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Original Research

Experimental maxillary sinus augmentation using a highly bioactive glass ceramic

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Abstract Physicochemical characteristics of a biomaterial directly influence its biological behavior and fate. However, anatomical and physiological particularities of the recipient site also seem to contribute with this process. The present study aimed to evaluate bone healing of maxillary sinus augmentation using a novel bioactive glass ceramic in comparison with a bovine hydroxyapatite. Bilateral sinus augmentation was performed in adult male rabbits, divided into 4 groups according to the biomaterial used: BO—particulate bovine HA Bio-Oss® (BO), BO+G—particulate bovine HA + particulate autogenous bone graft (G), BS—particulate glass ceramic (180–212 μm) Biosilicate® (BS), and BS+G—particulate glass ceramic + G. After 45 and 90 days, animals were euthanized and the specimens prepared to be analyzed under light and polarized microscopy, immunohistochemistry, scanning electron microscopy (SEM), and micro-computed tomography (μCT). Results revealed different degradation pattern between both biomaterials, despite the association with bone graft. BS caused a more intense chronic inflammation with foreign body reaction, which led to a difficulty in bone formation. Besides this evidence, SEM and μCT confirmed direct contact between newly formed bone and biomaterial, along with osteopontin and osteocalcin immunolabeling. Bone matrix mineralization was late in BS group but became similar to BO at day 90. These results clearly indicate that further studies about Biosilicate® are necessary to identify the factors that resulted in an unfavorable healing response when used in maxillary sinus augmentation.

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1 Introduction

Cell and tissue response to a biomaterial basically depends on its class, chemical composition, and morphology. When bone tissue is the target, a wide range of materials can be used, from autogenous bone to specific metals, depending on the purpose of their use [1, 2]. The necessity of recovering bone quality and quantity is required when oral rehabilitation with endosseous implants is aimed, demanding favorable tissue condition for success, and that for years it has been achieved with synthetic biomaterials, which include ceramics, glasses, glass–ceramics, amongst others [3, 4]. In order to interact in a satisfactory biological way, it is important that these materials are not only biocompatible, but also present a high bioactive level that

means the time that is needed for a living bone tissue to be more than 50 % attached to a material surface ($I_B = 100/t_{0.5bb}$), through the formation of a layer of a biologically active hydroxycarbonate apatite (HCA) at material/tissue interface that is quite similar to bone tissue apatite [5]. It is known that among the previous cited materials, glasses exhibit the highest bioactive levels, reaching $I_B = 12.5$, whereas hydroxyapatite (HA) is about 3.1 [6]. This information would be enough to elect this material as bone substitute; however, glass materials present low mechanical property, not desired when trying to reestablish a load bearing bone area. In an attempt to surpass this deficiency, a new Na_2O–CaO–SiO_2–P_2O_5 quaternary system highly bioactive glass ceramic ($I_B > 8$) was developed named Biosilicate® [7], fully crystallized, presenting modulus of elasticity similar to cortical bone. The increase in crystallinity of a glass material is what basically turns it into a glass ceramic [8].

Therefore, along with chemical properties, it is known that biomaterial microarchitecture interferes cells response [9]. In craniomaxillofacial surgery, particulate biomaterials are usually indicated to fill in specific cavities such as dental sockets and maxillary sinus, aiming further local rehabilitation with dental implants. For this, bovine HA and β-tricalcium phosphate have been the materials of choice presenting favorable results, associated or not with particulate autogenous bone grafts [10, 11]. For years, Bio Oss®, a slow-resorbable ultraporous bovine HA, has been considered the "gold standard" biomaterial for bone reconstruction due to its physical and chemical similarity with human bone [12]. It can be found as scaffold and particles, from 250–1000 and 1000–2000 μm, which present around 10 nm HA crystals with large-mesh interconnected pores that facilitate angiogenesis process and cell migration. This particular microstructure may be responsible for direct osteogenic proteins, enhancing its osteoconductivity [12]. Particulate Bio Oss® is indicated to fill in bone cavities as dental sockets, for maxillary sinus augmentation, peri-implantar and periodontal defects, amongst others [3, 13, 14].

