Effect of Local Rosuvastatin Administration on Calvarial Bone Defects

Akif Türer, PhD, Çiğdem C. Türer, PhD,† Umut Balli, PhD,† Mustafa C. Durmuşlar, PhD,* Mehmet E. Önger, PhD,‡ and Hakan H. Çelik, PhD§*

Abstract: The purpose of this study is to investigate the potential of the local administration of different doses of rosuvastatin (RSV) on autogenous grafted critical-sized cortical bone defects. Twenty-four rats were divided into 3 groups: Group C (control), Group RSV-0.1, and Group RSV-1. A 5-mm diameter critical-size defect was created in the calvarium of each animal. In Group C, the defect was filled by autogenous graft and sterile saline–treated absorbable collagen sponge (ACS) was applied. Defects in the experimental groups (groups RSV-0.1 and RSV-1) were grafted by autogenous graft and ACS with saline solution containing 0.1- and 1-mg RSV were applied. All animals were euthanized at 28 days after operation. Stereologic and micro-computed tomography (μCT) analyses were performed. New bone area and connective tissue volumes were measured. Stereologic analysis showed that the difference between group RSV-1 with a mean bone formation of $1.79 \pm 0.06\,mm^3$ and groups RSV-0.1 and control (C) was statistically significant ($P \leq 0.05$) with a mean bone formation of $1.29 \pm 0.28\,mm^3$ and $1.08 \pm 0.12\,mm^3$, respectively. Connective tissue volume was also significantly higher in 1-mg RSV applicated group. Micro-CT results were similar with stereologic analyses. Local administered 1-mg RSV enhances bone regeneration in critical-size calvarial rat defects filled with autogenous graft.

Key Words: Bone formation, μCT, rat, rosuvastatin

(*J Craniofac Surg* 2016;27: 2036–2040)

The osteoinductive and osteoconductive properties of bone grafts result in the stimulation of new bone formation. Osteoinductive grafts induce the differentiation of primitive cells into osseous-forming cells, whereas osteoconductive grafts provide a scaffold. Autologous bone grafts are therefore the gold standard in the restoration of large bone defects because they have both osteoinductive and osteoconductive properties.[1,2]

From the Departments of *Maxillofacial Surgery; †Periodontology, Bülent Ecevit University, Zonguldak; ‡Department of Histology and Embryology, Ondokuz Mayis University, Samsun; and §Department of Anatomy, Hacettepe University, Ankara, Turkey.

Received January 6, 2016.

Accepted for publication April 14, 2016.

Address correspondence and reprint requests to Akif Türer, PhD, Bülent Ecevit Üniversitesi, Diş Hekimliği Fakültesi Ağiz, Diş ve Çene Cerrahisi AD. 67600 Zonguldak; E-mail: akifturer@gmail.com

This research was supported by Scientific Research Project Fund of Bülent Ecevit University with number 2014–68370268–01.

The authors report no conflicts of interest.

ISSN: 1049-2275

DOI: 10.1097/SCS.0000000000002763

An important factor in the repair of bony defects is the stimulation of local bone formation to shorten the healing time. Such stimulation can be achieved by the topical application of biological growth factors that interact directly with bone-forming cells during bone regeneration. Bone morphogenic proteins (BMPs) are among the biological growth factors that mediate bone repair. BMPs increase the transcription of osteoinductive genes and stimulate the expression of genes required for osteoblast differentiation.[3–5] However, although BMPs have positive effects on new bone formation, their therapeutic use is expensive, which has led to the search for more economical bone repair options.[6]

Statins are specific, competitive inhibitors of 3-hydroxy-2-methyl-glutaryl coenzyme A reductase. They are used widely to lower cholesterol, as they provide an important and effective approach to the treatment of hyperlipidemia and arteriosclerosis.[7] In addition to their cholesterol-lowering activity, the beneficial effects of statins on bone formation, by increasing the expression of BMPs, were reported by Mundy et al.[8] The statin rosuvastatin (RSV) is used widely to prevent cardiovascular disorders. Its long half-life and powerful effects are comparable to those of simvastatin and atorvastatin.[9] However, RSV also has pleiotropic effects, including bone stimulation.[10] In the present study, we examined the effects of different doses of RSV, administered in an absorbable collagen sponge (ACS), on rat calvarial bone defects filled by autologous grafts.

