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# Bone Response to Biosilicates® with Different Crystal Phases

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The aim of this study was to investigate the histological and histomorphometrical bone response to three Biosilicates with different crystal phases comparing them to Bioglass®45S5 implants used as control. Ceramic glass Biosilicate and Bioglass®45S5 implants were bilaterally inserted in rabbit femurs and harvested after 8 and 12 weeks. Histological examination did not revealed persistent inflammation or foreign body reaction at implantation sites. Bone and a layer of soft tissue were observed in close contact with the implant surfaces in the medullary canal. The connective tissue presented few elongated cells and collagen fibers located parallel to implant surface. Cortical portion after 8 weeks was the only area that demonstrated significant difference between all tested materials, with Biosilicate 1F and Biosilicate 2F presenting higher bone formation than Bioglass®45S5 and Biosilicate® vitreo ($p=0.02$). All other areas and periods were statistically non-significant ($p>0.05$). In conclusion, all tested materials were considered biocompatible, demonstrating surface bone formation and a satisfactory behavior at biological environment.

Key Words: biosilicate, bioglass, biocompatibility, bone response.

## INTRODUCTION

Glasses and ceramics are routinely used in medical and dental treatments as bone substitutes, and their use has been greatly improved in the last 30 years (1,2). Biomaterials such as silicate, ceramics and calcium silicate glasses have been used aiming to improve bone repair and promote hard tissue substitution. These materials are able to interact with body tissue stimulating osteogenesis favoring undifferentiated mesenchymal cells mitogenesis, leading to osteoblast and new bone formation. Bioglass®45S5 and Biosilicate® demonstrated similar bioactivity and biocompatibility with significant increase of matrices of mineralized bone compared to a ceramic material (3). When bioglass implants are used *in vivo*, its osteocondutivity promotes deposition of a calcium-phosphate layer surrounding the implant, thus leading to osseointegration due to the interaction of the biomaterial in physiological environment through biodissolution/biodegradation, apatite crystal precipitation, and bone formation around implant (4,5).

Ideally, a glass-ceramic system for substitution and bone augmentation should be osteoconductive and have reasonable mechanical properties (6), such as Bioglass®45S5, known as the material with the highest bioactivity index (7). Recently, a 100% crystallized glass-ceramic based on the general formula $SiO_2$. $P_2O5.Na_2OCaO$ was developed and named Biosilicate® (Patented 0300644-1; Federal University of São Carlos, Brazil). Its crystallinity significantly changes the fracture characteristics of the glass. Roriz et al. (8) analyzing alveolar defects filled by Biosilicate® demonstrated that this material presents higher amount of mineralized tissue on its surface compared to the control group, suggesting that Biosilicate® has excellent biocompatibility and satisfactory mechanical properties. These results stimulate the development of new materials, such as bioactive glass to be used as scaffolds. The aim of this

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study was to evaluate bone response and quantify the bone-to-implant contact (BIC) of three different crystal phases of Biosilicate® implants comparing them to Bioglass®45S5, used as control material.

## MATERIAL AND METHODS

This study was approved by the Ethics Committee for Animal Research of Ribeirão Preto Dental School, University of São Paulo, Brazil (Protocol- 07.1.975.53.0).

Sixteen 3-month-old New Zealand male rabbits weighting between 2.5 and 3.0 kg were used in the study with two implants placed bilaterally in the femur of each animal (64 implants). Bioglass®45S5 implants, based on the $SiO_2$.CaO.$Na_2O$.$P_2O_5$ composition were used as a control material due to their high efficiency in surface bone formation (2).