Biosilicate® also can be found as particles and scaffold [15, 16], and has been studied as bone substitute in a number of situations, especially for long bone defects repair [17, 18]. Although its chemical characterization has been well described, its physical characteristics remain less explored [5]. The maintenance of alveolar ridge volume after tooth extractions followed by dental implants installation at experimental level has already been performed using Biosilicate® with satisfactory results [19]. Repair of calvarial defects of rabbits has already been studied, also resulting in interesting bone tissue repair, which was quite similar to autogenous bone graft in relation to bone formation and VEGF and Runx-2 immunolabeling [20].

Considering that satisfactory results also depend on the integrity, viability, and environment of the recipient site, since revascularization and cell migration from recipient bone are fundamental for the quality of tissue formation, the site where a biomaterial is to be tested should be carefully selected, counting on its anatomical and physiological particularities. Maxillary sinus is a natural sterile bone cavity, ventilated by air change that flows through sinus *ostium* from nasal cavity, which represents a common target for reconstructive procedures aiming dental implants rehabilitation [21]. Differently from dental sockets and artificial bone cavities created to be filled with a number of biomaterials, it is lined by respiratory mucosa named Schneiderian membrane, constituted by pseudostratified epithelium, lamina propria and by a *periosteum-like* layer [22] that seems to be different from cortical bone periosteum in regard its osteogenic potential. Studies about anatomical and histological analysis of rabbits' maxillary sinus reveal a quite similar aspect as humans [23, 24]. Because of its anatomical proximity with maxillary premolar and molar teeth, their absence can compromise dental rehabilitation with endosseous dental implants, since they can undergo alveolar pneumatization, resulting in insufficient bone for implant installation. In order to turn this anatomical site proper to be rehabilitated, maxillary sinus lifting surgical procedure is performed, as extensively described in literature [25]. Considering its anatomical features, maxillary sinus present a different environment when compared to dental sockets and bone defects, which probably interfere in bone grafting healing. Aging and general bone resorption can also contribute to decrease the number and size of local maxillary sinus vascularization. In this way, when analyzing biomaterial behavior it is important to observe in which anatomic site it is tested. The present study aimed to analyze maxillary sinus lifting repair using particulate Biosilicate® in a rabbit model, focusing on the quality of the newly formed bone.

2 Materials and methods

All experimental protocols involving animals performed in this study followed the Principals of Laboratory Animal care (NIH), as well as of Brazilian Society of Laboratory Animal Science (COBEA), and the project was approved by the Ethical Committee for Animal Care (protocol 007/13) of Sagrado Coração University—USC.

2.1 Study design

Forty male New Zealand rabbits with mean weight of 4 kg, 5 months of age, underwent bilateral sinus lift surgeries and were divided into four groups, according to the biomaterial

used: BO—particulate bovine HA (1000–2000 μm,) (Bio-Oss®, Geistlich Pharma AG, Wohlhusen, Switzerland), BO+G—particulate bovine HA + particulate autogenous bone graft (G), BS—particulate glass ceramic (180–212 μm) (Biosilicato®, Vitrovita, São Carlos, Brazil), and BS+G—particulate glass ceramic + G. The animals were maintained in controlled environment of 21–22 °C and 12-h light–dark cycles during all experimental period, with access to food and water ad libitum. It was determined that the animals would have their right-side sinuses filled with BO or BS, and the left ones with the biomaterials associated (1:1) to G. After 45 and 90 days, all animals were euthanized for specimen's removal ($n = 5$).

