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Effects of local simvastatin–alendronate conjugate in preventing periodontitis bone loss

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Background and Objective: Local host-modulation therapy is an emerging approach to prevent disease progression in sites with moderate periodontitis. The combination of simvastatin and alendronate would be an intriguing host-modulatory strategy because of the bone-anabolic properties of simvastatin and the antiresorptive/bone-targeting characteristics of alendronate. The objective of this study was to evaluate the effects of local administration of a simvastatin–alendronate–β-cyclodextrin (SIM–ALN–CD) conjugate for preventing experimental periodontitis bone loss.

Material and Methods: Twenty-four mature female Sprague–Dawley rats were treated with three, 12 μL injections, administered one week apart, bilaterally into the palatal/interproximal gingiva. The injections contained: (i) a conjugate of 0.5 mg of SIM and 3.75 mg of ALN–CD in H_2O; (ii) H_2O alone; or (iii) no treatment. One week later, the same sites were subjected to induction of experimental periodontitis by three injections (i.e. one injection administered every other day for five d) of 0.01 mg of *Escherichia coli* endotoxin [lipopolysaccharide (LPS)] in phosphate-buffered saline (PBS) or PBS alone. After an additional week, the rats were killed, the palates were harvested and interproximal bone volume and adjacent thickness were calculated using microcomputed tomography. Subsequently, specimens were decalcified, and interproximal histologic sections were stained with hematoxylin and eosin for evaluation of alveolar crest osteoclasts and surrounding inflammation. Values were compared among treatment groups using analysis of variance and the Kruskal–Wallis test.

Results: Interproximal bone volume was reduced by LPS injections ($p \leq 0.04$), yet when experimental periodontitis was preceded by treatment with SIM–ALN–CD, more bone was preserved than after treatment with carrier alone ($p = 0.007$). While LPS caused a significant loss in bone thickness over the palatal roots ($p \leq 0.04$), the injection protocol (PBS) also caused a significant loss of palatal bone thickness ($p \leq 0.03$). However, prophylactic SIM–ALN–CD injections resulted in no further loss of bone thickness during experimental periodontitis. LPS injections gave histologic evidence of increased osteoclasts and subsulcular inflammation, both of which were reduced when preceded by treatment with SIM–ALN–CD ($p \leq 0.0002$).

Conclusion: The primary conclusion of this study was that locally applied SIM–ALN–CD has the potential to prevent episodes of periodontitis bone loss.

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Traditional methods used to treat periodontitis and maintain periodontal health are aimed at the removal of bacterial plaque (biofilm) and calculus from around the teeth, thereby reducing inflammation caused by the bacteria. Variations in microbial plaque and host competency may occur in the same individual over time (1). Therefore, patients more susceptible to periodontal infections are placed on strict maintenance intervals. Periodontal maintenance appointments serve to evaluate progression of the disease, remove plaque and calculus, implement further treatment if necessary and reinforce the importance of adequate home care. Owing to the difficulty in establishing a bacteria/calculus-free environment, local and systemic antibiotics may be used as adjuncts during or after mechanical periodontal therapy to reduce the bacterial load and improve clinical health (2,3). Local antibiotics are placed into periodontal pockets and released over time, with the intention of targeting bacteria within the pathological sulcus.

Host modulation is another method used to treat periodontitis. The goal of this therapy is to alter the way in which the host responds to the bacteria, thereby decreasing the progression of the disease. Nonsteroidal anti-inflammatory agents, subantimicrobial doses of doxycycline, bone morphogenic proteins, and growth factors have all been studied (1,4–6). Because prevention of periodontal bone loss is so important in maintaining a stable periodontium, the use of simvastatin (a cholesterol-lowering drug with bone-anabolic properties) and bisphosphonates (antiresorptive agents) have been evaluated for their effects on inhibiting bone loss resulting from periodontitis. Systemic statins have shown little impact on preserving periodontal bone, presumably because of their rapid removal from the bloodstream by the liver (7). Although, bisphosphonates have been found to reduce bone loss associated with periodontitis (8–10), concerns about vulnerability to osteonecrosis of the jaw have dampened the enthusiasm for this approach (11).

