Early alveolar bone regeneration in rats after topical administration of simvastatin

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Objectives. The aim of this study was to ultrastructurally examine the influence of simvastatin on bone healing in surgically created defects in rat mandibles.

Study design. Bone defects 0.8 mm in diameter were created in the buccal aspect of first mandibular molar roots and filled with 2.5% simvastatin gel, while the controls were allowed to heal spontaneously. The rats were humanely killed 7, 9, 11, or 14 days postoperatively, and the specimens were processed for scanning and transmission electron microscopy, as well as for colloidal gold immunolabeling of osteopontin.

Results. The regenerated alveolar bone in the simvastatin-treated defects presented smaller marrow spaces, and the collagen fibrils were regularly packed exhibiting a lamellar bone aspect. Osteopontin was present through the bone matrix during the wound healing and alveolar bone regeneration.

Conclusion. The present study provides evidence that a single topical application of 2.5% simvastatin gel improves the quality of the new bone and decreases bone resorption. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112:170-179)

Most surgeries involving maxilla and mandible are performed in the alveolar process that is the part of the jaw that contains the teeth. It is composed of 2 thick outer layers of cortical bone at its labial/buccal and lingual surfaces. A spongy cancellous bone with numerous marrow spaces forms the interior of the alveolar process, except for the wall of the alveolar socket that is composed of alveolar bone. The bone tissue at this area is formed by few thin layers with a parallel orientation to the cervical-apical direction of the root and anchors the collagen bundles of the periodontal ligament, which are also called Sharpey’s fibers. Although fundamentally comparable to other bones in the body, the alveolar bone is a specialized part of the mandibular and maxillary bones that forms the supporting periodontium of the teeth. It undergoes continuous and rapid remodeling in response to the high functional demands of mastication. Despite its high renewal potential, the alveolar bone and the alveolar process as a whole can be lost as a consequence of periodontal diseases. Thus, the use of drugs that might regulate the factors that stimulate alveolar regeneration is an interesting possibility in oral surgery.

Statins are effective lipid-lowering drugs widely used as reductors of the risk of cardiovascular disease given their capability to inhibit the 3-hidroxy-3-methylglutaryl coenzyme A reductase that is the rate-limiting enzyme in the synthesis of endogenous cholesterol. Statins inhibit the enzymes of the mevalonate pathway, reducing prenylation of GTPases like ras and Rho, which are important in the maintenance of cellular integrity, cytoskeleton, and vesicle traffic.2 Recent studies have demonstrated that statins may have pleiotropic effects in bone metabolism. Their effects on bone formation seem to be associated with the increase of bone morphogenetic protein-2 mRNA expression3-6 and with the enhancement of vascular endothelial growth factor expression in osteoblastic cells that are important factors for their differentiation.7 Additionally, statins appear to reduce bone resorption by decreasing the expression of RANKL and cathepsin K,6 the fusion of osteoclast precursors and by disrupting the actin ring of osteoclasts that result in a decrease in the number of active osteoclasts.8

These putative mechanisms of action could represent the rationale for the use of statins in alveolar bone regeneration. Thus, surgical procedures would take advantage of a local application because it allows a focused effect into specific bone defects. Recent studies tested the effect of the local application of simvastatin associated with different carriers, such as gelatin,9 polylactic/polyglycolic acid,10 and calcium sulfate,11 and

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have demonstrated potential to increase bone formation. However, there is not a consensus of an ideal carrier for the drug on bone defects and further studies are necessary.12 Additionally, ultrastructural aspects of the bone healing under simvastatin treatment are unknown.

To test the hypothesis that the topical administration of a single dose of simvastatin may improve alveolar bone regeneration, a periodontal window wound model in rats13,14 was used, by which a space to retain simvastatin-containing gel is created while preventing bacterial infection and epithelial down growth. Aiming to detect the areas of mineralized bone matrix formed inside the defect, a suitable scanning electron microscopy (SEM) approach was used. In addition, immunocytochemistry for the noncollagenous bone protein osteopontin (OPN), which is present at early bone regeneration,15 combined with transmission electron microscopy (TEM) was also carried out to evaluate the type of newly formed bone.