METHODS

The 6- to 8-week-old Wistar rats (n = 24) used in this study were housed in standard cages in rooms with a relative humidity of 40% to 60% and a temperature of $22 \pm 1^{\circ}C$. The illumination system of the room was configured to automatically provide 12 hours of light and 12 hours of darkness. This study was approved by the Animal Experimentation Committee of Bülent Ecevit University, Zonguldak, Turkey.

Experimental Groups

The rats were divided randomly into 3 groups of 8 animals each, which received different RSV treatments: no RSV [control (C)], 0.1-mg RSV (RSV-0.1), and 1.0-mg RSV (RSV-1). All rats were euthanized after 28 days. Bone formation was evaluated by computed tomography and stereological analyses.

Surgical Procedure

All surgeries were performed under sterile conditions in the surgical suite of an animal laboratory. Each rat was anesthetized with an intramuscular injection of 3-mg xylazine hydrochloride (Rompuns; Bayer, Leverkusen, Germany) per kilogram and 35-mg ketamine hydrochloride (10% Ketasol; Richter Pharma AG, Wels, Austria) per kilogram. Under general anesthesia, the rat's calvarium was shaved, and the cutaneous surface was disinfected with povidone-iodine solution. A semilunar incision was then made and a full-thickness flap was reflected, exposing the parietal and frontal bones.

A 5-mm-diameter (critical-size) calvarial defect was made with a trephine used in a low-speed handpiece under continuous sterile saline irrigation. The defect included a portion of the sagittal suture. Care was taken during the surgery to avoid damage to the dura mater.

Defects in all 3 groups were filled by autogenous grafts. Autogenous bone grafts were harvested from left tibia of rats. Medial surface of the left legs of the subjects were shaved, and the area was disinfected with povidone-iodine solution. The legs were given the flexion position and longitudinal incisions of 20 to 25 mm were made periosteally to reach the medial surfaces of the tibia. The medial surfaces of the tibia were exposed with blunt dissection, and soft tissues were excluded. Autogenous bone graft, covering the cortex and medulla layers of the bone, was obtained by using round-tipped, stainless steel drill with a diameter of 3 mm under sterile saline solution. In the C group, a sterile saline-treated ACS of 2-mm thickness and 5-mm diameter was applied to defect. In the RSV-0.1 and RSV-1 groups, ACSs were instead immersed in, respectively, 0.1- and 1-mg RSV, dissolved in 0.2-mL saline. Defects were filled with autogenous grafts, and the respective treated ACSs were applied on the top of autogenous graft. Flaps in all 3 groups were sutured with resorbable 4/0 polyglactin 910 sutures (Vicryl; Ethicon, Somerville, NJ). For postoperative infection control and analgesia, respectively, each animal was injected with 10-mg cefazolin sodium (Sefazol; M Nevzat, Istanbul, Turkey) per kilogram and 200-mg metamizol sodium (Novalgin; Aventis, Istanbul, Turkey) for 5 days after the operation. Four weeks after surgery, the animals were euthanized by lethal anesthetic injection. After skin dissection, the calvaria were removed and immersed immediately in a 10% tempered formaldehyde solution.

Stereology

The calvarial samples were decalcified in formic acid (5%) for 21 days and then fixed in 10% formaldehyde, dehydrated in a graded alcohol series, and cleared in xylol for light microscopy examination. The dehydrated specimens were then embedded in fresh paraffin. Each paraffin block was serially cut into 7-μm-thick sections using a microtome (Leica RM 2135; Leica Instruments, Nussloch, Germany). For volume estimation, every 20th section from a set of consecutive paraffin sections from each sample was selected, with the first section chosen randomly. The selected sections were stained with hematoxylin and eosin and photographed using a stereology analysis system (Stereo Investigator 9.0; Microbrightfield, Williston, VT) and a light microscope (M4000 B; Leica Instruments) equipped with a digital color camera (Microbrightfield).