Local application of simvastatin and the bisphosphonate, alendronate, have been evaluated *in vivo*. Locally applied simvastatin has been shown to be a potent stimulator of bone formation and bone morphogenic protein-2 synthesis (12). When used on maxillary/mandibular bone, simvastatin increased bone thickness and defect repair in animals (13–17) and in humans (18,19). Similarly, bone morphogenic protein-2 stimulation was seen when topical simvastatin was applied *in vivo* (20–22). Local application of alendronate to oral bone has also been shown to preserve or stimulate bone growth (23–31). Alendronate can be conjugated to beta-cyclodextrin, resulting in a bone-targeting delivery system that has high affinity for hydroxyapatite in bone and forms a carrier for bone-anabolic agents (32), such as simvastatin acid. This combination would be intriguing, potentially allowing the locally applied complex to be focused and retained on bone, releasing a bolus of bone-anabolic simvastatin, and then, as the cyclodextrin degenerates, the antiresorptive capabilities of alendronate could become active to preserve the new bone.

The current study was designed to evaluate the effects of a simvastatin and alendronate conjugate as a preventative agent against a future episode of periodontitis. The complex, or its carrier alone, was applied before the initiation of experimental periodontitis induced by lipopolysaccharide (LPS) injections [or phosphate-buffered saline (PBS) injection controls] into the interproximal gingival tissues of rat molars (33). Micro-computed tomography (μCT) was used to evaluate bone volume and bone thickness in the areas of the injections. In addition, histologic specimens were subsequently processed to evaluate osteoclast activity and inflammation. Our hypothesis was that the simvastatin/alendronate conjugate would inhibit bone loss in LPS-induced periodontitis in rats.

Material and methods

Twenty-four mature retired-breeder female Sprague Dawley rats (Harlan Teklad, Madison, WI, USA) were used for μCT analysis of bone responses to prophylactic drugs in the experimental periodontitis model. The rats were allowed to acclimate for 1 wk before the first procedure. All animals were treated and housed in the University of Nebraska Medical Center (UNMC) College of Dentistry Animal Facility under the auspices of the UNMC Animal Care and Use Committee (IACUC # 09-071-09-FC).

Experimental periodontitis

Escherichia coli endotoxin (LPS) was given to induce experimental periodontitis using a protocol modified from Ramamurthy *et al.* (33). All rats with experimental periodontitis received palatal interproximal injections of 10 μL of *E. coli* endotoxin (1 mg/mL of LPS in PBS) palatally between the maxillary first and second molars (M1/M2) and between the maxillary second and third molars (M2/M3) every other day for five d. The same injection protocol, but of PBS only, was given to rats as a non-periodontitis control. Some animals had an untreated side in the maxilla followed by treatment with PBS, so the contralateral side (H_2O/PBS) could be compared with the drug/PBS groups to determine any crossover effects of the drug treatments on the opposite side of the arch.

Experimental groups

The experimental periodontitis rats were divided into one of three groups of eight animals each and injections were performed interproximally between M1/M2 and M2/M3 according

to the timetable shown in Table 1. Group (i): 12-μL injections containing 0.5 mg of simvastatin and 3.75 mg of alendronate β-cyclodextrin in H_2O (SIM–ALN–CD) on the right side and H_2O alone on the left side; then 10 μL of PBS on both right and left sides. Group (ii): SIM–ALN–CD on the right side and H_2O on the left side; then 10 μL of *E. coli* LPS in PBS on both the right and left sides. Group (iii): no treatment on the right side, H_2O on the left side; then PBS on both the right and left sides. Before each procedure, the rats were weighed to monitor any changes in weight. Drugs were injected once a week for 3 wk, and PBS/LPS was injected every other day for five d.

