

Simvastatin and biphasic calcium phosphate affects bone formation in critical-sized rat calvarial defects¹

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ABSTRACT

PURPOSE: To investigate the effects of locally applied simvastatin plus biphasic calcium phosphate (BoneCeramic[®]) or collagen sponge on bone formation in critical-sized bone defects.

METHODS: Thirty defects of 5mm in diameter were created bilaterally with a trephine bur in the calvariae of fifteen Wistar rats. The defects were divided into five groups: group 1 - control, no treatment; group 2 (BoneCeramic[®]); group 3 (BoneCeramic[®] + 0.1mg simvastatin); group 4 (collagen sponge); and group 5 (collagen sponge + 0.1mg simvastatin). After eight weeks the animals were euthanized and their calvariae were histologically processed. Hematoxylin and eosin-stained sections were subjected to histological and histomorphometrical analyses. The area of newly formed bone was calculated and compared between groups.

RESULTS: The greater amount of a bone-like tissue was formed around the carrier in group 3 (BoneCeramic[®] + 0.1mg simvastatin) followed by group 2 (BoneCeramic[®]), and almost no bone was formed in the other groups. Group 3 was significantly different compared to group 2, and both groups were significantly different compared to the other groups.

CONCLUSION: Simvastatin combined with BoneCeramic[®] induced significantly greater amounts of newly formed bone and has great potential for the healing of bone defects.

Key words: Simvastatin. Osteogenesis. Calcium Phosphates. Rats.

Introduction

Important structural changes on bone such as reduction on bone height and width occur as a consequence of periodontal disease and tooth extraction. Such bone changes may compromise the functional and esthetic oral rehabilitation of the patients and require bone grafting procedures^{1,2}. Autogenous bone is an osteogenic, osteoinductive and osteoconductive material that has been considered the gold standard material for bone graft. It is totally biocompatible and rich on factors essential for osteoblast differentiation such as bone morphogenetic proteins (BMPs), especially BMP-2 which is a potent bone inductive growth factor, and vascular endothelial growth factor (VEGF) which has angiogenic property^{3,4}. On the other hand, autogenous bone grafts have a few disadvantages such as its resorption potential and the need for a donor area as a second surgical site which may be limited in certain clinical situations⁵.

Calcium phosphate ceramic materials have been commonly used as autogenous bone substitutes due to their excellent biocompatibility. These ceramic materials have also been used as carriers for drugs and growth factors such as BMPs^{6,7}. Among the several calcium phosphate materials reported in the literature, Straumann BoneCeramic[®], a biphasic calcium phosphate (BCP), is a totally synthetic material composed of 60% hidroxyapatite (HA) and 40% β -tricalcium phosphate (β -TCP)⁸. A clinical advantage of this chemical composition is that, while resorption of the β -TCP component occurs at a faster rate allowing early bone formation, the HA component is slowly resorbed, thus providing a long-term space maintenance for the late formation of new bone within a bone defect⁸. Furthermore, BoneCeramic[®] has been shown to be safe, biocompatible, and an effective scaffold for new bone formation in several types of bone defects on the alveolar ridge and maxillary sinus^{9,10}. Despite its positive results in terms of new bone formation, BoneCeramic[®] is an osteoconductive material that lacks osteoinductive properties. Several recent reports have pointed out to the fact that the use of biologically active molecules with osteoinductive activity could be associated with scaffolds used in bone defects, resulting in osteoconductive and osteoinductive bone substitute materials¹².

Statins are drugs that have been widely used to lower blood cholesterol levels in the past several years. However, many studies have investigated the systemic and local effects of statins on bone metabolism and healing of bone defects^{13,14}. When locally applied, statins affect bone healing through osteoinduction by increasing angiogenesis and modulating proteins and growth factors^{15,16}. Simvastatin, one of the statins, has been locally applied

in different concentrations with different carriers to induce bone formation in bone defects, but the ideal combination of drug concentration/type of carrier is still uncertain¹⁷. Nevertheless, lower concentrations of simvastatin (0.1mg) have been shown to be better when ceramic carries are utilized¹⁸.