### *Sample Preparation and Surface Characterization*

Materials were prepared as previously described (9,10). Briefly, the Biosilicate® materials based on the general formula $SiO_2$.$P_2O_5$.$Na_2O$.CaO, and Bioglass®45S5 were obtained after weighing and mixing in rubber bottles high pure silica, calcium carbonate, sodium carbonate, and sodium phosphate during 30 min. Materials fusion took place between 1250 and 1380ºC after 3 to 4 h in an electric furnace (Rapid Temp 1710BL; CM Furnaces Inc., Bloomfield, NJ, USA) at the Vitreous Material Laboratory of the Federal University of São Carlos, São Carlos, SP, Brazil and the Vitrovita – Instituto de Inovação em Vitrocerâmicos Ltda, São Carlos, SP, Brazil. Implants (2 x 4 $mm^2$) were produced using the lost-wax casting technique. Plastic profiles of 2 x 4 $mm^2$ were invested in a refractory material (Whip-mix gypsum bonded investment, Whipmix Corp., Louisville, NY, USA), which once set was heated to 700ºC to burnout the plastic profile. The investment was then cooled to 590ºC prior to casting. The glass was melted at 1200ºC using an electrical furnace and then centrifugally cast into the investment mould (Degussa TS3 casting machine, Hanau, Germany). After grit blast the glass surface with 50 µm alumina, the rods were heat treated for 1 h at 520ºC and 1 h at 860ºC. These two different thermal treatments are essential to favor an initial crystallization at first cycle, and total material crystallization at a higher temperature. Biosilicate® composition and the thermal treatment protocol are detailed described at EP1601623B1 patent (Brazil). A very well domain on kinetic crystallization, nucleation, and growth allowed obtaining two glass ceramics fully crystallized: first showing only one crystalline phase containing 1$Na_2O$.2CaO.3$SiO_2$ and $P_2O_5$ in solid solution (named F1), and the second having two crystalline phases containing 1$Na_2O$.2CaO.3$SiO_2$ and a calcium phosphate phase (named F2).

The cast rods of glass measuring 2.2 x 4.0 mm (Fig. 1) were examined at the time of processing for defects such as porosity. Any specimen showing signs of porosity was discarded. Samples were immersed in acetone to remove any debris and abundantly irrigated with saline before implantation.

### *Implantation Procedures and Sample Harvesting*

Each animal was submitted to bilateral femur surgery at same session with two implants being inserted in each femur (Fig. 2) (2). Fenaren (sodium diclofenac - 2 mg/kg) and Tramal (tramadol chloride - 1 mg/kg)

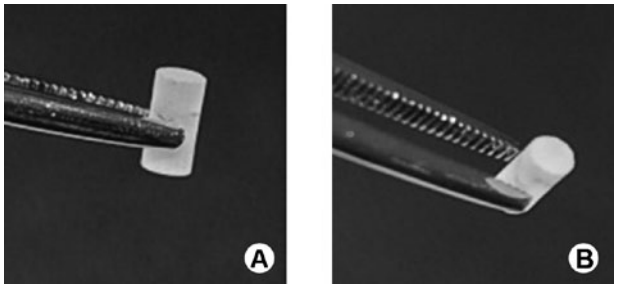


Figure 1. Glass implants used in the study measuring 2.2 x 4.0 mm.

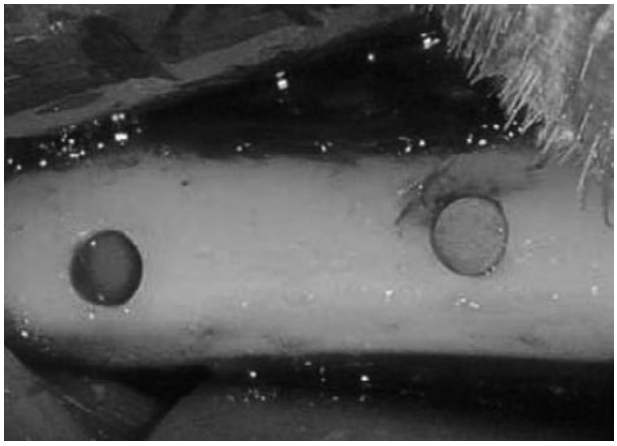

Figure 2. Femur exposed with two implants inserted after bone perforation.

were administered immediately and 24 h after surgery. The animals were maintained in cages with free access to food and water, and the wounds were inspected daily for clinical signs of complications or adverse reactions, and to monitor healing. At 8 and 12 weeks after implantation the animals were euthanized with a lethal dose of sodium thiopental. The femurs were harvested, radiographed to define implant location, and processed for morphological and histomorphometrical analyses. The periods of harvesting were selected based on previous studies (2,11,12).

