

Topical Simvastatin Improves the Pro-Angiogenic and Pro-Osteogenic Properties of Bioglass Putty in the Rat Calvaria Critical-Size Model

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Objective was to describe the effect of bioactive glass putty with and without topical simvastatin on new bone formation in critical-sized defects of rat calvaria. A calvarial bone defect was created in 20 male Wistar rats and filled with bioactive glass alone (n = 10) or combined with simvastatin (n = 10). After 4 weeks, the defects were histomorphometrically evaluated for volume fraction (Vv) of woven bone, vessel density, bioglass quantity, and inflammation. Compared to the bioglass-only group, rats treated with simvastatin had greater Vv of blood vessels ($3.3\% \pm 0.7$ vs $1.6\% \pm 0.1$, $P = .0002$) and new bone ($2.3\% \pm 0.2$ vs $1.8\% \pm 2.5$, $P = .003$). The Vv of the bioglass remnants in the bioglass-only group was higher than in the group treated with simvastatin ($2.4\% \pm 0.08$ vs $1.7\% \pm 0.3$, $P < .0004$). Chronic inflammation was noted in 1 rat from each group. Topical simvastatin seems to improve the pro-angiogenic and pro-osteogenic properties of bioglass putty in rat calvaria critical-size defects without significant inflammation.

Key Words: *bioglass putty, simvastatin, angiogenic, osteogenic inflammation, rat calvaria*

INTRODUCTION

With the expanding application of dental implants to rehabilitate edentulous and partially edentulous patients, the need for efficient biomaterials for bone augmentation is increasing. Autogenous bone is regarded as the graft of choice as it is osteoconductive and contains autologous inductive factors. However, its use is often complicated by limited stock and donor-site morbidity. Allograft bone may supply an alternative, but it also has limitations, such as partial loss of osteoinduction due to the harvesting procedure, and risk of disease transfer.

Consequently, various synthetic bone graft alloplasts have been developed.

Among these are the family of calcium phosphate ceramics¹ and glasses.² Chemically similar to bone mineral, these materials provide an appropriate framework on which direct bone apposition and bonding may occur.^{3,4} Bioactive glass (bioglass), a calcium phosphosilicate, has been reported to release ions into the physiologic environment,^{5,6} activate osteoblast gene expression,^{7,8} and enhance osteoblast proliferation.⁹⁻¹⁵ Studies found bioglass to be well absorbed and to enhance new bone growth.^{16,17} Compared to other alloplasts, bioglass leads to rapid new bone formation all through the grafted site, already 2 weeks after grafting.¹⁸ Originally available in particulate form, bioglass has recently been further improved to a putty, that can be applied alone or in combination with autograft bone, reducing the need for extensive bone harvesting. These characteristics make bioglass a candidate material for the surgical recon-

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struction of complex 3-dimensional craniofacial defects. Furthermore, according to *in vivo* studies, bioglass putty has superior bone-simulative properties compared to bioactive glass particulates, and it is associated with less inflammation.^{19,20}

To support the osteogenic process, alloplasts have been combined with osteoinductive molecules such as bone morphogenic protein (BMP) 2. However, problems such as insufficient shelf life, inefficient delivery to target cells, and high price are still an obstacle.²¹ The possibility that a pharmacologic compound will up regulate the body's production and secretion of intrinsic growth factors as BMP, combined with an osteoconductive bone substitute, appears to be a promising solution. Studies on mouse calvaria model have shown that topically applied simvastatin (SMV), a cholesterol-lowering drug (3-hydroxy-3-methylglutaryl coenzyme; HMG-CoA reductase inhibitor), stimulates bone growth by stimulating BMP-2.^{22,23} Additionally, local application of statins with different carriers induced osteogenesis^{24–29} as well as graft vascularization.^{30–34} However, others found that the doses required to induce optimal bone regeneration may increase inflammation.^{27,35}

Our electronic search of the literature yielded no reports on the addition of SMV to bioglass putty. This combination might theoretically improve the bone regeneration achieved solely by bioglass.

The purpose of this study was to histomorphometrically evaluate the added effect of SMV to bioglass putty on the amount of bone growth and vascularization, the amount of residual material, and the presence of chronic inflammation in critical-size defects in rat calvaria.