In the present study we hypothesized that the combination of BoneCeramic[®] and simvastatin would result in improved bone formation in the widely used critical-sized rat calvarial bone defect model¹⁹ when compared with BoneCeramic[®] alone. Furthermore, in order to verify whether simvastatin could overcome the lack of osteoconductive or osteoinductive activities in a carrier, the combination of simvastatin with a collagen sponge was also tested.

Methods

The study protocol was approved by the Committee for the Use of Animals on Research, Universidade de Brasilia, (protocol UnB doc 44299-2012).

Fifteen female Wistar rats (eight weeks of age, average weight of 300g) were used in this study. The animals were housed in groups of five per cage, kept under standard conditions with food and water *ad libitum*, room temperature, light/dark cycle of 12 hours (06:00 to 18:00 h).

Preparation of simvastatin solution

The simvastatin solution was prepared and applied to the bone defects as previously described¹⁹. Briefly, a solution containing 0.1mg of simvastatin diluted in 15 μ l ethanol (Farmogral, Brasilia-DF, Brazil) was applied to each bone defect created on rat calvaria as described below. Two different carriers were used for the simvastatin: Straumann BoneCeramic[®] (400-700 μ m) (Institut Straumann AG, Basel, Switzerland) or a collagen sponge (CollaTape[®], Zimmer Dental, Carlsbad, CA, USA). The bone defects treated with simvastatin and BoneCeramic[®] received 14mg of the carrier soaked in 15 μ l of simvastatin solution. The defects treated with CollaTape and simvastatin, received a round piece of the collagen sponge of the size of the defect soaked in 15 μ l of simvastatin solution.

Surgical procedures

The animals were anesthetized with a combination of ketamine (80mg/kg) and xylazine (10mg/kg) by an intramuscular injection. An antiseptic (povidone-iodine) was applied to the surgical sites, a skin incision was performed, and a flap was raised

exposing the calvarial bone. Two critical-sized bone defects of 5 mm in diameter just lateral to the sagittal plane were carefully prepared with a trephine bur (Neodent, Curitiba-PR, Brazil) under irrigation with saline solution and slight pressure to avoid damage to the dura mater of the brain. A total of 30 defects were created on fifteen 15 rats and divided in 5 groups that included 6 defects for each group treated as follows: group 1, control group (C) - no treatment; group 2, BoneCeramic® (BC); group 3, BoneCeramic® + Simvastatin (BCS); group 4, collagen sponge - CollaTape® (CS); group 5, CollaTape® + Simvastatin (CSS) (Figure 1). The flaps were then sutured with a 5-0 nylon suture (Ethicon®, São Paulo-SP, Brazil). Aspirin (150mg/kg) was given orally to the rats every 6 hours on the first day after surgery. The animals were observed daily for signs of inflammation. For the histological and histomorphometrical analyses, the animals were euthanized by decapitation eight weeks after surgery.

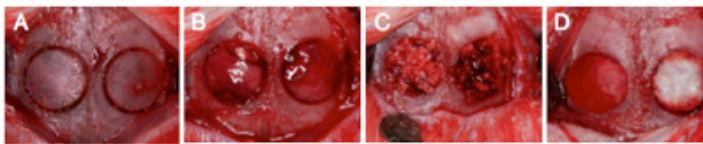


FIGURE 1 - Representative photographs of the surgical procedures. (A) Osteotomies created with a trephine on the parietal bone. (B) Bone defects exhibited after removal of cortical bone. (C) Bone defects treated with BoneCeramic®+Simvastatin (BCS, right side) and BoneCeramic® (BC, left side). (D) Bone defects treated with CollaTape®+Simvastatin (CSS, right side) and CollaTape® (CS, left side).

Histological preparation

After euthanize, the calvarial bones were dissected out, the soft tissues were carefully removed, and the specimens were then fixed in neutral 10% formalin for 24 hours. The specimens were then washed in water for 24 hours and decalcified with a solution of 50% formic acid and 20% sodium citrate for 30 days. The calvarial bones were longitudinally divided in half and each half containing one treated defect was embedded separately in paraffin according to standard protocols. The embedded specimens were sectioned into 5 µm serial slices with a microtome. All sections were stained with hematoxylin and eosin for later microscopic and histomorphometrical analyses.