2.2 Surgical procedures

Before surgery, all animals were sedated with intraperitoneal administration of 1 % ketamine (0.20 ml/kg) and 2 % xylazine (0.30 ml/kg) (Francotar, Virbac Ltda., São Paulo, Brazil). After trichotomy of the region to be manipulated, antisepsis was performed with 1 % polyvinylpyrrolidone aqueous solution. In addition, 2 % mepivacaine plus epinephrine 1:100.000 local anesthetic was already used in order to improve hemostasis. Maxillary sinus lifting procedures were performed according to Xu et al. [26]. After careful elevation of Schneiderian membrane, the biomaterials were introduced according to previous description [27]. Autogenous bone was retrieved from the animals' calvarial bone using a 0.5 cm trephine bur under copious irrigation with 0.9 % saline solution. Soft tissues were repositioned and sutured with 5–0 nylon. All animals were immediately medicated with 0.2 ml/kg of enrofloxacine (Flotril 2.5 %®, Schering-Plough S.A., Rio de Janeiro, Brazil) and 0.10 ml/kg of tramadol (Tramal®, Schering-Plough S.A., Rio de Janeiro, Brazil), both for 3 consecutive days by intramuscular administration. One specimen of each period was sent to image analysis (μCT scanning and scanning electronic microscopy—SEM), in order to reveal the morphology of the glass–ceramic particles and also characteristics of bone/biomaterial contact.

2.3 Histological procedures and analysis

The animals were euthanized after 45 and 90 days, and the sinuses immediately fixed in 10 % buffered formalin. After, the specimens were washed in tap water for 24 h, and immersed in 10 % ethylenediaminetetraacetic acid (EDTA) for paraffin embedding processing and Goldner Trichrome (GT) and Picrosirius-red staining. The histological slices stained with GT were analyzed under light microscopy in order to observe the healing events as inflammation, granulation tissue, new bone formation and remodeling, and the interaction among the biomaterial, bone graft, and newly formed bone. Histological slices stained with Picrosirius-red, revealed their organization and amount of the collagen fibers bundles considering the greater birefringence intensity of the collagenous matrix, the better its organization.

2.4 MicroCT

Specimens were subjected to μCT scanning (SkyScan, Kontich, Belgium) at an X-ray energy level of 50 kVp, and 800 μA current. Images were captured with 14.1 μm voxel, 1.1° at each pace, and 360° rotation, and further reconstructed using NRecon v1.6.4.8 software (SkyScan, Kontich, Belgium), with the same reconstruction parameters for all the specimens. After, the tridimensional images and the reconstructed ones were realigned using DataViewer 1.4.4.0 software, to be reconstructed in 3D in CTvox 2.3 software, using volume renderization.

2.5 Scanning electron microscopy (SEM)

The specimens were taken from the formalin and let to dry for 30 min to be scanned using PSEM eXpress (Aspex Corp, Belmont, USA), which do not demand metal coating. For the analysis of bone–biomaterial interface, the images were captured at ×250, in order to observe bone tissue adaptation and integrity of the biomaterial.

2.6 Picrosirius-red polarization method

To make possible the identification and analysis of collagen amount and quality by the birefringence of its fiber bundles organization, sagittal histological slices of the maxillary sinuses were stained with Picrosirius-red. Four central fields of the defects were analyzed under polarized light microscope with ×10 magnification (Fig. 1a). To quantify only thin (greenish) and thick (yellow and red) fibers corresponding to the newly formed collagenous matrix, bone grafts particles (BO+G and BS+G groups) were removed in Adobe Photoshop CS6 Software to delimit the region of interest (Fig. 1b, c). The intensity of birefringence from greenish to yellow and red color collagen fibers was measured using *ImageJ* (version 1.36) software to define corresponding area (pixel^2) of these fibers, as well as total birefringent fibers (Fig. 1d) [28].

2.7 Immunohistochemistry

For immunohistochemistry analysis of bone repair, 3 μm sections of each specimen were deparafinized in xylene and rehydrated in a graded series of ethanol to distilled water and immersed in 0.01 M citrate-buffer at pH 6.0 to be heated in a steamer for 30 min. Histological slices were