### *Histological Processing*

The bone segments containing the implants were ground sectioned for light microscopy (13). The bone and implants were immediately fixed in 3% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.3) for 3 h. After fixation, the specimens were dehydrated through a series of graded ethanol solutions and embedded in resin (LH White Hard Grade, London, UK) over a period up to 9 days. Each specimen was placed in a polyethylene cup and filled with fresh resin, which was polymerized in an oven at 60ºC for 18 h. After polymerization, resin blocks were sectioned with a diamond knife (Microslice 2; Ultratec Manufacturing Inc., Santa Ana, CA). The samples were polished using silicon carbide paper and diamond polishing pastes, and the polished block surfaces were stained and processed with Stevenel's blue and Alizarin red for light microscopy. The stained blocks were mounted on slides using glass bond and were cut using a diamond band saw to leave a section near 1 mm thick. The cut surfaces of the sections were polished and thinned down to 30-40 µm using silicon carbide paper without disruption of the implant tissue interface. Cover slips were used to protect the sections and the slides were examined under a standard light microscope.

The biocompatibility of the materials was based on their osseointegration and osteoconductivity capacity. For that, the length of the cross-sections of the samples was measured followed by measurement of the length of this perimeter in close contact with bone matrix at ×10 original magnification. BIC was expressed as percentage of the implant surface in direct contact with the mineralized bone matrix in the cortical and medullary areas. At least 6 slides *per* period of each material were used. This parameter was determined by using public domain image analysis software NIH Image (National Institutes of Health, Bethesda, MD, USA).

### *Statistical Analysis*

The data were subjected to the ANOVA and Tukey's test using the SPSS/WIN statistical analysis program (SPSS Inc., Chicago, IL, USA). Differences at $p \leq 0.05$ were considered statistically significant.

## RESULTS

### *Clinical Observations*

No intraoperative or postoperative complications were observed with the animals. The implantation sites appeared to have healed with no visible signs of inflammation or adverse tissue reaction. Radiographs confirmed that the implants had been placed successfully through cortex and maintained in femur marrow.

### *Histological and Histomorphometrical Analyses*

Histological evaluation demonstrated non-absorbable cylinders localized at implantation sites with no persistent inflammation or foreign body reaction (Fig. 3). In all experimental groups the bone healing resulted in the formation of lamellar bone trabeculae adjacent to or in direct contact with the implant surface (Fig. 4A-F). Considering the medullary area, Biosilicate® implants presented a close relationship with new bone formation,

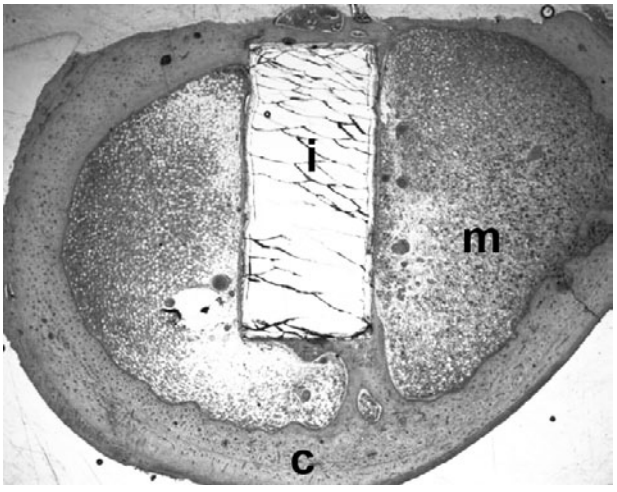


Figure 3. Histological aspect of Bioglass®45S5 implant into rabbit femur 8 weeks after surgical procedure (C, cortical bone, I, implant, M, medullary bone) (original magnification ×1.6).