The unbiased Cavalieri method was applied to the light microscopy images to stereologically estimate the volume of new bone using point-counting test grids. The point density of the point-counting grids was designed to obtain an appropriate coefficient of error (CE) for the area of interest in the images of the serial sections.[11] The grid, with its systematic array of points, was placed randomly on the image shown on the screen of a personal computer. The volume of each area of interest in each section was estimated with the following formula:

$$\text{Volume} = t \times a/p \times \sum p,$$

where t is the section thickness, a/p is the area of each point on the point counting grid, and $\sum p$ is the total number of points within the area of interest. The CE and coefficient of variation were estimated according to the formula by Gundersen and Jensen.[12]

Micro-Computed Tomography

The specimens were scanned using micro-computed tomography (μCT; SkyScan 1174; Micro Photonics Inc, Allentown, PA) at 50 kV and 800 μA. Scanning was performed at a spatial resolution of 15 μm, with the specimen rotated in 0.7-degree increments for a total of 180-degrees. All images were processed by three-dimensional reconstruction using the NRecon software (Bruker microCT, Skyscan, Kartuizersweg 3B, Belgium). The data were evaluated with CTAn software (Bruker microCT, Skyscan). Only the volume of mineralized new bone formation, without graft material, was calculated.

STATISTICAL ANALYSIS

The Shapiro–Wilk test was used to confirm the normal distribution of the data. The μCT and stereological parameters were analyzed using the Kruskal–Wallis nonparametric test, followed by post-hoc group comparisons with the Bonferroni-adjusted Mann-Whitney test, after a failed normality test of the data. For the Bonferroni correction, $\alpha = 0.05/3 = 0.016$ was considered to indicate statistical significance. All tests were performed using statistical software (SPSS version 19.0; SPSS Inc, Chicago, IL). $P < 0.05$ was considered to indicate statistical significance.

RESULTS

All animals tolerated the surgery well and survived the postsurgical period. No wound dehiscence, wound infection, or abscess formation was observed at any surgical site.

Histological Evaluation

Histological analysis showed new bone formation in all groups. In the C and RSV-0.1 groups, almost all defects were occupied by remnants of the bone graft particles. Newly formed bone was observed, mainly close to the original borders of the defect. Most bone graft particles showed empty osteocyte lacunae. In some specimens, the particles did not demonstrate new bone formation. In group RSV-1, however, most bone graft particles were surrounded by newly formed bone. Large areas of intense resorption of the bone graft particles were observed in most of these specimens (Fig. 1).

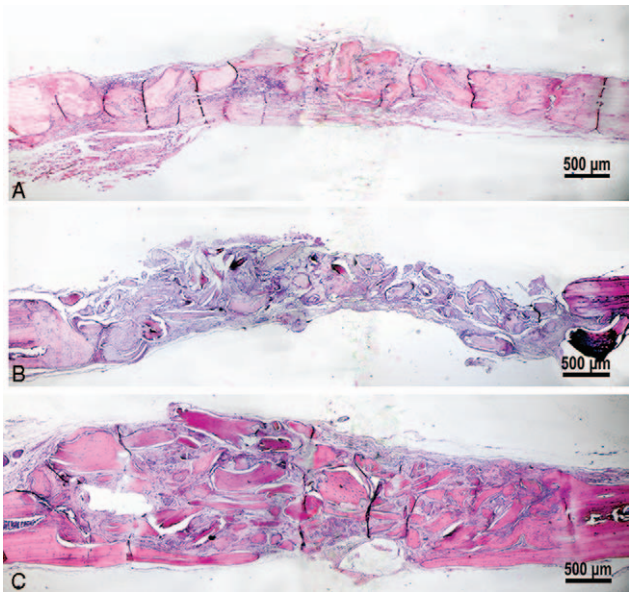

FIGURE 1. Panoramic views of the defects. (A) Group C, (B) Group RSV-0.1, and (C) Group RSV-1.

Stereological Analysis

Mean bone volume in group RSV-1 was $1.79 \pm 0.06\,mm^3$, whereas mean volumes in the RSV-0.1 and C groups were $1.29 \pm 0.28\,mm^3$ and $1.08 \pm 0.12\,mm^3$, respectively (Fig. 2). The differences between the RSV-1 group and the C and RSV-0.1 groups were statistically significant ($P \leq 0.05$). Although bone formation in the RSV-0.1 group was superior to that in group C, the difference was not statistically significant.