MATERIAL AND METHODS

Twenty-seven 2.5-month-old male Wistar rats weighing 200 to 230 g were used in this study. Principles of laboratory animal care (NIH Pub 1985:85-23) and national laws on animal use were observed and authorized by the local Ethical Committee for Animal Research. The animals were anesthetized with intramuscular injection of Rompun/Ketalar, and then the alveolar bone was exposed through an incision along the mucogingival junction. A bony defect approximately 0.8 mm in diameter was created through the alveolar bone over the mesiobuccal root of the mandibular first molar with a slow-rotating size half-round carbide bur driven at low speed by a dental drill that was cooled by copious irrigation with phosphate-buffered saline (PBS). The defect was drilled through the alveolar bone to gently expose the periodontal ligament (Fig. 1). In all animals, the defects were made on both right and left sides of the mandible at the first molar area. The right mandibular hole was filled with a gel containing 2.5% simvastatin, whereas the left site was used as control and, therefore, it was left to heal spontaneously. In both groups, soft tissues were closed with 8-0 interrupted sutures. All the intraoral procedures were carried out under a surgical stereomicroscope at magnifications ranging from ×8 to ×20. The animals were randomly divided into 4 groups, each one composed of 6 rats, and then humanely killed at day 7, 9, 11, or 14 after surgery. In addition, in 1 group sacrificed at day 9 after surgery, the hole on the right side was filled with plain gel to evaluate its effects in wound healing compared with the left side in which the defects were left with only blood clot.

The hemimandibles were dissected out and the soft tissues covering the bone gently removed, except at the buccal aspect of the surgical place. In addition, the tip of the incisors and the mandible at the third molar area were fractured using a bone-cutting forceps to facilitate the penetration of fixative. Tissues were quickly placed in a fixative containing 0.1% glutaraldehyde plus 4% formaldehyde (freshly prepared from paraformaldehyde) buffered at pH 7.4 with 0.1 M sodium cacodylate. Specimens were immersed in a beaker containing 40 mL of fixative at room temperature, which was subsequently put in a 20 × 20-cm glass recipient filled with ice and placed in a laboratory microwave oven. The oven temperature probe was submerged into the fixative and the specimens were immediately exposed to microwave irradiation at a 100% setting for 3 periods of 5 minutes, with the temperature programmed to a maximum of 37°C. After microwave fixation, specimens were transferred into fresh fixative and left overnight at 4°C. They were then washed in 0.1 M sodium cacodylate buffer, pH 7.4, for 1 hour.

For SEM analysis, specimens randomly taken from 3 animals of each group, including the group composed of the animals treated with plain gel, were treated with 2% sodium hypochlorite for 30 minutes in an ultrasonic apparatus to remove all the soft tissues covering bone and teeth. The hemimandibles were subsequently
washed with distilled water, transferred to 30% ethanol and dehydrated in increasing concentrations of ethanol. To avoid shrinkage of the specimens during air drying, the mandibles were immersed in 100% hexamethyldisilizane (HMDS) for 10 minutes and left under a fume hood equipped with an exhaust system for complete evaporation of HMDS. Specimens were mounted on aluminum stubs using colloidal silver adhesive and sputter-coated with gold in a Balzers SDC-050 apparatus (Bal-Tec AG, Principality of Liechtenstein). The specimens were examined with a JEOL 6,100 scanning electron microscope (Jeol Ltd, Tokyo, Japan), operated at 10 to 15 kV.

The specimens processed for TEM were randomly taken from 3 animals of each group and decalcified in 4.13% EDTA acid for 30 days. Then, the hemimandibles were washed extensively in 0.1 M sodium cacodylate buffer, pH 7.4, and the specimens processed for morphologic analysis were postfixed in 0.1 M cacodylate-buffered 1% osmium tetroxide for 1 hour at room temperature, whereas the decalcified samples destined to immunocytochemistry were left unosmicated. Then, samples of both groups were dehydrated in graded concentrations of ethanol and embedded in LR White Resin (London Resin Company, London, UK). Toluidine blue–stained 1-μm-thick sections were examined in a light microscope and the region containing the bone defects was selected for ultrathin sectioning. Sections 80-nm thick were cut using an ultramicrotome equipped with a diamond knife, collected onto 200-mesh copper grids, stained with uranyl acetate and lead citrate, and examined with a JEOL 1010 transmission electron microscope (Jeol Ltd) operated at 80 kV.