Histological and histomorphometrical analyses

Histological analysis was carried out with a light microscope (Zeiss, Jena, Germany) under x20 and x200 magnification and the morphology of the newly formed tissue in the bone defect area was examined. Tissue sections were screened

under a light microscope and the most central histological sections of each surgical defect was selected for the analyses.

The histomorphometrical analysis was carried out with the ImageScope® software (Leica Biosystems, São Paulo-SP, Brazil) and the area of the newly formed bone was calculated according to a previously described method²⁰. Briefly, the total area was delineated on the captured digital images of the entire surgical defects as follows: two vertical lines were drawn on each side of the defect that was limited by the original cortical calvarial bone. These two vertical lines on each side were connected by two horizontal lines, one on top and another at the bottom, forming a rectangle containing the entire newly formed tissue within the confines of this rectangle. The area of this rectangle was considered to be 100% of the area to be analyzed (total area). Then, only the newly formed bone was selected and its area calculated as a percentage of the total area.

Statistical analysis

For the statistical analysis, the area of the newly formed bone in the groups was evaluated by a commercial software (SAS 9.3) with One-way analysis of variance (ANOVA), and a significant difference between groups of $p < 0.05$ was established. The data were further analyzed by Tukey method for post hoc multiple comparisons test.

Results

Histological analysis

Figure 2 shows an overview of the entire healed defect of all groups at low magnification (x20) with the panels arranged from the thinnest to the thickest newly formed tissue. In group 1 (control), there was no evidence of new bone formation (Figure 2A). In group 2, which was treated with only BoneCeramic® (BC), a newly formed tissue suggestive of bone was observed with a more intense staining similar to the original bone tissue at the border of the defect (Figure 2D). The newly formed tissue observed in group 3 (BCS), that was treated with BC soaked with simvastatin, was more evident as compared to the other groups (Figure 2E). In group 3, the entire length of the defect was filled by a tissue with a width similar to that of the border of the defect. In both groups 2 and 3, spaces left by BC particles were present (Figures 2D and E, asterisks). In group 4, the defect was treated only with a collagen sponge (CS) and a fibrous tissue layer apparently thicker than that of the control group was observed, whereas in group 5, that was

treated with a collagen sponge soaked in simvastatin (CSS), the presence of a fibrous tissue thicker than the one observed in groups 1 and 4 (control and CS, respectively) was present (Figures 2B and C).

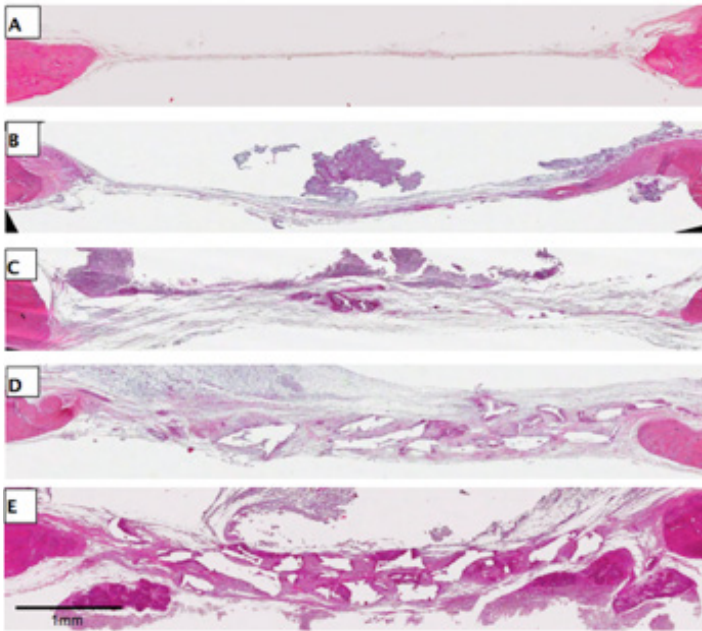


FIGURE 2 - Representative photomicrographs showing an overview of the entire healed defect of all groups, arranged according to their increased thickness. (A) Group 1: control group, no treatment. (B) Group 4: CollaTape®. (C) Group 5: CollaTape®+Simvastatin. (D) Group 2: BoneCeramic®. (E) Group 3: BoneCeramic®+Simvastatin (original magnification x20).

