J Mater Sci: Mater Med (2009) 20:2521–2526
DOI 10.1007/s10856-009-3824-z

Effects of biosilicate and bioglass 45S5 on tibial bone consolidation on rats: a biomechanical and a histological study

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Received: 1 March 2009 / Accepted: 14 July 2009 / Published online: 31 July 2009

Abstract The purpose of this study was to investigate the effects of Bioglass 45S5® and Biosilicate®, on bone defects inflicted on the tibia of rats. Fifty male Wistar rats were used in this study, and divided into five groups, including a control group, to test Biosilicate® and Bioglass® materials of two different particle sizes (180–212 μm or 300–355 μm). All animals were sacrificed 15 days after surgery. No significant differences ($P > 0.05$) were found when values for Maximal load, Energy Absorption and Structural Stiffness were compared among the groups. Histopathological evaluation revealed osteogenic activity in the bone defect for the control group. Nevertheless, it seems that the amount of fully formed bone was higher in specimens treated with Biosilicate® (granulometry 300–355 μm) when compared to the control group. The same picture occurred regarding Biosilicate® with granulometry 180–212 μm. Morphometric findings for bone area results (%) showed no statistically significant differences ($P > 0.05$) among the groups. Taken together, such findings suggest that, Biosilicate® exerts more osteogenic activity when compared to Bioglass® under subjective histopathological analysis.

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1 Introduction

Millions of fractures occur every year worldwide, with 6.2 million of them being reported per year in the United States [1]. Among those, 5–10% show delayed healing; many persist for more than 9 months, and thus are termed non-union fractures. Multiple factors can impair fracture consolidation, including bone loss caused by diseases, trauma, or tumor resection [2]. Hence, there remains a need to learn more about the biology of fracture healing as well as to develop strategies for ensuring normal repair of the skeleton [2].

Bioglasses inducing active biomineralization for bone regeneration have been a high demand in the development of clinical regenerative medicine. Recent development of biomaterials in the field of tissue regeneration includes bioactivity inducing cell adhesion, and differentiation to achieve early healing efficacy [3].

One of the most common and studied bioactive glasses is Bioglass 45S5®, which has been known as the bioactive

glass with the highest bioactivity index [4]. It was first introduced in the early 1970s by Hench and, since then, it has been used in many clinical applications, including ridge preservation, sinus augmentation, and the repair of periodontal bone defects [5]. It is a silica-based melt-derived glass characterized by a SiO_2 content of less than 60%, a high Na_2O and CaO content, and a high CaO: P_2O_5 ratio. Bioglass 45S5® has been shown to stimulate in vitro osteogenesis inducing proliferation and differentiation of human fibroblasts and osteoblasts [6–8]. Moreover, bioactive glass materials and bioactive glass composites may be applicable in load-bearing orthopedic applications [9].

Despite the stimulatory effects of Bioglass 45S5® on bone metabolism and on fracture consolidation, the use of this bioactive glass has been restricted due to its poor mechanical properties [10, 11]. In this context, our research group has developed a novel fully-crystallized bioactive glass-ceramic of the quaternary P_2O_5–Na_2O–CaO–SiO_2 system (Biosilicate®, patent application WO 2004/074199). Therefore, full crystallization of the material may lead to enhanced mechanical properties of the bulk material or less sharp and abrasive particles when the material is milled to a powder. The Biosilicate® has presented a stimulatory effect on bone cell metabolism [4]. Comparing the growth of osteogenic cells on Biosilicate® and Bioglass 45S5® disks for a period of up to 17 days, they found that, although no significant differences were detected in terms of protein content and alkaline phosphatase activity at days 11 and 17, Biosilicate® supported significantly larger areas of calcified matrix at day 17. Results indicate that full crystallization of bioactive glasses in a range of compositions of the system P_2O_5–Na_2O–CaO–SiO_2 may promote enhancement of in vitro bone-like tissue formation in an osteogenic cell culture system.