Drug preparation and application

Individual drug dosages for experimental periodontitis were based on: (i) previous studies showing that three, weekly injections of 0.5 mg of simvastatin caused significant growth of rat alveolar bone (15) and (ii) 3.75 mg is the maximum amount of alendronate in the cyclodextrin conjugate needed to incorporate 0.5 mg of simvastatin acid. Synthesis of the alendronate–cyclodextrin conjugate has been described previously (32). Briefly, β-cyclodextrin was first reacted with p-toluene-sulfonyl chloride to obtain mono-6-(azido)-β-cyclodextrin. Simultaneously, alendronate was modified to form 1-hydroxy-4-penta-4-ynamidobutane-1,1–diyldiphosphonic acid. A "click" reaction of these two products resulted in the final product of alendronate β-cyclodrextrin (ALN–CD) (32). To be inserted into the annulus of the ALN–CD conjugate, simvastatin was converted into simvastatin acid. Its complex with ALN–CD (SIM–ALN–CD) was then produced by mixing simvastatin acid and ALN–CD in deoionized water and filtration through a 0.22-μm nylon filter (34). All components of the conjugate were assessed, using high-performance liquid chromatography, to be > 95% pure.

Anesthesia/death

Anesthesia was induced by placing the rats in an induction chamber with 1–4% isofluorane/100% O_2 (1–3 L/min), followed by application of a nose cone with 0.5–2% isofluorane/100% O_2 (0.5–1 L/min) to maintain anesthesia during the injections.

One week following the final LPS/PBS injections, all rats were killed by CO_2 asphyxiation. The maxillary area was separated from the rest of the skull and the entire palate was placed in 10% formalin for storage before μCT scans were performed.

μCT measurements

The palates were scanned using a high-resolution μCT system (Skyscan 1172; Skyscan, Aartselaar, Belgium). The X-ray source was set at a voltage of 70 kV with current of 141 μA and a fixed exposure time of 110 ms. The resolution was 12 μm using a camera pixel size (1000 × 668) with a 0.5-mm aluminum filter. Five frames were averaged for each rotation with a rotation step of 0.7° following an angle of 180°. Three-dimensional reconstructions were performed using the system-reconstruction software (NRecon; Skyscan) (Fig. 1). All specimens were aligned on three axes until the cemento–enamel junctions (CEJs) were in an even plane, then coronal sections were used to obtain the measurements (Fig. 2). The raw images reconstructed by the program were enhanced by use of a histogram. By setting the upper and lower ranges on a bimodal graph, the histogram resulted in optimal image contrast (to define bone and root from other tissues) before taking measurements.

Bone-volume measurements were taken interproximally between the first- and second-molar roots and were analyzed in the total volume of interest, which was a 0.41 × 0.41-mm interproximal core extending through all horizontal sections. The bone thickness was evaluated at 0.2-mm intervals on the mesio-lingual root of second molars (between the two palatal injections) starting 0.2 mm from the CEJ (before first bone) and extending 2.0 mm apically (near apex) (NRecon; Skyscan).

Histologic analysis

Following the μCT scans, specimens were decalcified in 5% formic acid for at least 1 wk, then blocked parallel to the long axis of M1 and M2 on the lingual margin in order to allow subsequent histologic sections to show the interproximal region between the two teeth. Specimens were then dehydrated and embedded in paraffin, and 5-μm sections were coded and stained with hematoxylin and eosin.

The interproximal alveolar crest surface length was measured between M1 and M2 to where the periodontal

Table 1. Injection schedule for experimental periodontitis

Group	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
1	SIM–ALN–CD Right H_2O Left	SIM–ALN–CD Right H_2O Left	SIM–ALN–CD Right H_2O Left	No tx	PBS X3 Right/Left	Death
2	SIM–ALN–CD Right H_2O Left	SIM–ALN–CD Right H_2O Left	SIM–ALN–CD Right H_2O Left	No tx	LPS X3 Right/Left	Death
3	No tx Right H_2O Left	No tx Right H_2O Left	No tx Right H_2O Left	No tx	PBS X3 Right/Left	Death