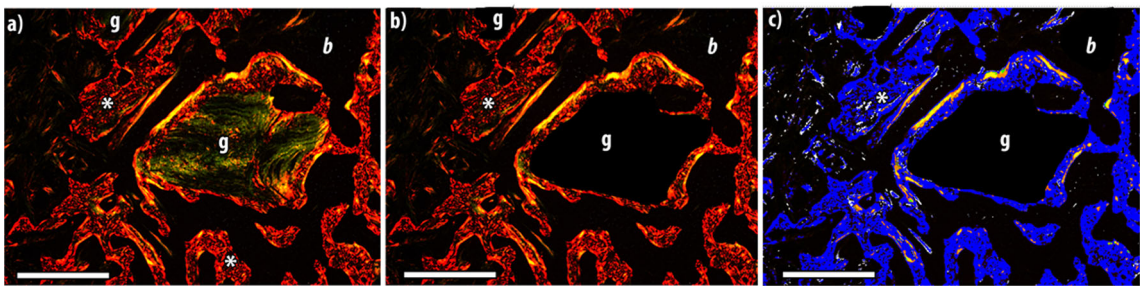

Fig. 1 **a–c** Example of image processing and analysis of collagen matrix. **a** Image captured under polarized light to identify new bone formation (*asterisk*) featuring *red* and *yellow* birefringence; bone graft (g) is identified by *greenish* fibers, while biomaterials **b** remain in a *dark* background field. **b** After image processing to exclude bone graft areas, the image was binarized (**c**), to quantify *greenish*, *yellow* and *red* fibers, as demonstrated by *red* fibers (*blue* binarization) (Picrosirius-*red staining*, original magnification ×10; *scale bar* = 100 μm) (Color figure online)

treated with proteinase K for 30 min in room temperature. Endogenous peroxidase was blocked with 2 % peroxide hydrogen for 10 min and washed with PBS (phosphate buffer solution). Then, primary polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, EUA) anti-OC and anti-OPN, were incubated overnight at 4 °C and washed for 30 min, three times. The slices were incubated with biotinylated secondary antibody (LSAB, Dakocytomation) for 30 min, washed in PBS and incubated with streptavidin-peroxidase conjugate (LSAB, Dakocytomation) for more 30 min. After, reaction was stained with 3,3′-diaminobenzidine tetra hydrochloride (Sigma Aldrich, St Louis, MO, USA) and counterstained with Harris hematoxylin. For negative control, primary antibody was omitted. Labeling levels for each antibody was determined by semi-quantitative analysis, considering the scores from "−"to "+++" (− = absent, + = mild, ++ = moderate, and +++ = intense) performed by two evaluations in a double-blind system [29].

2.8 Statistical assessment

Data sets from Picrosirius-red polarization method for comparison of all groups and all periods were statistically analyzed by One-Way analysis of variance (ANOVA) followed by Student's T test. For data that did not fit in distribution of normality, Kruskal–Wallis test followed by Dunns'test were used, considering $P < 0.05$).

3 Results

3.1 Histological, SEM, and μCT analysis

At day 45, SEM images presented tight adherence of BO particles and newly formed bone tissue. This condition was confirmed by light microscopy, when high-cellularized newly formed bone was observed in BO group deposited on the surface of biomaterial particles, surrounded by connective tissue. BO+G group presented non-viable G particles and maturing bone in contact with the biomaterial. Similar images were observed in BS and BS+G groups, showing close contact between bone and biomaterial (Fig. 2). However, in these groups, smaller amount of bone was observed with predominance of soft tissue, as revealed by light microscopy, which showed connective tissue with intense focal mononuclear leukocytes, especially around the degrading biomaterials particles. μCT images revealed important morphological differences between BO and BS, when BO particles seemed to keep their original dimensions in comparison to BS particles at 45 days.

At day 90, close contact between bone and biomaterial was maintained, with the biomaterial embedded in bone tissue especially in BO and BO+G groups. In light microscopy it was possible to see lamellar bone around the biomaterial and the medullary spaces constituted by adipose tissue. Although numerous particles of BS embedded in the formed bone were observed in BS and BS+G groups, soft tissue and even collagenous encapsulation was also noted in contact with the degrading biomaterial, identified by the intense macrophage activity and foreign body reaction (FBR) evidenced by light microscopy. μCT images revealed that, while BO particles, associated or not to bone graft, persisted at day 90, BS particles were mostly identified by the accumulation of chronic inflammatory cells that were attracted during degrading process (Fig. 2).