Notwithstanding the positive effects of Biosilicate® on bone cell proliferation, studies investigating its effects on bone healing are fairly limited in the literature. To the best of our knowledge, there is one study demonstrating in vitro osteogenesis on a highly bioactive glass-ceramic [4]. It is important to emphasize that in vitro studies do not consider the complex homeostatic situation that occurs in vivo. In order to progress our understanding of the physiological processes of the Biosilicate® on fracture consolidation, the goal of this study was to examine the mechanical and histological characteristics of bone defects filled with two different particle sizes of biosilicate materials (180–212 and 300–355 μm mean size) and to compare these characteristics to those obtained with a Bioglass® material of similar particle sizes. An additional control group, that remained empty, was included. Recently, we have applied this methodology with success in rats exposed to laser treated or not with anti-inflammatory drugs [12, 13].

2 Materials and methods

2.1 Biomaterials

High purity silica and reagent-grade calcium carbonate, sodium carbonate, and sodium phosphate were used to obtain glass compositions: Bioglass 45S5® and Biosilicate® parent glass. The chemicals were weighed and mixed for 30 min in a polyethylene bottle. Premixed batches were melted in a platinum crucible at a temperature range of 1250–1380°C for 3 h in an electric furnace (Rapid Temp 1710 BL, CM Furnaces Inc., Bloomfield, NJ, USA) at the Vitreous Materials Laboratory of the São Carlos Federal University (São Carlos, SP, Brazil). Samples were cast into a 10 × 30 mm^2 cylindrical graphite mold and annealed at 460°C for 5 h.

To obtain the fully-crystallized Biosilicate® glass-ceramic, Biosilicate® parent glass cylinders were submitted to cycles of thermal treatment to promote their crystallization. The first thermal cycle was performed at a relatively low temperature, just above the glass transition temperature to promote volumetric nucleation of crystals. Afterwards, the nucleated samples were submitted to further treatment at approximately 100°C above the nucleation temperatures. Detailed compositions and thermal treatment schedules to obtain the Biosilicate® glass-ceramic are described in the patent WO 2004/074199.

Biosilicate® and Bioglass 45S5® cylinders were crushed and the powders were sieved to select particles in the 180–212 m range, used for filling bone defects in the present study.

2.2 Experimental design and surgical procedures

Fifty male Wistar rats (aged 12 weeks and weighing 250–300 g) were used in this study. They were maintained under controlled conditions of temperature (24 ± 2°C), light-dark periods of 12 h, and with free access to water and commercial diet. All animal handling and surgical procedures were strictly conducted according to the Guiding Principles for the Use of Laboratory Animals. This study was approved by the Animal Care Committee guidelines of the São Carlos Federal University. Rats were divided into five groups ($n = 10$ per group): bone defect control group (CG)—bone defects without any fillers, and groups containing bone defect filled with: group Biosilicate® 180-212 (granulometry 180–212 μm) (G1); group Biosilicate® 300-355 (granulometry 300–355 μm) (G2); group Bioglass45S5® 180-212 (granulometry 180–212 μm) (G3); group Bioglass 45S5® 300-355 (granulometry 300–355 μm) (G4).

Bilateral non-critical size bone defects were surgically created at the upper third of the tibia (10 mm distal from the knee joint). Surgery was performed under sterile conditions

and general anesthesia induced by intra-peritoneal injection of Ketamine/Xylazine (80/10 mg/Kg). The medial compartment of the tibia was exposed through a longitudinal incision on the shaved skin. A standardized 2.5-mm-diameter bone defect was created by using a motorized drill under copious irrigation with saline solution. Holes were compressed with gauze for 5 min. Immediately afterwards, bone cavities were completely filled with the corresponding biomaterial of the treated groups. After implantation, the cutaneous flap was replaced and sutured with resorbable polyglactin, and the skin was disinfected with povidone iodin. Health status of the rats was daily monitored, and no clinical abnormalities were found.