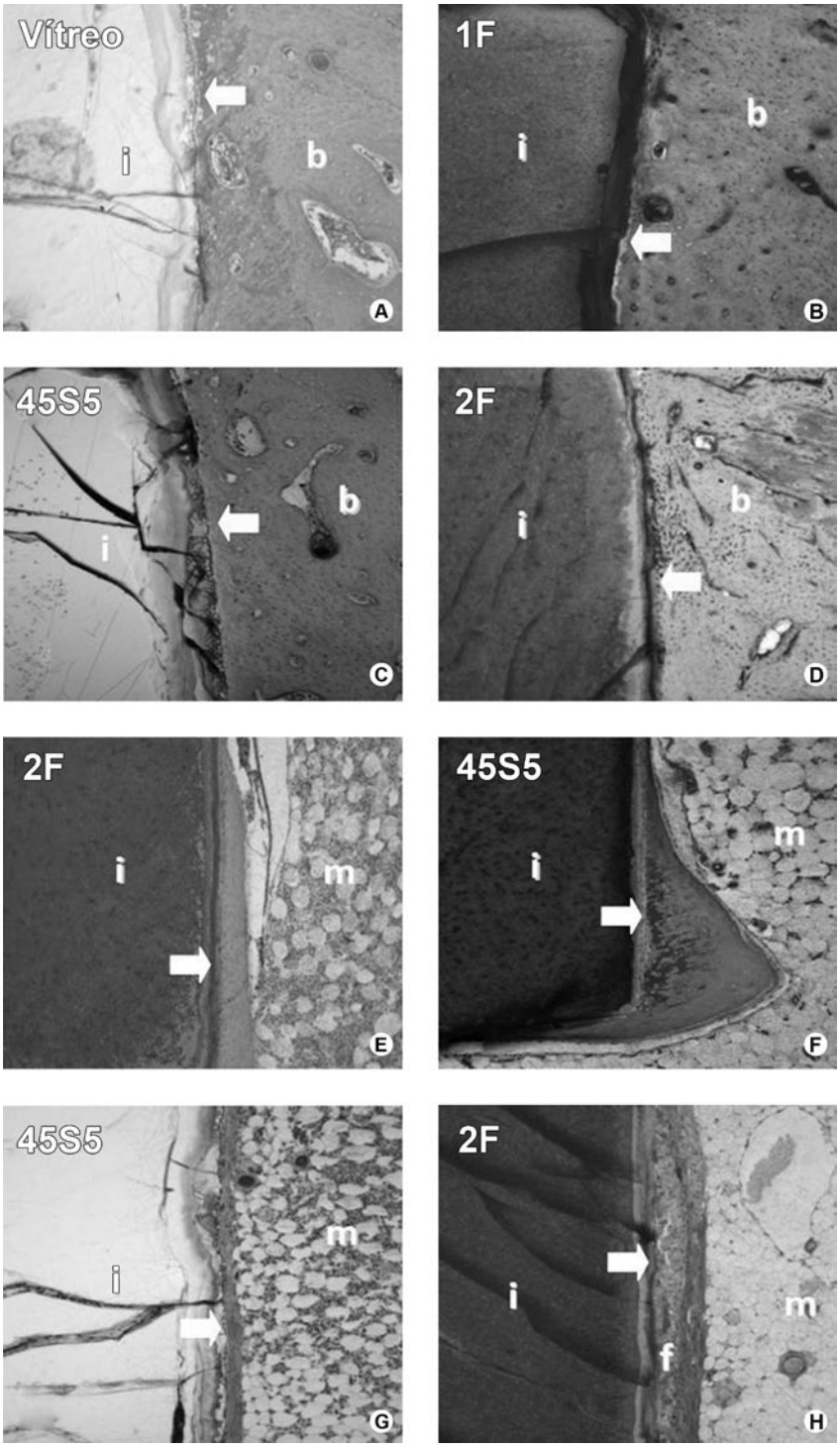


Figure 4. Ground sections of Biosilicate® cylinders after 8 and 12 weeks of implantation. A= Biosilicate®vítreo implant-tissue interface showing the cortical bone in close contact to the implant surface after 8 weeks; B= Biosilicate 1F - tissue interface showing bone formation between implant surface and the cortical bone at 8 weeks; C= Direct contact between the Bioglass®45S5 implant surface and cortical bone (12 weeks); D= Biosilicate 2F - direct contact at cortical region after 12 weeks; E= Implant - tissue interface showing bone formation in the medullary canal after 12 weeks (Biosilicate 2F); F= Bioglass®45S5 - arrow showing bone in close contact with the implant, in the medullary canal after 12 weeks; G= Bioglass®45S5 - tissue interface showing soft tissue in close contact with the implant, in the medullary canal after 8 weeks; H= Arrow showing soft tissue in close contact with Biosilicate 2F implant in the medullary canal after 12 weeks. b: cortical bone; i: implant; m: bone marrow; f: fibrous tissue. Original magnification: ×10.

demonstrating the biocompatibility and osteocondutivity of tested materials (Figs. 4E and 4F). A layer of soft tissue was observed in close contact to implant surfaces in the medullary canal. The connective tissue was composed of a few elongated cells and collagen fibers located parallel to implant surface (Figs. 4G, 4H - arrows). The mean percentages of BIC at medullary and cortical regions are summarized in Tables 1 and 2.