Left, left side of mouth; LPS, lipopolysaccharide; No tx, no treatment; PBS, phosphate-buffered saline; Right, right side of mouth; SIM–ALN–CD, simvastatin acid-alendronate β-cyclodextrin; X3, three injections over five d

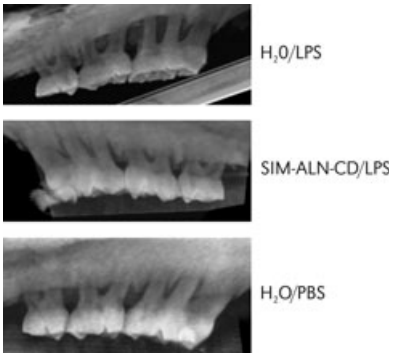

Fig. 1. Three-dimensional reconstructions of maxillary molars using microcomputed tomography (μCT). LPS, lipopolysaccharide; PBS, phosphate-buffered saline; SIM–ALN–CD, simvastatin–alendronate–β-cyclodextrin conjugate.

ligament was of uniform thickness using a light microscope, at 200× magnification, with digital camera/software (Prof Res C3; JENOPTIK Optical Systems, Jena, Germany). Osteoclasts were counted using osteoclast morphology (large, multinucleated cells located on the bone surface in resorption lacunae), and the adjacent surface length was measured, as previously reported (13). Osteoclast identification of similar cells was confirmed on additional sections using anti-TRAP goat polyclonal immunoglobulin G (1 : 50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by a goat HRP-DAB Kit (R & D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The severity of the inflammatory process was estimated by two investigators (R.A.R., N.N.) masked to the experimental conditions and using a scoring system modified from Coimbra *et al.* (35) where polymorphonuclear leukocytes, mononuclear leukocytes and blood vessels were ranked as follows: 0 = no inflammatory cells; 1 = few inflammatory cells or blood vessels; 2 = inflammatory cells and blood vessels scattered throughout the connective tissue; and 3 = predominance of inflammatory cells and blood vessels. Two areas were evaluated: connective tissue above the alveolar crest but below the transseptal fibers; and connective tissue subjacent to the sulcular epithelium.

Statistical analysis

For μCT analysis, eight rats per group were used in this study, which was based on histological detection of bone gain following administration of three injections of 0.5 mg simvastatin at one week intervals (15). Specimens were coded by animal number and side (right/left) and were measured without the knowledge of group designation. Analysis of variance was used for intergroup comparisons. Histologic parameters were compared among groups using the Kruskal–Wallis test. The level of significance was set at $p \leq 0.05$.

Results

The weight of the animals (Fig. 3) showed no significant change over the course of the study. Bone volume measurements (Fig. 4) showed that interproximal injections of H_2O followed by PBS (H_2O/PBS; drug carrier and nonperiodontitis controls) preserved significantly more bone volume than did carrier plus experimental periodontitis (H_2O/LPS, $p \leq 0.04$). In addition, when prophylactic SIM–ALN–CD injections preceded LPS (SIM–ALN–CD/LPS), significantly more bone volume was preserved than when LPS was preceded by H_2O (H_2O/LPS, $p = 0.007$). When SIM–ALN–CD injections were given on the right side followed by PBS (SIM–ALN–CD/PBS), bone volume on the left side (H_2O/PBS) was not different from H_2O/PBS contralateral to no treatment/PBS.

Palatal bone thickness over the mesio-lingual root of M2 also showed significant differences among groups (Fig. 5). Although 10 measurements were taken at 0.2-mm intervals from the CEJ to 2.0 mm from the CEJ, the results of only three levels are given here: 0.6 (first level where bone was consistently