The connective tissue volume data are shown in Figure 3. In the RSV-1 group, mean bone formation was $1.74 \pm 0.17\,mm^3$, whereas mean formation values in the RSV-0.1 and C groups were $1.28 \pm 0.04\,mm^3$ and $1.23 \pm 0.18\,mm^3$, respectively. The differences between the RSV-1 group and the other 2 groups were statistically significant ($P \leq 0.05$). Connective tissue volume was larger in group RSV-0.1 than in group C, but the difference was not statistically significant.

μCT Analysis

The μCT results are shown in Figures 4 and 5. The differences between group RSV-1 and groups RSV-0.1 and C were statistically significant ($P \leq 0.05$). The volume of new bone was greater in group RSV-0.1 than in group C, but the difference was not statistically significant.

DISCUSSION

The advantages of the rat calvaria model in studies of bone regeneration are well documented. The bone defects are easy to manipulate, simple, and do not require wound stabilization, nor are muscular layers involved in their formation. The compressive force exerted on the rat calvaria is similar to that used in the treatment of intraoral wounds. Moreover, rats are inexpensive and can be easily housed and fed.[13] In the present study, we used this model to test the ability of local RSV application to stimulate new bone formation. Rats with 5-mm calvarial defects were thus treated with autologous grafts and ACSs containing 0.1- or 1-mg RSV. Critical-size defects are defined as bone defects that do not heal spontaneously during the animal's lifetime.[14] In rats, calvarial defects 5 mm in diameter are regarded as critical-size defects.[15]

New bone volume was confirmed using 2 different methods of sample analysis: stereology and μCT. As histological examinations are limited to two-dimensional slices, the use of histology alone may have failed to identify small islands of bone formation. In 1984, Sterio[16] described several modifications of the approaches used to estimate the volume of objects in three-dimensional space. These techniques have since been applied to estimate microscopic parameters in three-dimensional space, including in μCT. The reliability of these morphological measurements has been reported.[17,18]

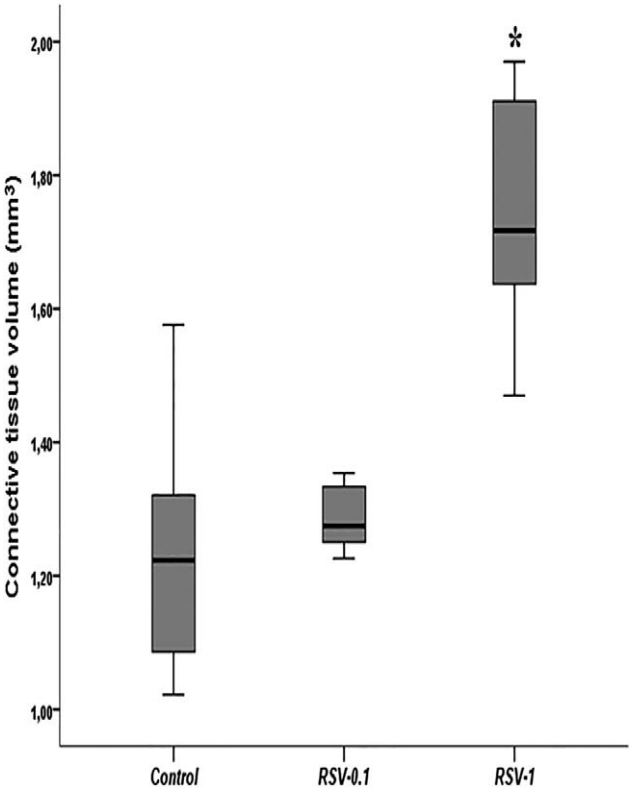

FIGURE 3. Connective tissue volume (mm^3) from stereological analysis in defects. *Statistically significant difference from the control group (Bonferroni-adjusted Mann-Whitney test).