3.2 Picrosirius-red polarization method

All groups presented birrefringence for greenish, yellow and red fibers, demonstrating the matrix maturation dynamics (Fig. 3). Considering total fibers, BO group presented an increase in matrix amount in comparison to BO+G and BS groups at day 45 (Fig. 4a). Although all groups presented an increase in these fibers at day 90, especially BO+G group, again no difference was detected

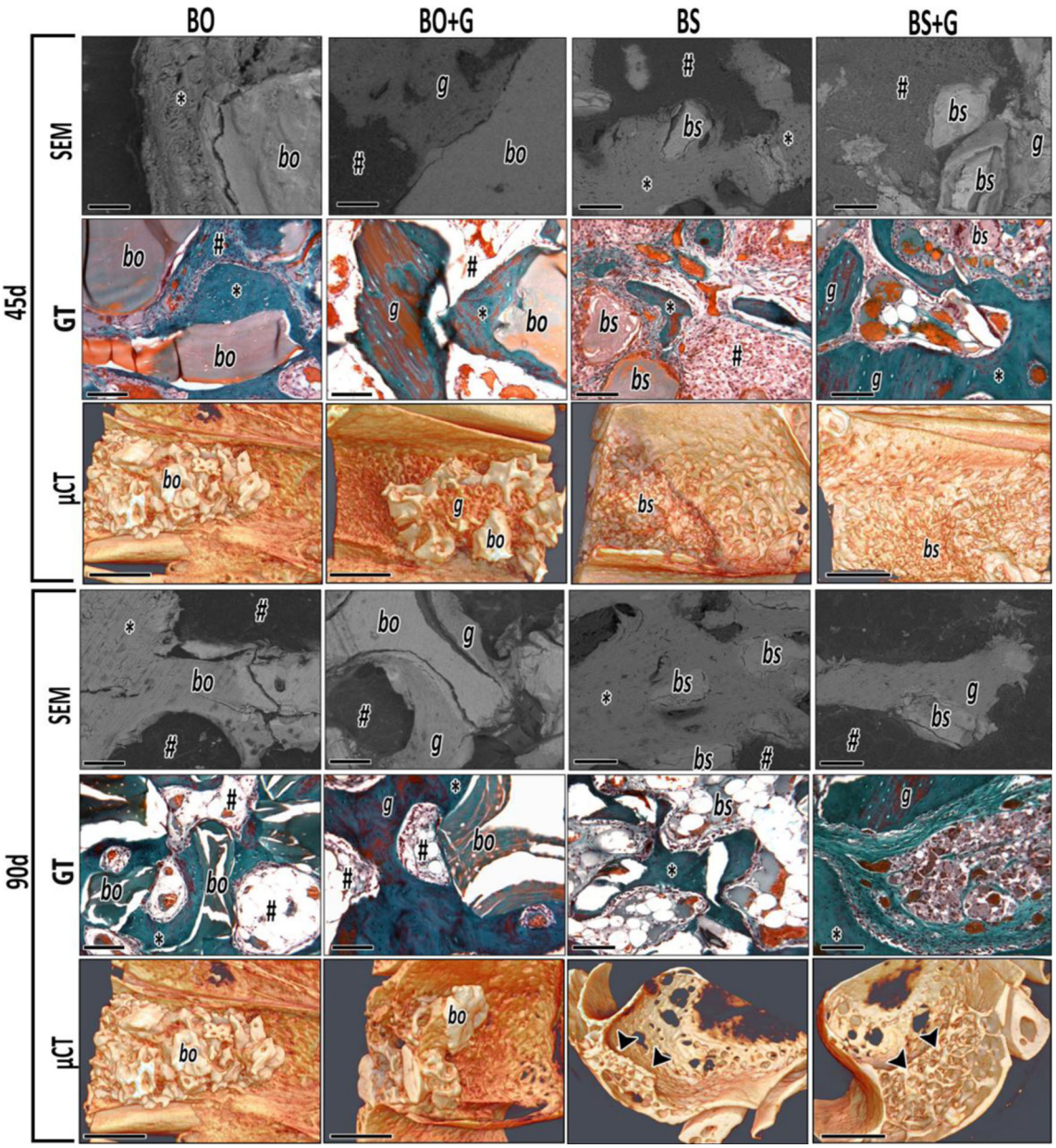

Fig. 2 Details of bone trabecullaes surface (*asterisk*), Bio Oss® (bo), bone graft (g), Biosilicate® (bs), and soft tissue (*hash*) shown by SEM images. Goldner trichrome stain (GT) evidences connective tissue (*hash*), newly formed bone (*asterisk*), Bio Oss® (bo), Biosilicate® (bs), and newly bone graft (g). uCT 3D reconstructions reveal morphological characteristics of the biomaterials, mainly at day 45. At day 90, BS and BS+G are identified by *arrow-heads* (SEM 250×; GT 25×, *scale bar* = 200 µm; µCT *scale bar* = 1.5 mm)

at this period. No statistically significant difference was detected among the groups considering greenish fibers in all periods (Fig. 4b). Considering yellow fibers, significant decreased was observed in BS group when comparing with BO group at day 45. In this same period, significant increase was also detected in BO red fibers in comparison to BO+G group (Fig. 4c). Interestingly, when bone graft (BS+G) was associated to the biomaterial BS, an increase was observed in red fibers at day 90, resulting in a significant difference between BS and BS+G (Fig. 4d).

3.3 Immunohistochemistry

At day 45, intense OPN and OC immunolabeling was observed in BO+G group, whereas BO alone presented moderate labeling. In contrast, BS showed mild labeling for both proteins, but when associated to bone graft, OPN presented moderate positivity. No differences were observed at day 90 comparing all groups. Despite this similarity, OC labeling semi-quantitative analysis revealed that when bone graft was associated to both biomaterials, it