MATERIALS AND METHODS

Animal model

This study was approved by the institutional committee for animal experiments. Surgery was performed on 20 albino male Wistar rats aged 30–35 weeks, each weighing 500 g. General anaesthesia was induced by intraperitoneal injection of ketamine hydrochloride (40 mg/kg) mixed with xylazine (10 mg/kg). The dorsal part of the cranium was shaved and prepared aseptically for surgery. A 20 mm-long incision was made in the scalp along the sagittal suture, and the skin, subcutaneous tissue, and periosteum were elevated, exposing the

parietal bones. A square full-thickness calvarial bone defect measuring 8 × 8 mm was created with an ultrasonic piezo bone scalpel, without dura perforation and under constant irrigation with sterile physiologic solution to prevent overheating. The wound was thoroughly irrigated with warmed saline to remove residual bone dust. The cranial defect was then filled with either bioglass putty (NovaBone, Novabone Products Jacksonville, Fla) alone (BG group, n = 10) or with a mixture of bioglass NovaBone putty and simvastatin (BGS group, n = 10). To prepare the mixture, simvastatin powder (5 mg) (Merck & Co Inc, Whitehouse Station, NJ) was mixed with sterilized distilled water in a ratio of 1:1 and then incorporated into the bioglass putty. The mixture was administered at a dose of 1 mg per 1 kg body weight (0.5 mg per animal).

After the defect was grafted completely to the plane of the cranial outer table, the periosteum was sutured with 4.0 Vicryl sutures, and the skin, with 5/0 nylon sutures. Animals were observed daily for clinical signs of inflammation. They were humanely killed 4 weeks after the procedure by carbon dioxide asphyxiation.

Histopathologic assessment and morphometric analysis

The rat skulls were sawed and the top part of the skulls (skull caps) were incubated in decalcifying solution (10% formic acid solution) at room temperature for 7–8 days, followed by washing under running tap water for 15 minutes. Skull caps were transversally cut, placed in plastic cassettes, dehydrated by an automated machine, embedded in paraffin block, and sectioned at approximately 3–5 μ. Sections were placed on glass slides and incubated at 82°C for 30 minutes. Slides were stained with hematoxylin and eosin by an automated machine and stained with Masson trichrome (Bio Optica, Milan, Italy) according to the manufacturer's instructions.

For a histopathologic assessment and a morphometric analysis, the entire slide was screened at low magnification (×40), and the region of interest located. A matched Masson trichrome staining helped to locate new ossification sites and was particularly useful for interpreting equivocal cases. The whole area of the original defect was assessed using the modified point counting method.³⁶ At ×100 magnification, each standard field defined by

a 100-square grid (Olympus, Tokyo, Japan) with a surface of 0.64 mm^2 , comprising 121 intersections between each horizontal and vertical line, was mounted on a BH2 Olympus microscope. The number of intersections containing blood vessels, woven bone, inflammation, and bioglass were counted for each case. When an intersection hit one of these elements, the specified element scored 1 point. In each section, the count of intersections was 1231, representing 12 consecutive nonoverlapping fields. Then, the sum of points overlying each specified element was calculated, divided by the total number of counted intersections in that section, and multiplied by 100. This was defined as the volume fraction (Vv) of each element.

Statistical analysis

Differences in the mean Vv of the various elements among the study groups were statistically analyzed using 1-way analysis of variance. Statistical significance was set at $P < .05$. The statistical package InStat (STATA 12 SE StataCorp, College Station, Tex) was used for computations.

RESULTS

Macroscopic observation

All animals recovered well after surgery. No macroscopic infection of the wounds was noted. There were no side effects such as paralysis, convulsions, respiratory distress, or signs of pain.

Microscopic observation

All animals had newly formed bone and vessels at 4 weeks after grafting. The new bone was located in the inner cortical plate area, the center of the lesion, and the outer cortical plate. It was identified by the haphazard organization of collagen-containing fibrous matrix (osteoid) and the presence of viable osteocytes within the matrix. The newly formed blood vessels, mainly capillaries, were identified by their fine circular structure of 5- to $10\text{-}\mu$ diameters, single layer of endothelium, and containment of red blood cells. Bioglass was present in all cases, as indicated by findings of hollow lacunae and separation lines (Figure 1). Chronic inflammatory infiltrate was present in 1 animal from each group (Figure 2); in the others, it was either mild or absent.