In a higher magnification (x200), only a thin fibrous tissue was present in the defect area in the control group (Figure 3).

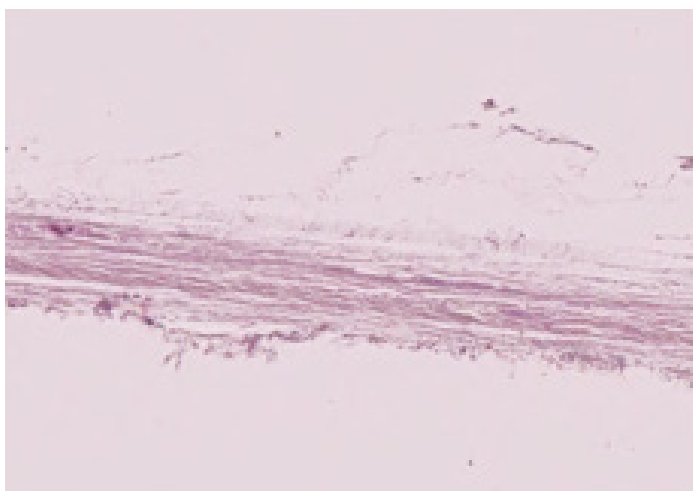


FIGURE 3 - Representative photomicrograph of group 1 (control, no treatment) at a magnification of x200 depicting a thin fibrous tissue formed in the wounded area.

In group 2, several cuboidal cells located at the surface of the BC particles (spaces left by the BC particles after demineralization, (Figure 4, asterisks) with morphological characteristics of osteoblasts were observed (Figure 4, arrows). These cells were surrounded by a bone-like matrix and in some areas, several osteoblast-like cell layers were present. The location of the cells on the surface of the BC, the bone-like matrix surrounding the cells, and their morphological appearance indicates that those cells are osteoblasts in bone matrix synthetic activity. Around the BC particles we also observed a connective tissue rich in fibroblastic cells and blood vessels (Figure 4).

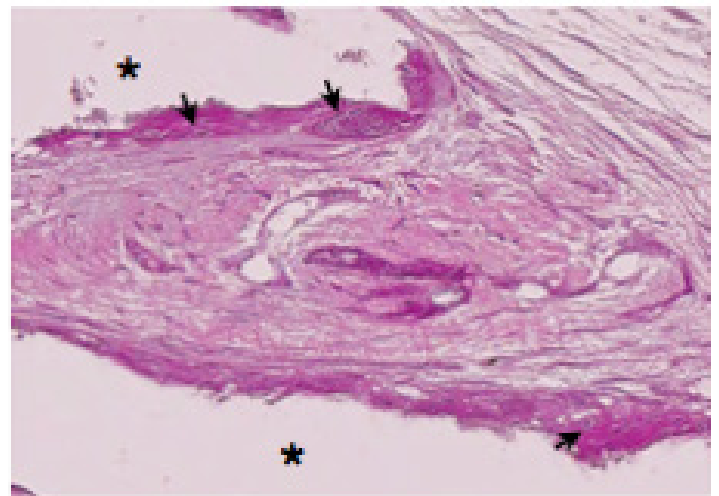


FIGURE 4 - Representative photomicrograph of group 2 (BC, BoneCeramic®) at a magnification of x200 showing several cuboidal cells with morphological characteristics of osteoblasts (arrows) located at the surface of the BC particles (spaces left by the BC particles after demineralization, asterisks).

In group 3 a newly formed bone-like tissue was observed in close proximity to the BC particles (spaces left by the particles after demineralization, (Figure 5, asterisks). This was also observed in group 2, however, in group 3 the staining of the tissue was more intense and the amount of the bone-like tissue in group 3 was visually greater than that of group 2 (Figure 5). When compared to group 2, a greater number of cuboidal cells aligned on the surface of the BC particles was observed in group 3, and in many regions, several layers of osteoblastic cells were present (Figure 5, arrows). Thick layers of a bone-like matrix around the osteoblastic cells were present, suggesting a great activity of bone matrix synthesis. A connective tissue surrounding the BC particles with many fibroblastic cells and blood vessels was also observed (Figure 5).