The BIC in bone marrow at 8 weeks was as follows: Biosilicate®vitreo > Bioglass®45S5 > Biosilicate 2F > Biosilicate 1F. BIC at 12 weeks presented Biosilicate 1F > Biosilicate 2F > Biosilicate®vítreo > Bioglass®45S5. No significant difference was found between the materials or tested periods (p=0.08 and p=0.14 respectively). Considering BIC at cortical portion after 8 weeks, Biosilicate 2F presented the highest bone formation followed by Biosilicate 1F, Bioglass®45S5, and Biosilicate®vítreo. Biosiliacate1F and 2F presented similar results with no significant difference (p=0.06). However, in this period, Biosilicate 1F and 2F demonstrated higher BIC compared to Bioglass®45S5 and Biosilicate®vítreo (p=0.02). At 12 weeks the BIC was similar between all tested materials without significant difference (p=0.08).

## DISCUSSION

The results of the present study showed an excellent biocompatibility of Bioglass®45S5, Biosilicate®vitreo, Biosilicate 1F, and Biosilicate 2F after bone implantation. It is important to emphasize that Bioglass®45S5 has shown high bioactivity for many years (7). Different studies have reported that the Biosilicate® glass exerts more osteogenic activity than Bioglass®45S5 under subjective histopathological analysis (8,14). Accordingly, our results showed higher bioactivity of Biosilicate 1F and 2F at the cortical portion after 8 weeks. The results after twelve weeks confirmed similar behavior among Bioglass®45S5 and all tested Biosilicate® glass ceramics. It is important to emphasize that the percentage of osseointegration of Biosilicate 2F was 100% after 12 weeks and that Biosilicate 1F osseointegration process was finished after 8 weeks. In contrast to cortical portion, the BIC in bone marrow showed no statistically significant differences among tested materials.

Our results suggest that new bone formation around the materials is influenced by their location within the bone, whether in the cortical bone as in the marrow bone. The osteogenic potential of the fibrous connective tissue at the bone marrow could be attributed to the migration of osteogenic cells from the endosteum during tissue repair. However, fragments of endosteum stripped off during surgical procedure that adhered to material surface could be the source of osteoblastic cells (2). Landry et al. (15) explain that activating periosteal or endosteal cells, as well as cells involved in the medullary repair system promotes an intense osteogenic response. This occurs at the initial repair phase, promoting new medullary bone formation.

In this study, considering that all the samples were prepared under the same conditions, the implant surface topography was likely similar for all rods. Therefore, the BIC could not have been influenced by factors other than surface chemistry. Moura et al. (3) showed that full crystallization of bioactive glasses from $P_2O5$.

Table 1. Bone-to-implant contact (BIC) of Biosilicate® samples and Bioglass®45S5 at the cortical area.

| Material | 8 weeks | 12 weeks |
|---|---|---|
| Bioglass®45S5 | 87(±8)[a] | 92(±8)[a,b] |
| Biosilicate®vítreo | 84(±15)[a] | 92(±4)[a,b] |
| Biosilicate 1F | 93(±6)[b] | 90(±9)[a,b] |
| Biosilicate 2F | 96(±4)[b] | 100(±0)[a,b] |

BIC is expressed as percentage of the implant surface in direct contact with the mineralized bone matrix in cortical and medullary area. Parentheses numbers represent the standard deviation. Number of samples of each tested biomaterial in each period of time (n=8). Different letters in columns indicate statistically significant difference (ANOVA and Tukey's post hoc test).

Table 2. Bone-to-implant contact (BIC) of Biosilicate® samples and Bioglass®45S5 at the medullary area.

| Material | 8 weeks | 12 weeks |
|---|---|---|
| Bioglass®45S5 | 57(±15)[a] | 52(±18)[a] |
| Biosilicate®vítreo | 66(±5)[a] | 53(±4)[a] |
| Biosilicate 1F | 44(±4)[a] | 56(±8)[a] |
| Biosilicate 2F | 48(±7)[a] | 56(±6)[a] |

BIC is expressed as percentage of the implant surface in direct contact with the mineralized bone matrix in cortical and medullary area. Parentheses numbers represent the standard deviation. Number of samples of each tested biomaterial in each period of time (n=8). Different letters in columns indicate statistically significant difference (ANOVA and Tukey's post hoc test).