On day 15, after the surgical procedure, rats were sacrificed with an overdose of intra-peritoneal injection of Ketamine. Soft tissues were removed from the tibiae to allow analysis of the defect.

2.3 Mechanical test

Biomechanical properties of the left tibia were determined by a three-point bending test with a 1 kN load (USA, 4444 model, 1 KN load cell). Tibiae were placed on a 3.8-cm metal device, which provided a 1.8-cm distance between the two supports. The load cell was perpendicularly positioned in the anterior-posterior direction at the exact site of the bone defect. A 5-N pre-load was applied in order to avoid specimen sliding. Finally, the bending force was applied at a constant deformation rate of 0.5 cm/min until fracture occurred. From the load-deformation curve, the maximum load at failure (N), structural stiffness (N/mm), and energy absorption (J) were obtained.

2.4 Histopathological analysis

For the histopathological analysis, the right tibiae were removed, and then fixed in 10% buffer formalin (Merck, Darmstadt, Germany) for 48 h and decalcified in 4% EDTA (Merck), and embedded in paraffin blocks. Five-micrometer slices were obtained in a serially sectioned pattern and stained with hematoxylin and eosin (H.E stain, Merck).

Histopathological evaluation was performed under a light microscope (Olympus, Optical Co. Ltd, Tokyo, Japan.). Any changes in the bone defect, such as presence of woven bone, inflammatory process, granulation tissue, or even tissues undergoing hyperplastic, metaplastic, and/or dysplastic transformation were investigated per animal.

2.5 Histomorphometry

To confirm the interpretation of the subjective morphological analysis (semi-quantitative method) of the bone defects, we used 64 square reticules (30×30 cm^2) to perform morphometric assessment of the photomicrographies, the details of which have been described previously [14, 15]. A total of three representative areas from each specimen were analyzed by systematic sampling at a nominal magnification of 400×. Point counting was performed on the bone formation inside the defect. When all fields of bone defects were analyzed, the volume density was calculated, based on the principle that each fraction is equal to the mean volume density occupied by its related component [14]. Results were presented as the mean volume density in each examined group. This analysis was established in a previous study conducted by our team [16].

2.6 Statistical analysis

The normality of all variables' distribution was verified using Shapiro–Wilk's W test. For the variable that exhibited normal distribution (energy absorption), comparisons among the groups were made using one-way analysis of variance (ANOVA), complemented by Tukey HSD post-test analysis. Kruskal-Wallis test followed by post-hoc Dunn's test were performed for variables not exhibiting normal distributions (maximum load and stiffness). STATISTICA version 7.0 (data analysis software system—StatSoft Inc.) was used to carry out the statistics analysis. Values of $P < 0.05$ were considered statistically significant

3 Results

3.1 General findings

Neither postoperative complications nor behavioral changes were observed. Lameness was noticed at the first and second days after surgery only. The rats returned rapidly to their normal diet and showed no loss of weight during the experimentation. None of the animals died during the experiment.

3.2 Biomechanical analysis

Table 1 shows the means and SD of the Biomechanical test for all groups. After the statistical analysis, no statistically significant differences ($P > 0.05$) were found when the values for Maximal load were analyzed. Yet, groups G2 and G3 (Bioglass®-treated groups) and group G4 (Biosilicate®-treated group) had increased 10 and 20%, respectively, when compared to the control group (CG). Energy Absorption or Structural Stiffness were not different when statistically compared among the groups ($P > 0.05$) as well.