Hollowed structures in different stages of bone

formation were noted: some were relatively empty, some contained acellular material that stained red with Masson trichrome (bioglass), and some contained material that stained both red (bioglass) and blue (new bone). Hollowed structures that were relatively occupied by osteoid and new bone suggested final stages of elimination (Figures 3 through 9).

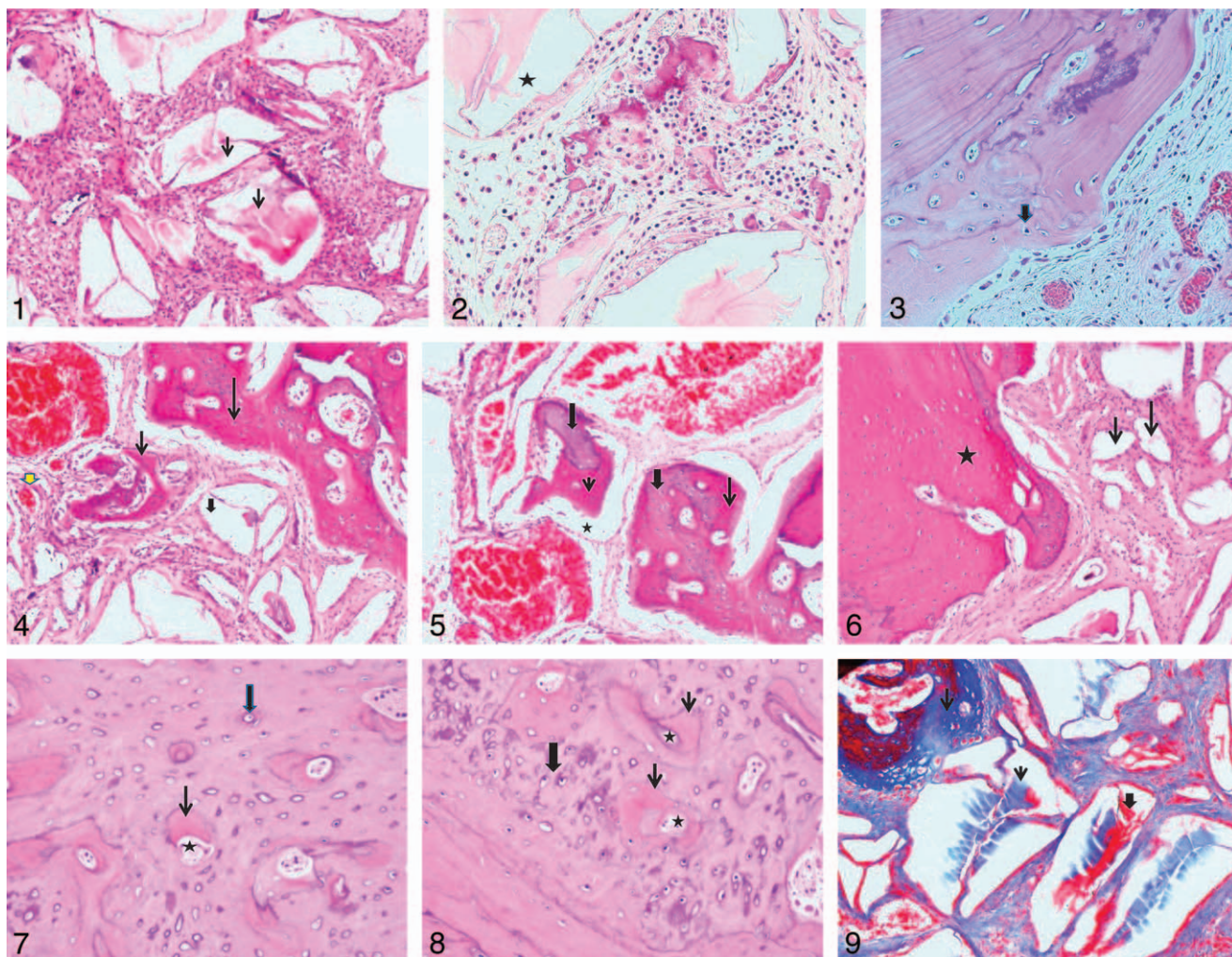
Morphometry

The histomorphometric data at 4 weeks after grafting are presented in the Table and in Figure 10. Statistical analysis revealed significant differences between the 2 groups. Compared to the BG group, the BGS group had a higher Vv of blood vessels ($3.3\% \pm 0.7$ vs $1.6\% \pm 0.1$, $P = .0002$) and new bone ($2.3\% \pm 0.2$ vs $1.8\% \pm 2.5$, $P = .003$). The Vv of the remaining bone filler material was higher in the BG group ($2.4\% \pm 0.08$ vs $1.7\% \pm 0.3$, $P = .0004$).

DISCUSSION

This study aimed to use the body's own reservoir to induce bone formation, triggered by topical pharmacologic stimuli (SMV) combined with a scaffold material (bioglass putty). New blood vessels and bone formed in all of the critical-size defects at 4 weeks after grafting. The density of both new blood vessels and bone was significantly greater with bioglass and SMV compared to bioglass alone. The amount of the residual graft material was higher in the bioglass-only group. Except for 2 cases (1 from each group), no significant inflammation was found in the defect or the adjacent soft tissue. Taken together, these findings suggest that SMV may have added value over bioglass putty alone in the treatment of critical-size defects.

The 4-week sampling time was chosen since studies proved that the osteogenic and angiogenic effects of bioglass may be identified as early as 2 weeks after grafting¹⁸; it was also short enough to appreciate the added value of SMV. We used the NovaBone putty rather than the classic particulate bioglass because it better suits the surgical challenges in the craniofacial area. The advantages of the rat calvaria model, as reported by Schmitz and Hollinger,³⁷ include the large number of specimens that can be used at relatively low cost, the low quantities of bone substitute needed, and the possibility to use particulate and gelatinous mate-