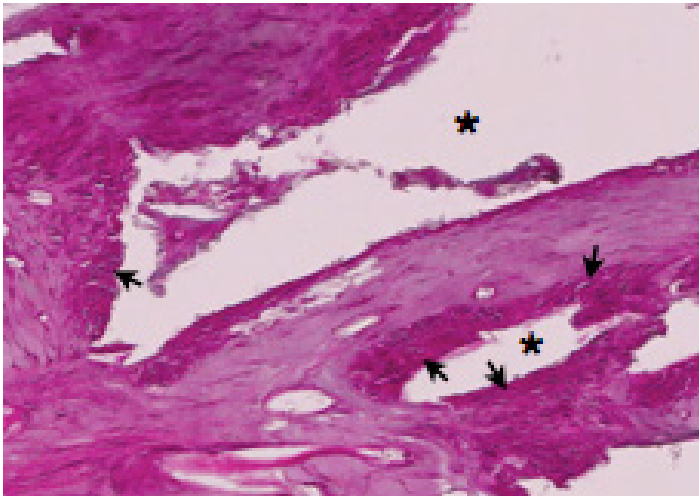


FIGURE 5 - Representative photomicrograph of group 3 (BCS, BoneCeramic®+Simvastatin) at a magnification of x200 showing a great number of cuboidal cells with morphological characteristics of osteoblasts aligned on the surface of the BC particles (spaces left by the BC particles after demineralization, asterisks) and thick layers of a bone-like matrix around the osteoblastic cells (arrows).

In group 4, in the region close to the center of the defect, the presence of a tissue with a more intense staining and a different characteristic than the fibrous tissue was observed. Remnants of the collagen sponge were not present and a few cells and blood vessels were observed (Figure 6). In group 5, in the center of the defect, a small island of a bone-like tissue was observed with the presence of cuboidal cells aligned on the surface, which is characteristic of osteoblasts surrounded by a bone-like matrix (Figure 7, arrows).

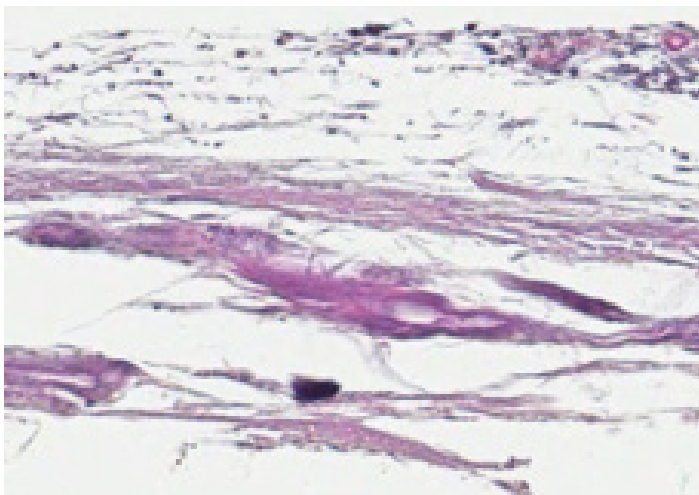


FIGURE 6 - Representative photomicrograph of group 4 (CollaTape®) at a magnification of x200 showing a fibrous tissue and no evidence of bone formation.

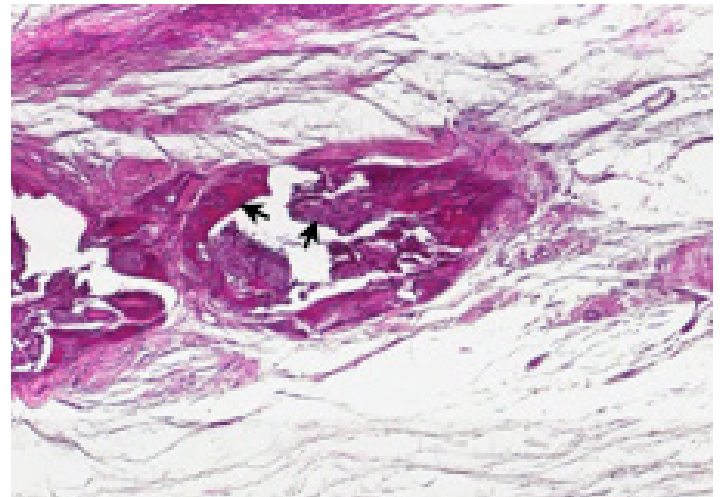


FIGURE 7 - Representative photomicrograph of group 5 (CollaTape®+Simvastatin) at a magnification of x200 depicting a small island of a bone-like tissue with cuboidal cells aligned on the surface (arrows).