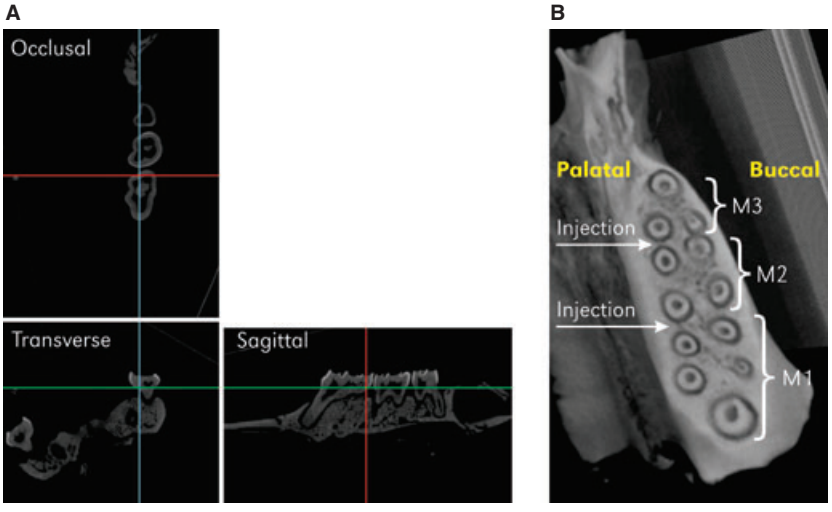

Fig. 2. (A) Alignment of specimens on three axes at the level of the cemento–enamel junction before obtaining measurements. (B) Orientation of the coronal section through the roots that were used for measurements.

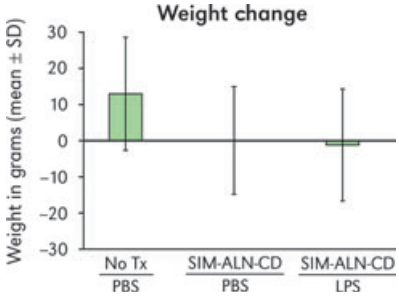

Fig. 3. Weight change (in g) in experimental periodontitis groups from baseline to death. Results are given as mean ± standard deviation. LPS, lipopolysaccharide; No TX, no treatment; PBS, phosphate-buffered saline; SIM–ALN–CD, simvastatin–alendronate–β-cyclodextrin conjugate.

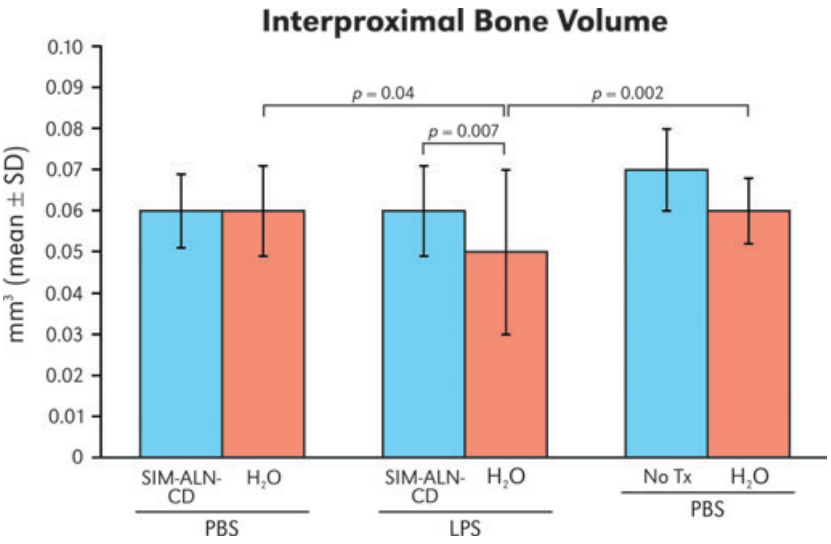

Fig. 4. Effect of prophylactic administration of the simvastatin–alendronate–β-cyclodextrin conjugate (SIM–ALN–CD) on interproximal bone volume (mm^3). The bars show mean ± standard deviation. Statistically significant differences among groups are noted. LPS, lipopolysaccharide; No TX, no treatment; PBS, phosphate-buffered saline.