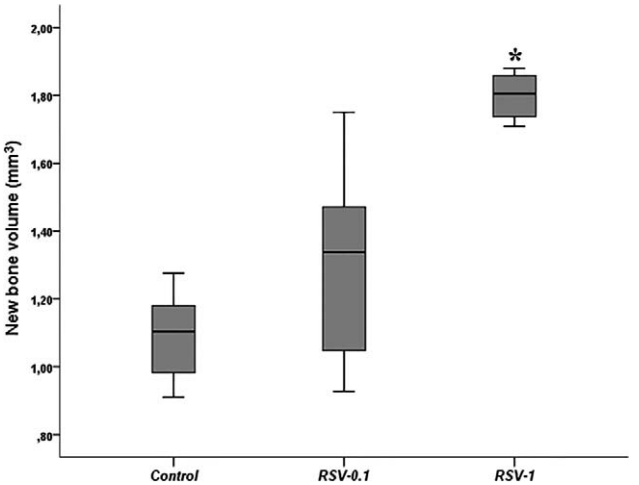

FIGURE 2. New bone volume (mm^3) from stereological analysis in defects. *Statistically significant difference from the control group (Bonferroni-adjusted Mann-Whitney test).

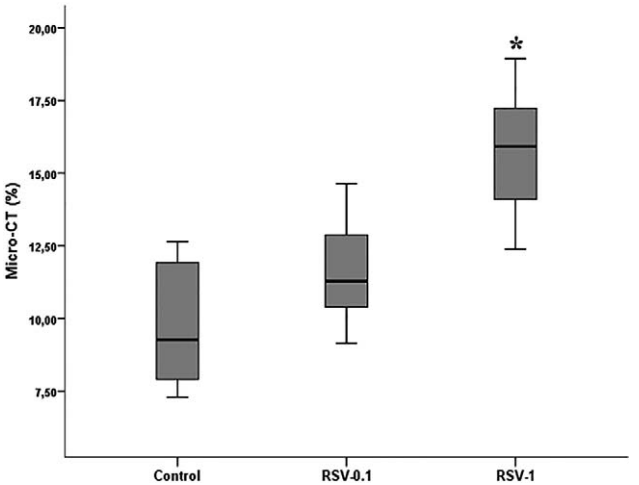

FIGURE 4. Percentage of new bone area from the micro-computerized tomography measurements in defects. *Statistically significant difference from the control group (Bonferroni-adjusted Mann-Whitney test).

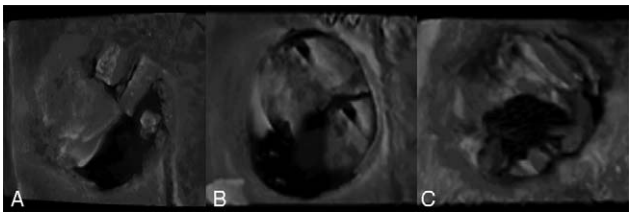

FIGURE 5. Micro-computerized tomography views of the defects. (A) Group C, (B) Group RSV-0.1, and (C) Group RSV-1.

Among the biological growth factors that augment bone growth and thereby promote bone healing in the repair of bony defects, BMPs are the most potent. These proteins stimulate osteoprogenitors and other specific cell types to differentiate into mature osteoblasts and osteoblast lineage cells, respectively.[19] Mundy et al[8] showed that statins enhance the expression of BMP-2. Wong and Rabie[20] investigated the early healing of bony defects grafted with simvastatin. They found that vascular endothelial growth factor was expressed on day 3 after grafting, and BMP-2 was expressed on day 4; new bone was formed on day 5. Thus, the time course of healing induced by statins is similar to that of BMP. Like other statins, RSV is a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor that reduces low-density lipoprotein cholesterol levels. RSV is the most potent of the currently marketed statins,[21] but its positive effects on bone formation have not been well studied. Therefore, in this study we evaluated the potential of RSV, applied locally to the autologously grafted area, to promote the repair of calvarial defects.

Statins have been used in different ways to treat bony defects. Gutierrez et al[22] showed that, with respect to bone formation in rats, topically applied statin was 50 times more active than oral administration of the drug, and local administration was important in optimizing its osteoinductive effect. In a study of the efficacy of local statin application to healthy bone, Ozeç et al[23] showed that a simvastatin gelatin sponge enhanced the healing of a bone defect in the rat mandible. Al-Obaidi et al[10] reported that the administration of ellagic acid and RSV accelerated tooth socket healing in diabetic rats after tooth extraction. Monjo et al[24] created critical-size cortical bone defects adjacent to titanium implants in rabbits and evaluated the use of ACSs as RSV carriers. They found that RSV, when applied locally, stimulated bone formation. Thus, based on these studies, we chose local application of RSV using ACSs to treat the rat calvarial defects. Consistent with other studies, our results demonstrated the beneficial effects of locally applied RSV in bone formation induced by autogenous grafts.