Histomorphometrical analysis

The mean values for the area of newly formed bone tissue (ANB) was higher in group 3 (BCS) as compared to all the other groups. The mean values of the ANB in groups 3 (BCS), 2 (BC), and 5 (CSS) were significantly higher than that of group 1 (control group). The mean value of the ANB in group 3 (BCS) was significantly higher than that of group 2 (BC). The mean value of ANB of group 4 (CS) was not significantly different from that of group 1 (control group). Similarly, the mean value of ANB of group 5 (CSS) was not significantly different from that of group 4 (CS) (Table 1).

TABLE 1 - Comparison of new bone formation among all groups.

Groups	% ANB±SD	P-value	P-value
1 (C) (n = 6)	0.00 ± 0.00		
2 (BC) ^{a,b} (n = 6)	9.04±0.86	< 0.0001 ^a	
3 (BCS) ^{a,b} (n = 6)	12.71±1.21	< 0.0001 ^a	< 0.001 ^b
4 (CS) ^{a,c} (n = 6)	0.91± 0.86	0.4188 ^a	
5 (CSS) ^{a,c} (n = 6)	2.25± 0.61	0.0024 ^a	0.1062 ^c

ANB, area of new bone; C, control; BC, BoneCeramic®; BCS, BoneCeramic®+Simvastatin; CS, CollaTape®; CSS, CollaTape®+Simvastatin; SD, standard deviation.

^aComparison of all groups with the control group, significant difference at $P<0.05$. All groups significantly different from control.

^bComparison between groups 2 (BC) and 3 (BCS), significant difference at $P<0.05$. Group 3 significantly different from group 2.

^cComparison between groups 4 (CS) and 5 (CSS), significant difference at $P<0.05$. Groups 5 not significantly different from group 4.

Plus-minus values are means ±SD.

Analysis performed by one-way analysis of variance (ANOVA) and by Tukey method for post hoc multiple comparisons test.

Discussion

The results of studies describing the application of simvastatin for the formation of bone in different bone defects have been encouraging and its effects on bone metabolism may have a great potential in medicine and dentistry^{13,17,21}. In the present study, simvastatin was applied with two distinct carriers in critical-sized calvarial defects and its impact on new bone formation was evaluated. Similar to several previous reports, the results of the present study show that the use of this drug in fact results in greater new bone formation when applied to the bone defects created in rat calvariae. Taken together, our results and those published by others suggest that simvastatin may have a great potential to improve the results of bone reconstructive procedures. Mundy *et al.* 1999¹⁶ were the first to report that simvastatin stimulated bone formation. More recently, other *in vitro* and *in vivo* studies have described the positive effects of simvastatin in bone tissue, especially when applied locally^{22,23}. According to those studies, simvastatin can be locally applied at higher concentrations without side effects, and thus reach an ideal concentration at the local site. Therefore, in the present study, simvastatin was also applied locally with a ceramic carrier (BoneCeramic®) and its bone-inducing capabilities were evaluated.

Several animal studies have focused on determining the ideal concentration of simvastatin for the induction of bone formation. Stein *et al.*²⁴ demonstrated that 0.1mg of simvastatin in methylcellulose gel in a polylactic acid membrane resulted in minimal local inflammation, but this concentration/carrier did not stimulate significant bone growth. On the other hand, other studies verified that 0.1mg of simvastatin associated with a ceramic carriers induced the greater bone formation with little inflammation^{17,25,26}. Thus, 0.1mg could be considered the ideal concentration for simvastatin to be locally applied with a ceramic carrier. Therefore, in our study we applied this concentration of simvastatin and, similar to other studies, a greater induction of bone formation was observed. On the other hand, studies have also reported that higher local doses of simvastatin (2.2mg) result in significant inflammation of the skin over the defects without additional gain on the amount of the newly formed bone, whereas very low doses (0.01mg) are not capable to stimulate bone formation²⁶. In the present study we also verified clinically the presence of a slight inflammatory response on the skin over the surgical site in the groups treated with simvastatin (BCS and CSS), which disappeared after approximately 10 days. This result is consistent with that of other studies, that reported a similar inflammatory response that was resolved after about