present), 1.0 and 1.4 mm (one-half and two-thirds of the root length) from the CEJ. This allows easier interpretation of the data and to focuses on the area where the injections were applied. Consistent findings at all three levels were: (i) LPS injections caused decreased bone thickness (H_2O/LPS vs. H_2O/PBS); (ii) injections themselves caused decreased bone thickness [no treatment (No Tx) /PBS vs. H_2O/PBS]; (iii) additional LPS injections did not cause significant further bone loss after SIM–ALN–CD prophylaxis (SIM–ALN–CD/PBS vs. SIM–ALN–CD/LPS); and (iv) bone thickness on the opposite side from SIM–ALN–CD generally showed no differences in bone thickness than when opposite the no-treatment side (H_2O/PBS comparisons).

Histologic evaluation indicated a uniform absence of inflammation next to the interproximal alveolar crest, below the transseptal fibers, in all groups. Osteoclasts on the alveolar crest also were rare, the only exception being in the H_2O/LPS group (Table 2, Fig. 6). Likewise, the H_2O/LPS group had a higher mean inflammation score adjacent to the gingival sulcus compared with SIM–ALN–CD/LPS and other groups.

Discussion

Experimental drugs and LPS/PBS were injected into the palatal side of M1/M2 and M2/M3 interproximal gingiva. The experimental periodontitis model was modified from that of Ramamurthy *et al.* (33), in which injections of *E. coli* LPS were given buccally and palatally between only M1/M2 to induce bone loss. Therefore, the effects of injections in the current study would have been more concentrated on the palatal aspect of M2. This prompted us to focus our μCT measurements on the palatal and interproximal surfaces of the mesio-lingual root of M2. Previously, we have also used *Porphyromonas gingivalis* LPS (36) because *P. gingivalis* is an important pathogen in periodontitis (37). However, *E. coli* LPS is more typical of the LPS of the majority of periodontal pathogens because *P. gingivalis* LPS lacks the traditional heptose and 2-keto-3 deoxyoctonate in the core sugars and has no myristic acid in the lipid A (38).

Measurements of interproximal bone volume showed that LPS injections caused a decrease in bone volume (H_2O/PBS vs. H_2O/LPS), supporting the validity of the experimental periodontitis model using μCT volumetric analysis. When the right side was treated with SIM–ALN–CD/PBS, no difference was seen on the left side (H_2O/PBS), relative to when no treatment was applied to the right side. This supports the lack of cross-over effects from local injections of SIM–ALN–CD to the contralateral side. This is in accordance with the study of Yaffe *et al.* (39), who found small amounts of alendronate on the contralateral side and even lower amounts at sites distant from the drug application. The amounts found were

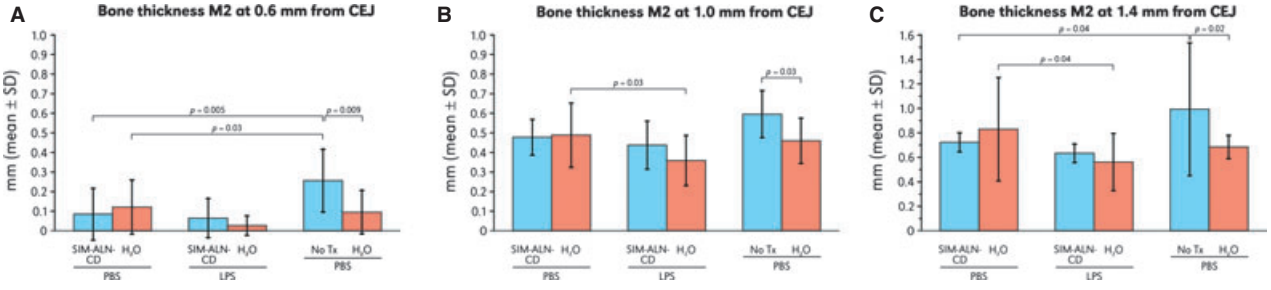

Fig. 5. (A–C) Effect of prophylactic administration of the simvastatin–alendronate–β-cyclodextrin (SIM–ALN–CD) conjugate on bone thickness over the mesio-lingual root of M2 at three levels from the cemento–enamel junction (CEJ). The bars show mean ± standard deviation. LPS, lipopolysaccharide; No Tx, no treatment; PBS, phosphate-buffered saline.