The minimum local concentration of RSV required to stimulate bone formation has yet to be systematically determined. Monjo et al[24] evaluated the effects of 3 different doses of locally applied RSV (0.1, 0.5, and 2.5 mg) on bone defects. Pradeep et al[25] used 1.2-mg topical RSV to treat mandibular degree II furcation defects. In this study, we tested the ability of 2 different doses of RSV (0.1 and 1 mg), applied via ACSs, to promote the repair of critical-size bone defects.

In the topical administration of a drug, the delivery system plays an important role. This is also the case in the local application of statins to induce controlled bone formation. The ACS was chosen as the carrier in this and other studies because its 3D structure acts as a scaffold. The ACS is also resorbable and has been tested clinically in humans. The use of other carrier materials has also been described in the literature. Wu et al[26] used polylactic acid/polyglycolic acid copolymer as a carrier to apply simvastatin into the extraction sockets of the mandibular incisors of rats. Stein et al[27] used methylcellulose gel in a polylactic acid membrane to evaluate the effect of local simvastatin on tissue inflammation and bone growth in rats. Mukozawa et al[28] compared 2 different carriers, hydrogel and the ACS, for the local application of simvastatin to nasal bone defects in rabbits. Similar BMP-2 expression and new bone formation were achieved with the 2 carrier materials.

Based on our stereological analyses of the calvarial defects and consistent with the findings of previous studies of statins, the local application of RSV had a positive effect on bone formation,[10,25] with better results achieved in the RSV-1 than in the RSV-0.1 group. In contrast to Monjo et al,[24] we found no statistically significant difference between the RSV-0.1 and C groups. The values obtained by μCT and stereological evaluation were similar, with both showing more new bone formation in defects treated with locally applied RSV than in those treated with saline. Connective tissue volumes correlated positively with RSV application and dose, with the highest connective tissue volume achieved in the RSV-1 group. This situation may be related to the inflammatory effect of statins, as noted in previous studies.[24,29]

CONCLUSION

According to the results of our study, 1-mg locally applied RSV promotes bone healing in experimental calvarial defects in rats. Further investigations aimed at establishing the optimal dose and carrier to maximize the anabolic actions of RSV on BMP regulation and bone are needed.

ACKNOWLEDGMENTS

The authors thank the research assistants Gamze Yayla and Burcu Delibaş for supporting stereological examination.

REFERENCES

1. Young S, Patel ZS, Kretlow JD, et al. Dose effect of dual delivery of vascular endothelial growth factor and bone morphogenetic protein-2 on bone regeneration in a rat critical-size defect model. *Tissue Eng Part A* 2009;15:2347–2362
2. Marden LJ, Reddi AH, Hollinger JO. Growth and differentiation factors: role in bone induction and potential application in craniofacial surgery. *J Craniofac Surg* 1990;1:154–160
3. Deschaseaux F, Sensebe L, Heymann D. Mechanisms of bone repair and regeneration. *Trends Mol Med* 2009;15:417–429
4. Schachter M. Chemical, pharmacokinetic and pharmacodynamic properties of statins: an update. *Fundam Clin Pharmacol* 2005;19: 117–125
5. Barrios-González J, Miranda RU. Biotechnological production and applications of statins. *Appl Microbiol Biotechnol* 2010;85:869–883
6. Axelrad TW, Steen B, Lowenberg DW, et al. Heterotopic ossification after the use of commercially available recombination human bone morphogenetic proteins in four patients. *J Bone Joint Surg Br* 2008;90:1617–1622
7. Hunninghake DB. Therapeutic efficacy of the lipid-lowering armamentarium: the clinical benefits of aggressive lipid-lowering therapy. *Am J Med* 1998;104:9–13
8. Mundy G, Garrett R, Harris S, et al. Stimulation of bone formation in vitro and in rodents by statins. *Science* 1999;286:1946–1949
9. Karlson BW, Palmer MK, Nicholls SJ, et al. Doses of rosuvastatin, atorvastatin and simvastatin that induce equal reductions in LDL-C and non-HDL-C: Results from the VOYAGER meta-analysis. *Eur J Prev Cardiol* 2016;23:744–747
10. Al-Obaidi MM, Al-Bayaty FH, Al Batran R, et al. Impact of ellagic acid in bone formation after tooth extraction: an experimental study on diabetic rats. *Sci World J* 2014;2014:908098
11. Odaci E, Sahin B, Sonmez OF, et al. Rapid estimation of the vertebral body volume: a combination of the Cavalieri principle and computed tomography images. *Eur J Radiol* 2003;48:316–326
12. Gundersen HJG, Jensen EB. The efficiency of systematic sampling in stereology and its prediction. *J Microsc* 1987;147:229–263