For the immunocytochemical analysis, ultrathin sections were incubated with a chicken egg yolk antirat OPN antibody diluted 1:150 for 5 hours, and subsequently with a rabbit antichicken immunoglobulin G (IgG) secondary antibody (Sigma Chemical Co, St Louis, MO, USA) diluted 1:2000 for 1 hours. The antibody-antigen binding sites were revealed with a protein A–gold complex incubated for 30 minutes. After this, the grids were rinsed with PBS followed by distilled water. All steps were carried out at room temperature. Negative controls for labeling specificity consisted of incubating the sections with the secondary antibody followed by protein A–gold or protein A–g-gold alone. Finally, the sections were stained with uranyl acetate and lead citrate.

RESULTS
Nine days after surgery the effects of application of plain gel into the hole were analyzed by SEM. This period was chosen because it was an intermediate time in bone regeneration, and bone formation was evident in the defect that was filled by the blood clot when compared with the defect filled with gel alone (Fig. 2).

SEM analysis of the control side revealed at 7 days of wound healing that the bone defects were empty without any evidence of bone formation (Fig. 3, A and C). In contrast, when the bone defects from the simvastatin side were examined at high magnification, a narrow layer of new bone was observed arising from the border of the defects (Fig. 3, B and D). Furthermore, the TEM showed few osteoclasts at the border of the defect only on the control side and macrophage enclosing the gel used as vehicle on the simvastatin side (data not shown).

After 9 days of healing, the new bone formed at the border of all defects was more evident than in the previous period, although it was somewhat wider on the simvastatin side (Fig. 4). TEM examination of the
areas of new bone formation on the control side revealed secretory osteoblasts surrounding collagenous extracellular matrix in which wide interfibrillar spaces were evident among irregularly disposed collagen bundles (Fig. 5, A). In contrast, the specimens from the simvastatin side showed some areas with more packed...
parallel collagen fibrils (Fig. 5, B). On the control side, the intact alveolar bone near the edge of the defect revealed OPN labeling concentrated in the lamina limitans around the cell processes (Fig. 5, C), whereas on the simvastatin side the OPN labeling was present within the newly formed alveolar bone among the collagen fiber bundles (Fig. 5, D).

Although some differences were noticed in the previous time points analyzed, at 11 days of healing no differences were discerned between the control...
specimens and the experimental groups of SEM preparations (Fig. 6). However, TEM analysis of the bone matrix on the control side showed the defects filled with immature bone with several spaces among the collagen fibrils, whereas the simvastatin side revealed areas with more compacted parallel collagen fibrils (Fig. 7, A and B). The immunocytochemical analysis showed accumulation of OPN in cement lines on the control side, as well as on the simvastatin side (Fig. 7, C and D).

After 14 days of healing, all the bone defects appeared completely filled with new bone. The specimens from the control side exhibited extensive areas of resorption at the outer surface of the alveolar process and into the bone defects themselves (Fig. 8, A and C). In contrast, the margins of the simvastatin-treated defect appeared regular, and the bone formed in the center of the defect generally exhibited a smooth surface (Fig. 8, B and D). Examination of the ultrathin sections showed that the bone of the control specimens contained collagen bundles randomly oriented, whereas they were generally parallel and densely packed in the experimental ones (Fig. 9). In addition, the bone formed facing the periodontal ligament was covered by a continuous osteoblast layer in the control group, whereas the osteoblasts were interrupted by some collagen bundles inserted into the alveolar bone in the experimental specimens (Fig. 10).

DISCUSSION
The results of the present study suggest that simvastatin stimulates bone regeneration when it is locally administered into defects created in the rat alveolar process. As the ultrastructural and immunocytochemical approaches have shown, bone formation started earlier in the simvastatin-treated rats than in the controls. Additionally, the laid down matrix presented a lamellar appearance.

Numerous studies have investigated aspects of...
bone regeneration by creating defects in several bone—especially calvaria and tibia—from different animal species. In the present study, we created a windowlike model defect in the mandibular alveolar bone. Although numerous studies have used this model to analyze several aspects of bone repair, this is the first investigation in which repair of alveolar bone in the vicinity of the periodontal ligament has been ultrastructurally investigated. In addition, recent studies reported positive results during the regeneration of bone.