Table 1 Mechanical properties of rat tibias

Group	Maximal load (KN)	Energy absorption (J)	Structural stiffness (N/mm)
CG	0.048 ± 0.020	0.025 ± 0.015	103.40 ± 47.22
G1	0.054 ± 0.021	0.032 ± 0.017	124.45 ± 53.90
G2	0.053 ± 0.022	0.039 ± 0.012	130.98 ± 32.93
G3	0.048 ± 0.018	0.029 ± 0.018	114.77 ± 31.20
G4	0.060 ± 0.014	0.140 ± 0.307	144.77 ± 36.89

Data are expressed as means ±SD

3.3 Histopathological analysis

Regarding the CG, all the defects were filled by highly cellularized woven bone composed of osteogenic cells inside the bone defect after 15 days (Fig. 1a). Most of the specimens showed osteogenic activity in the bone defect, and also in the medullary region, due to the presence of bone fragments resulting from bone defect. No inflammatory process was noticed to all specimens of this group, because no acute inflammatory cells were present. In specimens treated with Biosilicate® presenting granulometry 300–355 μm (G1), the bone defect was filled with biomaterial (Fig. 1b). However, woven bone was in apposition to the surface of the biomaterial (Fig. 1b). Therefore, it seems that the total amount of fully-formed bone was higher (10%) when compared to the control group. The same picture occurred regarding Biosilicate® with granulometry 180–200 μm (G2), i.e., the amount of fully-formed bone was found higher (10%) for this group as well (Fig. 1c). In the group exposed to Bioglass 45S5® either 300-355 (G4) or 180–200 granulometry (G3), bone defects were also filled with biomaterial (Fig. 1d) containing granulation tissue throughout the bone defect. In this group, there was also the presence of woven bone circumjacent to the biomaterial (Fig. 1e).