10 days of healing^{17,26}. Therefore, the inflammation observed in the present study was considered of little importance because it did not negatively affect bone formation, on the contrary, the defects treated with 0.1mg of simvastatin exhibited greater bone formation, especially when combined with the biphasic calcium phosphate carrier (BoneCeramic®).

BoneCeramic® (BC) is widely used as a bone substitute grafting material and its osteoconductive properties are well established⁸⁻¹⁰. However, the collagen sponge (CS) is not considered an ideal bone grafting material since it is not osteoconductive, it is quickly resorbed (about one week), and it is not capable of maintaining the space necessary for new bone formation²⁷. This was evident on the histological analysis of the present study in which CS was incapable to induce or conduct new bone formation or even induce the formation of a tissue with a similar width as the borders of the defect. Despite that, CS was used as a carrier for simvastatin to verify if the presence of the drug could overcome the deficiencies of the CS. According to our results, a slow resorbing and space keeping material is ideal as a carrier for simvastatin, and the CS lacked those properties. Thus, since BC has those properties, it proved to be an ideal carrier for simvastatin. As pointed out, BC is an osteoconductive material that lacks osteoinductive properties. The combination of BC and simvastatin may have resulted in an osteoconductive and osteoinductive material, capable of being at the same time a scaffold and inducing greater new bone formation.

Mukozawa *et al.*²³ observed significant new bone formation after eight weeks of healing when a collagen sponge soaked in 2.5mg of simvastatin solution was placed in 5mm bone defects on the nasal bone of rabbits. These results suggested that collagen sponge could be a good carrier for simvastatin. In the present study, the collagen sponge used resulted in minimal new bone formation, which differed from the results shown by Mukozawa *et al.*²³ This difference can be attributed to several methodological differences between the studies, since Mukozawa *et al.* treated the defects with 2.5mg of simvastatin dissolved in water as opposed to 0.1mg dissolved in ethanol. Furthermore, although the collagen sponges used in our study and that used by Mukozawa *et al.* are both of bovine origin, their composition differs. While CollaTape® is composed of type I collagen from bovine tendon, the collagen sponge used by Mukozawa *et al.* was composed of 85% to 95% type I collagen and 5% to 15% of type III collagen. Moreover, their results showed that this collagen sponge alone had the capacity to induce some bone formation, which is not the case of CollaTape®²⁸. Therefore, all the differences mentioned above may explain the conflicting results found by us and by Mukozawa *et al.*²³

Simvastatin is a hydrophobic drug, and therefore it is not soluble in water. Similar to other studies, we used ethanol to dissolve the simvastatin powder. According to Morris *et al.*²⁹, dissolving simvastatin in ethanol facilitates its application in small spaces/defects when compared to a methylcellulose gel containing simvastatin. On the contrary, Tanigo *et al.*³⁰, argue that the incorporation of simvastatin into a biodegradable gelatin of hydrogel favors its release with lesser or no inflammation. In fact, the simvastatin solution used in the present study facilitated the combination with BoneCeramic®, which was easily soaked by the solution. The osteoconductive properties of BC was demonstrated in the present study by the results of groups 2 and 3, whose defects exhibited a greater bone formation and were totally filled with a newly formed tissue of a similar width as the borders of the wound as compared to the groups in which BC was not applied. The combination of simvastatin and BoneCeramic® improved even further the properties of BC, and we hypothesize that the addition of 0.1mg of simvastatin to BC resulted in an osseoconductive and osseoinductive grafting material. Thus, this combination may be useful as an alternative grafting material to autogenous bone and may have a great potential to be applied in various bone defects in a clinical setting. Further studies are necessary to test this combination in other types of bone defects.

Conclusion

BoneCeramic® is a suitable carrier for simvastatin and that their combination induced significantly greater amounts of newly formed bone as compared with BoneCeramic® alone or a collagen sponge carrier.

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