13. Choi JY, Jung UW, Kim CS, et al. The effects of newly formed synthetic peptide on bone regeneration in rat calvarial defects. *J Periodontal Implant Sci* 2010;40:11–18
14. Schmitz JP, Hollinger JO. The critical size defect as an experimental model for craniomandibulofacial nonunions. *Clin Orthop Relat Res* 1986;205:299–308
15. Vajgel A, Mardas N, Farias BC, et al. A systematic review on the critical size defect model. *Clin Oral Implants Res* 2014;25: 879–893
16. Sterio DC. The unbiased estimation of number and sizes of arbitrary particles using the disector. *J Microsc* 1984;134:127–136
17. Novaes RD, Penitente AR, Talvani A, et al. Use of fluorescence in a modified disector method to estimate the number of myocytes in cardiac tissue. *Arq Bras Cardiol* 2012;98:252–258
18. Power SM, Matic DB, Holdsworth DW. The effects of nonvascularized versus vascularized bone grafting on calvarial defect healing. *J Craniofac Surg* 2015;26:290–295
19. Wozney JM, Rosen V. Bone morphogenetic proteins. In: Mundy JR, Martin TJ, eds. *Physiology and Pharmacology of Bone.* New York, NY: Springer-Verlag; 1998:725–748
20. Wong RW, Rabie AB. Early healing pattern of statin-induced osteogenesis. *Br J Oral Maxillofac Surg* 2005;43:46–50
21. Toth PP. An update on the benefits and risks of rosuvastatin therapy. *Postgrad Med* 2014;126:7–17
22. Gutierrez GE, Lalka D, Garrett IR, et al. Transdermal application of lovastatin to rats causes profound increases in bone formation and plasma concentrations. *Osteoporos Int* 2006;17:1033–1042
23. Ozeç I, Kiliç E, Gümüş C, et al. Effect of local simvastatin application on mandibular defects. *J Craniofac Surg* 2007;18:546–550
24. Monjo M, Rubert M, Wohlfahrt JC, et al. In vivo performance of absorbable collagen sponges with rosuvastatin in critical-size cortical bone defects. *Acta Biomater* 2010;6:1405–1412
25. Pradeep AR, Karvekar S, Nagpal K, et al. Rosuvastatin 1.2 mg in situ gel combined with 1:1 mixture of autologous platelet-rich fibrin and porus-hydroxyappatite bone graft in surgical treatment of mandibular degree II furcation defects: a randomized clinical control trial. *J Periodontol* 2015;5:1–15
26. Wu Z, Liu C, Zang G, et al. The effect of simvastatin on remodelling of the alveolar bone following tooth extraction. *Int J Oral Maxillofac Surg* 2008;37:170–176
27. Stein D, Lee Y, Schmid MJ, et al. Local simvastatin effects on mandibular bone growth and inflammation. *J Periodontol* 2005;76:1861–1870
28. Mukozawa A, Ueki K, Marukawa K, et al. Bone healing of critical-sized nasal defects in rabbits by statins in two different carriers. *Clin Oral Implants Res* 2011;22:1327–1335
29. Calixto JC, Lima CE, Frederico L, et al. The influence of local administration of simvastatin in calvarial bone healing in rats. *J Craniomaxillofac Surg* 2011;39:215–220