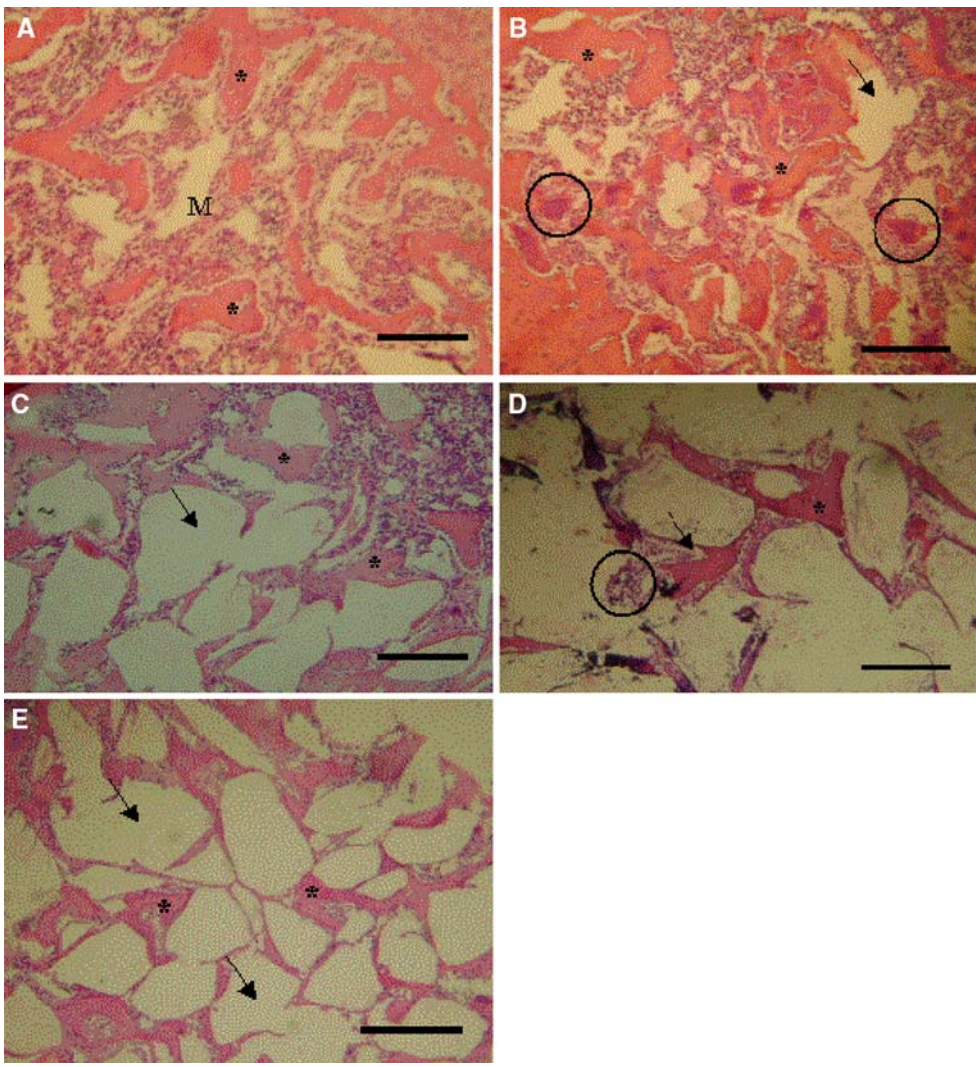

Fig. 1 Bone defects from control group (CG) (**a**) displaying high celullarized woven bone inside the defect (*asterisk*) and medullar region (M); G1—Biosilicate 300–355 μm group showing woven bone (*asterisk*) and biomaterial (*arrow*) (**b**). Note the presence of biomaterial inside the bone (*circle*). In **c**, G2—Biosilicate 180–212 μm group containing formed bone (*asterisk*) and the presence of biomaterial (*arrow*). G3—Bioglass 180–212 μm showing woven bone (*asterisk*), biomaterial (*arrow*) and granulation tissue (*circle*) (**d**). G4—Bioglass 300–355 μm with granulometry 180–200 showing new induced-bone (*asterisk*), and biomaterial (*arrow*) (**e**). H.E. stain, Bar = 36 μm

3.4 Morphometry

The area of newly formed bone was measured using the photomicrographies stained with H.E at a magnification of 400×. The results showed no statistically significant differences ($P > 0.05$) among the groups (treated with either Biosilicate® or Bioglass 45S5®) after 15 days, when compared to negative control. Such findings are shown in Fig. 2.

4 Discussion

The main purpose of the present work was to study the biological performance of Biosilicate® and Bioglass 45S5® on the bone consolidation process through a biomechanical and histopathological analysis. Our main results indicate that both biomaterials studied were not able to produce any significant increase in the biomechanical properties of the defect compared to the control group. Also, there was no significant difference concerning area of newly-formed bone between the control group and either biomaterial-treated groups (Biosilicate® or Bioglass®). However, the qualitative histopathological analysis suggested that Biosilicate®-treated groups presented higher area of bone inside the defect when compared to negative control considering the end-point established by this experimental design (15 days).

A wide variety of biodegradable polymers, bioactive glasses, and glass-ceramics have been used as a graft to heal large bone defects [17], mainly due to their ability to adapt to the defect's shape, their potential to stimulate osteogenesis and their capability to influence bone repair [18]. However, the success of the biomaterial implant and the improvement of fracture consolidation are dependent on many characteristics of the material as well as the composition [18].

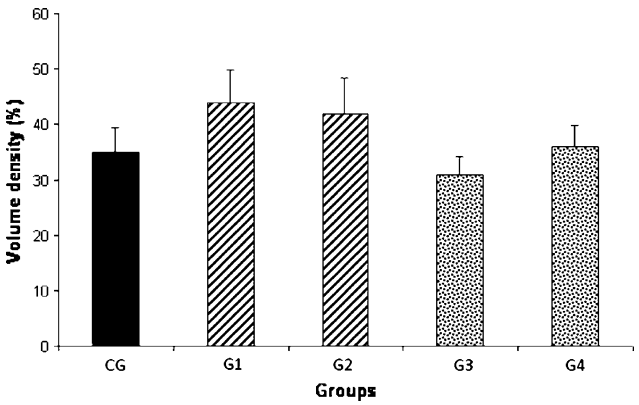

Fig. 2 Mean ± standard deviation of volume density (%) of newly formed bone in defects of rat tibias exposed to Biosilicate® or Bioglass®. CG—Control Group; G1—Biosilicate 300–355 μm; G2—Biosilicate 180–212 μm; G3—Bioglass 180–212 μm; G4—Bioglass 300–355 μm. $P > 0.05$