Fig. 8. Scanning electron micrographs of specimens from rats 14 days postsurgery. On the control defects A and C, the secretion of new bone trabeculae (arrows) was concomitant to bone resorption around the wounded area (R, resorbed area). On the experimental defect B and D, significant new bone formation (arrows) can be observed. At this time point, the simvastatin-treated defect seems shallower than the control, as observed in the higher magnification in E. Bar A = 200 μm; bar B = 400 μm; bar C, D = 100 μm; bar E = 10 μm.

Fig. 9. Transmission electron micrographs of specimens from rats 14 days postsurgery showing the bone regeneration matrix into the defects. In A, disorganized collagen fibrils (Col) typical from primary bone are seen in the new bone filling the control defect, whereas in B, the collagen fibril arrangement is parallel, resembling mature bone. Bar A = 5 μm; bar B = 3 μm.
defects after application of simvastatin with different carriers. Given the availability of a confined space in contact with the periodontal ligament, we used in our model a simvastatin-containing gel, which had been placed into the defect to evaluate its potential effect on the early stages of bone repair.

Once the orally administered simvastatin was processed in the liver, yielding low amounts of the drug available for bone, a gel was chosen as a carrier to topically deliver simvastatin into the bone defects created. We tested the possibility that the gel itself could positively modify the bone regeneration, acting as an osteoconductor material by applying only the vehicle into the defect. However, there was a delay in bone regeneration at this condition. The same negative effect was reported by Stein et al., who observed significant inflammatory signs in specimens that were only dealt with gel.

Only the simvastatin-treated specimens showed the onset of bone formation at the periphery of the defects after 7 days of healing. Later, at the ninth day, the newly formed bone was observed in both groups, but simvastatin-treated defects exhibited a more organized bone matrix than the control specimens. In this manner, bone formation and the improved characteristics of its matrix seem to have been triggered by simvastatin in the experimental defects, whereas the controls presented immature bone filling with numerous spaces throughout the collagen mesh where noncollagenous proteins are usually present.

Interestingly, osteoclasts and, therefore, resorptive activity were not observed in the simvastatin-treated defects, despite the lamellar-like matrix secreted. Thus, the osteoblasts may have been stimulated by simvastatin to secrete a lamellar bone matrix without the initiation of the resorptive phase. Immature (primary) bone remodeling requires osteoclast-mediated resorption followed by secretion of a more organized matrix by newly differentiated osteoblasts, thus forming the mature (lamellar) bone. In this experiment, osteoclasts were observed only at the edge of the immature bone formed in the control specimens, especially at the last time point. The absence of osteoclasts in the simvastatin-treated specimens could have been occasioned by the drug, which corroborates with the speculation that it might inhibit the fusion of clastic cell precursors or disrupt the actin ring of active osteoclasts.

Our ultrastructural immunolabeling study showed the noncollagenous protein OPN dispersed in mineralizing areas of the new bone matrix at simvastatin-treated defects, which may reflect its increased speed of deposition. Previous light microscopy immunolabeling studies on the same model reported OPN distribution restricted to a discrete line delimiting the edge of the defect where the bone was initially cut, and also at the adjacent periodontal ligament. The dispersed immunolabeling detected in the periodontal ligament adjacent to the defects of both groups suggests that the periodontal ligament cells may have an osteogenic potential, a concept that is supported by studies that demonstrated their high alkaline phosphatase expression. Indeed, TEM and especially SEM clearly revealed that bone formation in the defects started at the bottom of the defect facing the periodontal ligament in both groups. In addition, the alveolar bone deposition observed in the simvastatin-treated defects may be a...
reflection of the potential of this drug to stimulate cell proliferation and osteoblast differentiation reported in previous in vitro experiments performed with human periodontal ligament cells, besides its capacity to increase angiogenesis and strengthen bone at high doses.

In summary, our data suggest that simvastatin stimulates an earlier regeneration of rat alveolar bone with additional improvement of bone quality, and propose an advantageous local use of this drug with potential indication to regenerative attempts. Moreover, topical application is a convenient procedure in oral surgery. However, further studies regarding the optimal dose of the drug, different vehicles, or association to scaffolds for bone growth are still necessary.

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REFERENCES

1. Zeichner-David M. Regeneration of periodontal tissues: cemen
22. Rajshankar D, McCulloch CA, Tenenbaum HC, Lekic PC. Oste
26. Parfitt AM. The cellular basis of bone remodeling: the quantum concept reexamined in light of recent advances in the cell biol
27. Arana-Chavez VE, Bradaschia-Correa V. Clastic cells: mineral


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