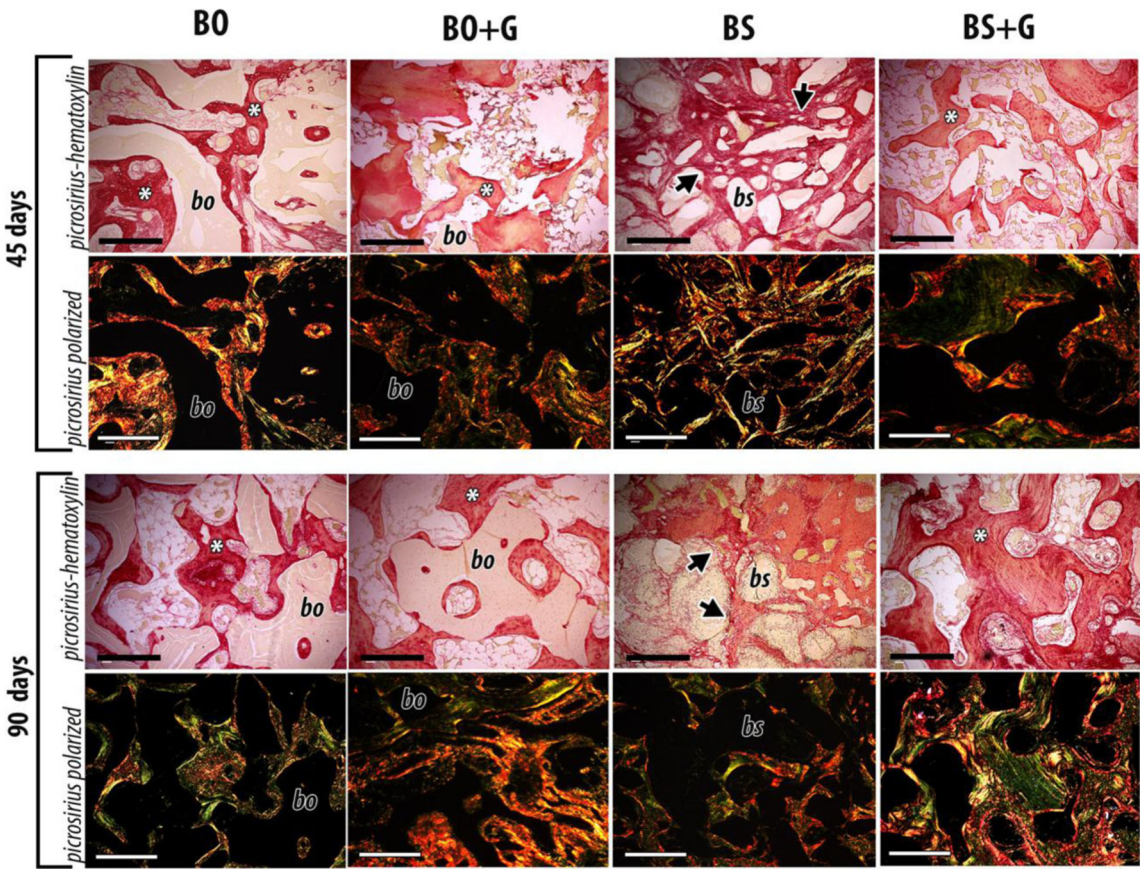

Fig. 3 At day 45, BO group seems to show lamellar matrix (*asterisk*) coincident with picrosirius polarized images, similar to BS+G group, whereas in BS picrosirius-hematoxylin reveals connective fibrous tissue around the biomaterial particles (*arrows*). At day 90, mature bone matrix (*arrows*) and organized bone tissue can be seen in all groups, shown by picrosirius-hematoxylin except in BS, due to the persistence of areas constituted by connective tissue fibers (*arrows*) associated to degrading biomaterial. (Bio Oss®, *bo*; Biosilicate®, *bs*). (Picrosirius-*red staining*, original magnification ×10; *scale bar* = 100 μm) (Color figure online)

was more evident in the mature bone matrix. When biomaterials were observed alone, immunolabeling was predominant in bone cells cytoplasm. The most frequently observed scores in all analyzed specimens are shown in Table 1 and Fig. 5.

4 Discussion

Previous studies have exhaustively proven Bio Oss® efficacy as bone substitute material in maxillary sinus augmentation [3], whereas Biosilicate® has still not. In a comparison between Biosilicate® and autogenous bone graft in rabbits'calvaria, similar results were found considering Runx2 and VEGF immunolabeling, when both materials permitted satisfactory bone formation [20]. However, in the present study glass ceramic showed a distinct histological feature when used for maxillary sinus augmentation. From day 45 it was possible to observe deficiency in bone formation and the presence of thin trabecullaes, although a tight adherence to Biosilicate® could be observed by SEM images. Evident poorer bone formation persisted at day 90, in comparison to BO group, which can probably be explained by the degradation pattern of the glass ceramic. Bio Oss® presented minimal resorption by eventual flat foreign body cells, but inciting no inflammatory infiltrate as also described by Hürzeler et al. [30], permitting a direct deposition of the newly formed bone on its surface, previously reported [31] and here confirmed by SEM analysis. Slow resorption of the bovine HA resulted in a better quality of collagenous organic matrix of the formed bone mainly at day 45, as shown by polarized analysis when yellow and red fibers were significantly higher in BO group in comparison to BS. However, at day 90, significant increase in red fibers was detected in BS+G group in comparison to BS alone (Fig. 4d), and by the analysis of Fig. 3, it was observed that the presence of bone graft gradually improved collagen I synthesis.

In the analysis of immunohistochemistry day 45, it was clear that both OC and OPN were more intensely marked in