The positive effects of Bioglass 45S5® on the osteogenesis and on osteoblasts cell proliferation are well known. Moreover, bioactive glass has been used successfully in the clinic, primarily for oral and maxillofacial applications, with minimal load requirements [9]. Vogel et al. found that Bioglass 45S5® was capable of accelerating bone consolidation on rabbit femur fracture, with the presence of an organized tissue in the implantation bed, 28 days after the surgery. Oonishi et al. [19] showed full restoration of the implantation site filled with Bioglass 45S5® within 2 weeks. The same results were found by Wheleer et al. [9] comparing the effects of the implants of Bioglass 45S5® and tricalcium phosphate and hydroxyapatite (HA) on rabbits' femur fracture consolidation. After 16 weeks of healing, more bone and thicker trabeculae were measured within the implant pores for the 45S5-coated implants compared to the HA-coated and CTL implants.

Interestingly, in our study, the biomechanical properties of the callus were not increased in any treated group. Our results corroborate those of Wheeler et al. [9], who also found no significant differences in the biomechanical properties of femur callus of rabbits receiving 45S5 implants 16 weeks after the implantation. Probably, the 15-day period after surgery was not long enough to induce modifications that could lead to an increase on bone strength.

Also, we found that the tissue response to Biosilicate® and Bioglass® for both particles sizes used in this work was similar. However, we observed a minor, though consistent, trend towards increased woven bone area in the Biosilicate® group, used at the lower granulometry (180–200 μm) (G3). It is well-known that the particle size is one crucial factor that influences bone growth behavior [10, 19, 20]. In general, the smaller the particle size, the faster the biomaterial resorption.

Under histopathological examination, the rats exposed to Biosilicate® showed higher newly formed bone when compared to control group for both granulometries used in this setting. Nevertheless, this was not confirmed in the case of the morphometric analysis as depicted by no differences found on the volume density data. It is important to emphasize that the measure adopted in this study is indirect, i.e., such data are presented in percentage (%), Certainly, this may influence in a positive response. Total area of newly formed bone is required to elucidate the issue. Some authors have revealed that full crystallization of Biosilicate® in a range of compositions of the system P(2)O(5)–Na(2)O–CaO–SiO(2) may promote enhancement of in vitro bone-like tissue formation in an osteogenic cell culture system [4]. Maybe, this could partially explain our

findings. Taken together, it seems that Biosilicate® is able to induce bone formation after 15 days of exposure. Further studies are necessary to elucidate the issue.

When specimens were exposed to Bioglass®, however, the presence of woven bone circumjacent to the biomaterial inside the bone defect was noticed under subjective morphological analysis. This was confirmed during the morphometric analysis, i.e., no statistically significant differences were noticed in all groups. By comparison, porous bioglass promoted bone formation over the entire extension of the defect independent of block size in comparison to control group in goats [21]. An earlier study conducted by Bretcanu et al. [22] have revealed that Bioglass®/P(3HB) scaffolds have potential as osteoconductive tissue engineering substrates for maintenance and normal functioning of bone tissue. Furthermore, other authors have assumed that the Bioglass 45S5®-derived glass-ceramic scaffolds proved to be biocompatible in terms of absence of inflammatory response at the in vitro implant site [23]. Our results agree with these findings since no inflammatory response was noticed to all biomaterials tested.

In summary, such findings suggest that although Biosilicate® exerts more osteogenic activity when compared to Bioglass® under subjective histopathological analysis. Further long-term studies should be developed to provide additional information concerning the late stages of the bone matrix synthesis and degradation induced by Biosilicate®. This additional investigation should focus on the final aim of the induced-regeneration of bones, which is the ability to restore the bone architecture with biological and mechanical properties similar to the uninjured one.

Acknowledgments The authors thank to Fapesp (grant 07/08189-9), CAPES (Nanobiotec network 2009), and CNPq for generous financial support in the period 2007–2009.

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