FIGURES 1–9. **FIGURE 1.** Bioglass remnants, represented by hollowed structures (arrows) in the defect area, are present 4 weeks after surgery (hematoxylin and eosin $\times 100$ magnification). **FIGURE 2.** Chronic inflammatory infiltrate composed mainly of lymphocytes and plasma cells was present in 2 cases, 1 from each group (hematoxylin and eosin [H&E] $\times 200$ magnification). **FIGURE 3.** Newly formed bone in the defect area in which viable osteocytes (thick arrow) are present as well as osteoblastic rimming (H&E $\times 200$ magnification). **FIGURE 4.** Bone neoformation in the BGS group: woven bone with abundant osteoblasts (thin arrows) and blood vessels (thick yellow arrow) in close proximity to bioglass hollows (thick black arrow) is noted (H&E magnification). **FIGURE 5.** Angiogenesis and osteogenesis. Woven bone with osteoblasts (thick black arrows) and osteoid (thin black arrows) seems to “emerge” in the center of the bioglass hollows (star), growing towards the periphery. Blood vessels are abundant (H&E $\times 100$ magnification). **FIGURE 6.** Remaining bioglass hollows (thin arrows) are seen in proximity to new bone (star) (H&E $\times 100$ magnification). **FIGURE 7.** Bone formation begins in the center of the hollowed structures (star) and progresses towards the periphery. Osteocytes (thick arrow) and osteoid (thin arrow) are noted (H&E $\times 400$ magnification). **FIGURE 8.** Bone neoformation merely occupies the hollowed structures (star). Osteocytes (thick arrow) and osteoid (thin arrow) noted (H&E $\times 400$ magnification). **FIGURE 9.** In the BGS group, the center of the hollowed structures contains either osteoid/woven bone, represented by blue staining (thin arrows), or bioglass remnants, in red (thick arrow). More mature bone (left) stains red as well (Masson trichrome $\times 100$ magnification).