Table 2. Histologic evaluation of groups

Group	Osteoclast surface (%)	Osteoclast (mm)	Subsulcular inflammation score
SIM–ALN–CD/PBS (n = 8)	2.6 ± 4.8*	0.0002 ± 0.0004	0.3 ± 0.5
H_2O/PBS (n = 8)	1.1 ± 3.1	0.0001 ± 0.0003	0.1 ± 0.4***
SIM–ALN–CD/LPS (n = 8)	1.1 ± 3.0	0.0002 ± 0.0007	0.6 ± 0.5***
H_2O/LPS (n = 8)	14.1 ± 7.6**	0.0014 ± 0.0007****	1.9 ± 0.4**
No tx/PBS (n = 6)	2.5 ± 6.1	0.0003 ± 0.0007	0.3 ± 0.5

LPS, lipopolysaccharide; No tx, no treatment; PBS, phosphate-buffered saline; SIM–ALN–CD, simvastatin acid-alendronate β-cyclodextrin.*Mean ± standard deviation
**Different from all other groups, $p \leq 0.0002$
***Different between groups, $p \leq 0.05$
****Different from all other groups, $p \leq 0.0008$

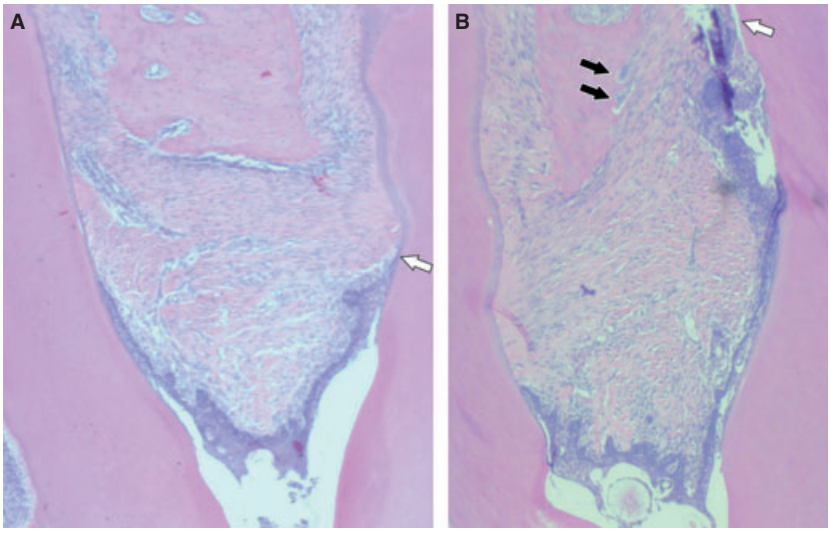

Fig. 6. Histophotomicrographs of M1/M2 interproximal lipopolysaccharide (LPS)-induced experimental periodontitis preceded by simvastatin–alendronate–β-cyclodextrin (SIM–ALN–CD) (A) or H_2O carrier alone (B). hematoxylin and eosin stain; original magnification × 100. Note that the apical epithelial migration (white arrows), bone resorption and osteoclasts (black arrows) seen after administration of H_2O/LPS (B) are absent after administration of SIM–ALN–CD/LPS (A).

not sufficient to inhibit resorption and the results confirm no effect from local crossover or systemic distribution of the drug. The finding that treatment with SIM–ALN–CD/LPS preserved more bone volume than did treatment with H_2O/LPS is consistent with the ability of SIM–ALN–CD to prevent periodontitis-induced interproximal bone loss and supports the main hypothesis of this research. Other pilot studies indicated that ALN–CD alone was not able to retard LPS-induced loss of interproximal bone volume (data not shown). The current findings are in accordance with other studies, in rats and humans, which have shown local application of simvastatin to be a stimulator of bone thickness and volume (12–19).