$Na_2O.CaOSiO_2$ system composition may enhance bone tissue formation in an osteogenic cell culture system. Granito et al. (14) suggest that Biosilicate® exerts more osteogenic activity when compared to Bioglass® under subjective histopathological analysis, with no significant differences between these two materials when maximal load, energy absorption, and structural stiffness are analyzed. Roriz et al. (8) found similar results for Biosilicate® and Biogran® in alveolar ridge reconstruction, demonstrating an excellent response of the bioactive glass ceramics. It is important to stress that *in vitro* conditions are different from the *in vivo* situation, due to the impossibility of simulating homeostasis in a complex situation. In the study by Roriz et al. (8), Biosilicate® presented more favorable results in both situations (*in vitro* and *in vivo*).

Previous studies have demonstrated that glass crystallization diminishes its bioactivity level (16) and some even suggested that crystallization might convert bioactive glasses into bioinert materials (17). Nevertheless, our results do not support this hypothesis. Xynos et al. (18) and Loty et al. (19) affirm that the total crystallization of Biosilicate® may alter some important properties of the material and consequently modify environment pH, turning it alkaline, which is favorable to osteoblast differentiation and function. The crystallization may even alter material properties like dissolution index, which might interfere on bone formation and be use to improve its biological properties. The different crystallization processes between Biosilicate®vítreo, Biosilicate 1F and Biosilicate 2F may have interfered with bone formation. Despite the identical composition of Biosilicate 1F and 2F (crystalline) compared to Biosilicate®vítreo, the materials might present different dissolution capacity, kinetics and osseointegration. As previously mentioned, a very well domain on kinetic crystallization, nucleation, and growth allowed to obtain two glass ceramics fully crystallized: first showing only one crystalline phase containing $1Na_2O.2CaO.3SiO_2$ and $P_2O_5$ in solid solution (named F1), and the second having two crystalline phases containing $1Na_2O.2CaO.3SiO_2$ and a calcium phosphate phase (named F2). Unfortunately, the phosphate phase was not identified until now using x-ray diffraction due to its very low phosphate concentration (less than 1.8%). However, it was possible to observe the phosphate phase on FTIR spectra machine using diffuse reflectance (3).

The results showed that, regardless of the chemical composition, all evaluated implants were biocompatible, as demonstrated by their capacity for osseointegration, which was calculated as the percentage of new mineralized bone tissue formed in close contact with the implant surface. The chemical composition was important as a determinant of residual glass solubility and hence stability in the biological environment. The regular appearance of the Biosilicate® implants in all micrographs suggests that all tested compositions were stable in the biological environment. The tested Biosilicate® formulation was therefore considered suitable for implant use. The absence of inflammatory cells or multinucleated cells after 8 and 12 weeks demonstrated the applicability of all tested materials favoring BIC. Vogel et al. (20) showed that the presence of multinucleated cells diminishes bone union to the surface of the implant. In addition, the absence of macrophage activity decreases the possibility of dissolution of glass-based materials, which might create an acidic pH on local tissue environment (7).

In conclusion, the results of the present study indicate that all tested materials are biocompatible and are suitable to be used in clinical dentistry.

## RESUMO

O objetivo deste estudo foi investigar histologicamente e histomorfometricamente a resposta óssea a três diferentes fases cristalinas do Biosilicato®, comparando-os aos implantes de Bioglass®45S5 utilizados como controles. Implantes de cerâmicas de Biosilicato® e implantes de Bioglass®45S5 foram inseridos bilateralmente em fêmures de coelho e avaliações histológicas realizadas após 8 e 12 semanas. As avaliações histológicas não revelaram inflamação persistente ou reação de corpo estranho nos sítios de implantação dos biovidros. A formação de tecido ósseo pôde ser observada em maior quantidade na porção cortical, com tecido conjuntivo sendo observado em íntimo contato com as superfícies dos implantes apenas na porção medular. O tecido conjuntivo apresentou células com forma alongada e fibras de colágeno localizado paralelamente à superfície do implante. A porção cortical (após 8 semanas) foi a única área que demonstrou diferença significante entre os materiais estudados, com o Biosilicato 1F e o Biosilicato 2F demonstrando maior formação de tecido ósseo em contato com a superfície quando compardos aos implantes de Bioglass®45S5 e Biosilicato®vítreo ($p=0,02$). As outras áreas estudadas nos diferentes períodos não foram consideradas estatisticamente significantes ($p>0,05$). Pode-se concluir que todos os materiais testados foram considerados biocompatíveis, com formação óssea na superfície e comportamento em ambiente biológico satisfatório.

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