rials. Experimental models with very young animals may be inappropriate to study osteopromotive materials. We used adult animals so that any bone regeneration could not be attributed to growth. SMV was chosen since it was shown to be one of the most potent statins in stimulating bone growth.²³

Simvastatin affects osteogenesis, angiogenesis, and inflammation

The biologic effects of statins on bone metabolism were first recognized by Mundy et al²² in 1999. Their study, along with others, suggests that statins, administered either locally or systemically, act as

TABLE			
Mean volume fraction (%) of woven bone, blood vessels and bioglass remnants in the bioglass + simvastatin group (BGS) compared to the bioglass group (BG)			
	Bioglass + Simvastatin	Bioglass	Statistical Significance
Woven bone	2.3 ± 0.2	1.8 ± 2.5	<i>P</i> = .003
Blood vessels	3.3 ± 0.7	1.6 ± 0.1	<i>P</i> = .0002
Bioglass remnants	1.7 ± 0.3	2.4 ± 0.08	<i>P</i> = .0004

potent stimulators of bone formation and regeneration. Either systemic or local administration was shown to promote bone growth and regeneration.^{25–27,33,38,39} SMV was found to up regulate BMP-2,^{38,39} vascular endothelial growth factor (VEGF) gene expression,⁴⁰ and alkaline phosphatase expression in osteoblasts.^{23,40,41}

In addition to osteogenesis, statins may contribute to bone angiogenesis by restoring reduced endothelial function via both their lipid-lowering effect and via actions independent of this effect. Statins up regulate endothelial nitric oxide synthase and inhibition of O₂ formation, thereby shifting the nitric acid-oxygen balance.⁴² Statins are known to elicit numerous pleiotropic effects, such as inhibiting the proliferation and migration of smooth muscle cells and inhibiting tumor growth and inflammatory activity. One study⁴³ documented a simvastatin-induced decrease in the production of interleukins 6 and 8 in epithelial cell lines and a reduction in nuclear factor-kappa B and activator protein 1 promoter levels, indicating an anti-inflammatory effect. The process apparently involves inhibition of Rac1 GTPase, a hydrolase enzyme that binds and hydrolyzes guanosine triphosphate. Statins can also depress the expression and function of leukocyte cell surface molecules, specifically the integrin heterodimer CD11b/CD18 required for monocyte adherence to endothelial monolayers.⁴³ In studies using other statins, mevastatin was found to reduce vascular inflammation by interfering with the BMP pathway at 2 ends: down regulation of pro-inflammatory BMP-4 followed by an increase in BMP endothelial cell precursor-derived regulator that suppresses the expression of the pro-inflammatory-cytokine-regulated intercellular adhesion molecule 1 (ICAM1).⁴⁴ In hypercholesterolemic rabbits, atorvastatin inhibited the neointimal contact of macrophages and activation of monocyte chemotactic protein-1 and nuclear factor-kappa B.⁴⁵ Statins may also have a

direct anti-inflammatory effect on monocytes and macrophages by causing a decreased expression of lipopolysaccharide-induced secretion of interleukin-1⁴⁶ in addition to ICAM1.⁴⁷ SMV was also found to prevent early and late joint inflammation and macrophage influx and suppressed periarticular bone destruction in experimental arthritis in rats.⁴⁸

Noteworthy, the effect of statins on inflammation may be contradictory. While its anti-inflammatory activities are well recognized,⁴⁹ researchers have found that in high doses SMV can actually induce inflammation around the site of local application, possibly in a dose-dependent manner.³⁵ For example, the local application of 2.2 mg SMV led to inflammation and scabbing of the skin overlying murine calvaria²⁶ and to the appearance of inflammatory signs in murine mandible when applied in methyl cellulose gel and polylactic acid membrane; these effects decreased when the dose was lowered.²⁷ Grafting calcium sulfate combined with 1.0 mg SMV in rat calvarial defects led to soft tissue inflammation over the defect without significant bone formation at 1–5 weeks, but once the inflammation subsided (8 weeks), a significant amount of bone was observed.⁵⁰ The authors