The use of interproximal bone volume, as determined by μCT, has advantages over defleshing the specimen, staining and then measuring the distance from the CEJ to the bone crest at multiple points, as has been traditionally done with the experimental periodontitis model (33,36). Consistently locating the CEJ or the bone crest on stained specimens was difficult with samples in our pilot studies, especially in interproximal crater defects. Furthermore, consistent alignment of defleshed or histologic specimens is also difficult, but critical for accurate measurements. μCT allowed easy identification of the CEJ and alignment in three planes, and volumetric evaluation of interproximal bone limited the variability of multiple point-to-point measurements. Park *et al.* (40) suggested that three-dimensional measurements were more sensitive in separating group effects. In addition, μCT allowed measurement of bone thickness, which cannot be accurately determined from defleshed specimens. Specimens used for μCT can also be subsequently decalcified for histologic evaluation, as was performed in the current protocol.

Bone thickness measured palatally to M2 roots consistently showed that injections both of LPS and of water caused bone loss. The LPS-induced reduction of bone thickness on the palatal root is expected in the experimental periodontitis model. Furthermore, trauma (injections) over thin bone has been shown to cause bone loss (41). It should be noted that SIM–ALN–CD followed by LPS resulted in no further significant bone loss than seen when followed by PBS. Simvastatin has been shown to be highly effective in augmenting bone thickness (15,17). Also, alendronate delivered locally has been shown to inhibit subsequent bone loss (23,26).

As injection of H_2O alone caused bone loss, perhaps it is the injection of 10–12 μL of fluid that is playing the major role, and not the drug itself. Therefore, other application methods for drug delivery should be explored. Methylcellulose gel containing simvastatin has been injected into human periodontal defects (18,19). Furthermore, local application of simvastatin in methocellulose gel in a polylactic acid membrane showed bone formation (13). Similarly, alendronate in a gel has been applied as an adjunct to scaling and root planing in mandibular Class II furcations (30) and in other intrabony defects (31), resulting in more bone compared with placebo gel. The incidence of osteonecrosis of the jaw following short-term local application would appear to be negligible (42). Subcutaneous and intravenous appli-

cations of alendronate have also been shown to inhibit alveolar bone resorption (8,10), but with higher osteonecrosis of the jaw concerns. SIM–ALN–CD appeared not to have a crossover effect on palatal bone thickness, as also was seen with interproximal bone volume.

Histologic evaluation of the specimens revealed that experimental periodontitis with three injections of LPS, preceded by three injections of water carrier, at one week intervals, resulted in more osteoclasts on the alveolar crest and subsulcular inflammation than with PBS injections. This confirmed that LPS induced experimental periodontitis, although the levels of osteoclast activity were lower than on bone lining the periodontal ligament (43). Most importantly, when SIM–ALN–CD preceded LPS, the levels of osteoclast activity and inflammation were significantly reduced. These findings were consistent with, and may partially explain, the preservation of interproximal bone volume and bone thickness in the SIM–ALN–CD/LPS group. These results were not unexpected because simvastatin previously has been shown to reduce inflammation (44) and osteoclast numbers (21) in rats. Alendronate has long been known to inhibit osteoclast activity in rats (45); however, local alendronate may have little effect on osteoclast numbers and inflammation (27,46).

Systemically applied parathyroid hormone (PTH) has recently been used to treat bone loss, including periodontal osseous regeneration (47). The potent anabolic effects of systemic and topical PTH have been demonstrated in rat experimental periodontitis models (48–50), making their combination with bisphosphonate another possible choice to prevent periodontitis. While the anabolic potential of PTH is probably superior to that of simvastatin, the need for intermittent application required most PTH protocols to use 12–30 injections of the protein, making use of PTH cost-prohibitive for the conventional prevention of periodontitis. Furthermore, the delivery of proteins uses a very different vehicle than used for small molecules such as simvastatin.

Taken together, the results of this study support the potential of locally applied SIM–ALN–CD to prevent episodes of periodontitis-induced bone loss.

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