and bone ash content without detrimental effects in chickens.[23] Supplemental boron as BA has also been shown to increase bone strength measures in rats.[24] In addition, boron has been found to be effective in early bone regeneration in rabbits after expansion of the midpalatal suture.[13] Also, it has been shown that boron increased the mRNA expression of collagen type I, osteopontin, bone sialoprotein, osteocalcin, and RunX2 and protein levels of bone morphogenetic protein-4, -6, and -7 *in vitro*.[10]

Boron might play a role in the maintenance of bone metabolism, especially in the case of certain vitamin and mineral deficiencies such as vitamin D, magnesium, and potassium. It is reported that when there is no nutritional deficiency or metabolic stress, the need for boron seems to be low.[25] Skeleton, kidney, and brain are the tissues most affected by boron deprivation. Daily boron intake with diet is in the range from 0.5 mg/d to 3.1 mg/d.[26] Recently, we have reported that 50 mg/kg BA decreased inflammation and alveolar bone loss and increased osteoblastic activity in diabetic Wistar rats.[15] Furthermore, in this study, BA administration decreased alveolar bone resorption via induction of osteoblastic activity in retinoic acid-induced osteoporotic rats with periodontitis.

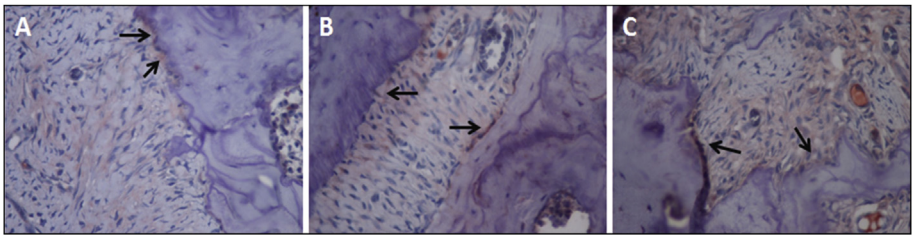

Figure 3 Representative receptor activator of nuclear factor kappa-B ligand staining in the groups. Arrows indicate receptor activator of nuclear factor kappa-B ligand positive staining areas. (A) Control group (×200); (B) osteoporosis and periodontitis group (×200); (C) boric acid 50 mg/kg/d group (×200).

Table 1 Osteoblast numbers and mandibular density measurements in the study groups. Data are given as mean ± standard deviation.

Groups	Osteoblast No.	Mandibular density
C	39.16 ± 20.59	0.39 ± 0.03
P	30.83 ± 6.64	0.38 ± 0.02
O	20.66 ± 6.68*,**	0.38 ± 0.02
O+P	29.16 ± 10.75*	0.36 ± 0.03
BA50	49.16 ± 18.00	0.39 ± 0.01

* $P < 0.05$ versus P group.
** $P < 0.05$ versus BA50 group.
BA50 = boric acid 50 mg/kg/d group; C = control group; O = osteoporosis group; O+P = osteoporosis and periodontitis group.

mediators mainly associated with bone remodeling is receptor of RANK, a very important cytokine for differentiation and activation of osteoclasts. It was reported that the administration of serum RANKL to mice promoted osteoclast growth and activation, leading to osteoporosis. Expression of RANKL is a good indicator of osteoclastic activity and bone loss. Recently it was found that boron is a physiological regulator of the normal inflammatory response,[28,29] inhibits the activities of specific enzymes involved in extracellular matrix turnover and metabolism, and reduces RANKL expression. However, we found that BA had no effect on RANKL expression in our study. In addition, systemically administered BA did not reduce TRAP+ osteoclast cell numbers but diminished tissue destruction. The percentages of the RANKL+ areas in periodontal ligaments and alveolar bone within the groups were similar.