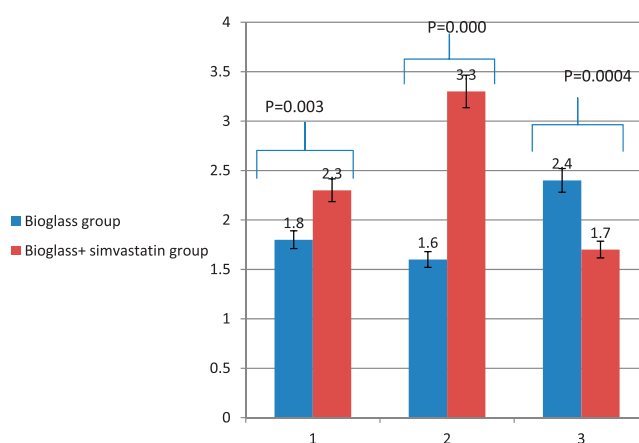


FIGURE 10. Mean volume fraction (%) of woven bone, blood vessels and bioglass remnants in the Bioglass + simvastatin group (red) compared to the Bioglass group (blue).

suggested that the burst release of SMV following early resorption of the filter induced the inflammation and caused a delay in bone formation.

In a recent study of alpha-tricalcium (bis)orthophosphate (α TCP), 0.1 mg SMV was found to be the optimum dose for maximum bone regeneration without inflammation.³⁵ Considerable bone regeneration occurred in unfilled empty defects that were adjacent to the treated ones, leading to the speculation that SMV may exert a paracrine effect. Nassar et al⁵¹ reported that SMV therapy can reverse cyclosporine A-induced alveolar bone loss in inflamed murine periodontal tissue. Adding SMV to conventional scaling and root planing therapy in patients with chronic periodontitis led to a greater decrease in gingival index and probing depth and greater gain in clinical attachment, with significant intrabony defect fill.⁵²

Lee et al⁵³ confirmed that a dose of 0.5 mg statin produced the best bone growth/inflammation ratio. In the present study, we administered 0.5 mg SMV (1 mg/kg body weight) to the grafting site along with bioglass putty. This led to bone regeneration without significant local tissue inflammation.

Bioglass is not only involved in osteogenesis, but influences angiogenesis and inflammation as well

Besides the osteopromotive properties of bioactive glasses, there is much evidence in recent literature that they (or their dissolution products) increase vascularization both in vivo and in vitro when incorporated in tissue-engineered scaffolds. Bioglass stimulated fibroblasts to secrete angiogenic factors such as VEGF and fibroblast growth factor and stimulated endothelial cell proliferation and the formation of endothelial tubules.^{54–57} This topic was thoroughly reviewed in a recent article.³⁴

Attention should be directed to the balance between the presence and elimination of bioglass. In the present study, the loss of the bioglass putty was more pronounced in the rats that were treated with SMV. Accordingly, this group had formed more bone. Similar results were noted by Nyan et al³⁵ using α TCP particles. Thus, bioglass, like α TCP, might be rapidly degraded and exchanged by new bone. It may have been the result of the continuous exposure of the osteoblasts to SMV due to its sustained release from the gradually degrading bioglass putty scaffold that led to an up regulation of BMP-2 and subsequent stimulation

of migrated mesenchymal cells to osteoblastic differentiation.

Some studies suggested that bioglass particles may contain cracks, surface irregularities, and internal cavities that hasten its resorption or dissolution by increasing its surface area.^{19,58} The present histologic study showed that the bioglass remnants are indeed hollowed in the center, but no inflammation was found, except in 1 case from each group.

In conclusion, within the limitations of this study, topical application of simvastatin to rat calvaria with critical-size defects treated with bioglass putty significantly improved the pro-osteogenic and pro-angiogenic properties, without increasing inflammation. This suggests that simvastatin may have added value in the regeneration of bone defects with bioglass. Both simvastatin and bioglass influence osteogenesis, angiogenesis, and inflammation. Further studies may corroborate these results.

ABBREVIATIONS

α TCP: alpha-tricalcium (bis)phosphonate
 BMP: bone morphogenic protein
 H&E: hematoxylin and eosin
 ICAM1: intracellular adhesion molecule 1
 SMV: simvastatin
 VEGF: vascular endothelial growth factor
 Vv: volume fraction

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