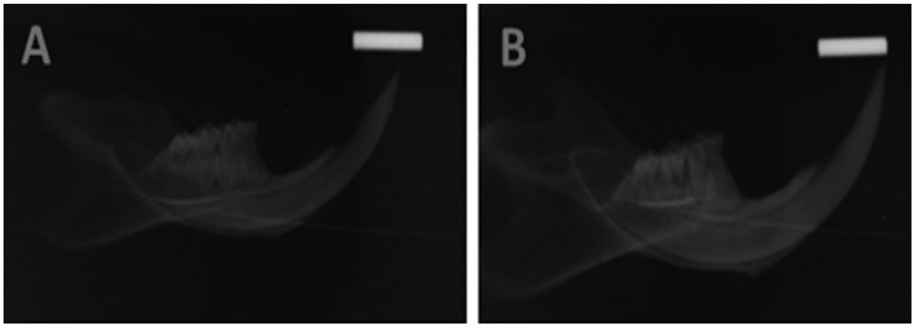

Figure 4 Representative radiographic images of mandibles. (A) osteoporosis and periodontitis group; (B) boric acid 50 mg/kg/d group.

Boron can be toxic when fed in higher amounts like all minerals. Boron, BA, and boron oxide are primarily irritants under exposure conditions. Biochemical symptoms of toxicity include riboflavinuria and riboflavin deficiency, along with the inhibition of the dehydrogenase enzymes. Toxic ingestions of boron may cause nausea, vomiting, and diarrhea.[27] A percentage of BA (17.48%) is boron and the fatal dosages of boron for humans and rats are 640 mg/kg and 2660 mg/kg, respectively. In our study we used the dosage of 50 mg/kg BA and this amount of BA contains 8.92 mg/kg boron. Considering the average weight of the rats in our study, the dose of boron given to the rats was 2.95 mg and this dose is in the limits of daily consumption.[12,26]

Bone remodeling is a physiological process resulting from the osteoblastic and osteoclastic activity. One of the

Retinoic acid-induced osteoporosis model involved minimal trauma, rapid assaying, and was sensitive and specific. Also, it was shown that serum calcium level, total body BMD, and isolated left femora BMD after ashing had all significantly decreased in retinoic acid-induced osteoporotic rats.[30] As an alternative for retinoic acid-induced osteoporosis model, it is reasonable to consider the ovariectomized female rat models for a postmenopausal osteoporosis study. However, the ovariectomized model should be curtailed due to its longer and more complicated experimental process.[16,30] Besides, there are some limitations of animal studies of osteoporosis. Osteoporosis is a chronic disease mostly resulting from postmenopausal hormone alterations.[1,31] The alteration of bone tissue observed in osteoporosis is a slowly progressive long process

and the etiopathogenesis of osteoporosis is different from other chronic inflammatory bone diseases. Unlike the slow nature of the disease seen in humans, retinoic acid-induced osteoporosis occurs in 15 days with a rapid change in rat bone, mimicking secondary osteoporosis in humans.

Ligature methods have been accepted as useful experimental models of periodontitis with alveolar bone resorption.[32–35] This ligature results in bacterial plaque accumulation and triggers an inflammatory response, reproducing human periodontal disease.[32,35] In the present study, ligature placement on the first molar tooth caused a significant amount of bone loss and also, the amount of bone loss was the highest in osteoporotic rats. However, there are several limitations of animal studies. Firstly, although molars in rats are similar in anatomic configuration and structure to humans, they are smaller making it difficult to perform any sort of periodontal treatment. Secondly, another limitation of the experimental model used is that the induced periodontitis follows an acute course, during which tissue trauma and adjacent microbial accumulation accelerate the destructive process. Such pathways of acute inflammation are likely to differ from chronic periodontitis.[32,34]

In conclusion, our results revealed that osteoporosis may lead to enhanced alveolar bone loss in experimental periodontitis. Furthermore, this study represents, within the inherent limitations between experimental animal and human disease interventions, the evidence that systemic administration of BA decreases alveolar bone loss in experimental periodontitis in osteoporotic rat models.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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