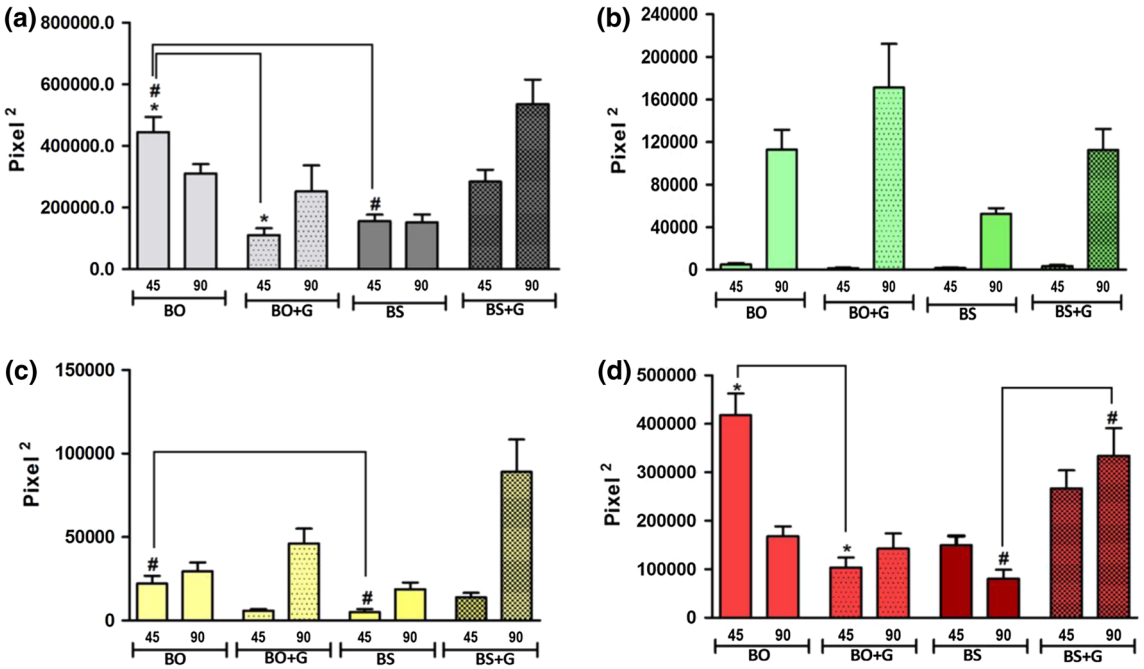

Fig. 4 Quantification of collagen fiber bundles by Picrosirius-polarization method along 45 and 90 days during biomaterials integration. Intensity of birefringence measured from total area of collagen fibers (**a**), as well as *greenish* (**b**), *yellow* (**c**) and *red* (**d**) collagen fibers. Results are presented as mean and SD of pixels^2. *Symbols* (*asterisk* or *hash*) indicate significant statistical difference ($P < 0.05$) between groups in the same time point (Color figure online)

Table 1 Mean scores assigned for OC and OPN according to the analyzed periods in all groups

	OPG	OC
45 days		
BO	++	++
BO+G	+++	+++
BS	+	+
BS+G	++	+
90 days		
BO	+++	+++
BO+G	+++	+++
BS	+++	+++
BS+G	+++	+++

the presence of the HA. Again, biological process of biomaterial resorption may have interfered in this response, revealing the optimal osteoconductive characteristic of Bio Oss®. However, at day 90, despite the lack of significant difference among the groups considering immunolabeling area, it was possible to observe predominant OPN and OC labeling in bone matrix when bone graft was associated to the biomaterials, whereas cytoplasm labeling was predominant in the absence of the autogenous graft. Although some controversies still exists, its association to synthetic biomaterials is a common clinical practice, probably in order to take the osteoinductive property of autogenous bone [3].

Despite the high bioactivity of the tested glass ceramic, it seems that degrading process surpassed bone cells stimulation at this specific anatomical site, creating a soft tissue barrier around some of the particles. Biosilicate® particles caused intense chronic inflammatory response with predominance of numerous foamy macrophages and FBR that persisted until day 90, resulting in collagen deposition around the resorbing particles of the biomaterial. Recent studies have focused on immune system playing a decisive role in the fate of a biomaterial in living tissues [9, 32], depending upon its physicochemical characteristics, which are responsible for controlling the quantity and conformation of adsorbed protein [33]. Adsorbed proteins regulates cell adhesion, proliferation and tissue formation, but they are also determinant in modulating host response for biomaterial/tissue integration, e.g. fibrinogen, activating epitopes that bind to phagocytes receptors leading to a proinflammatory condition [34]. Although the formation of HCA layer on Biosilicate® surface is well established, protein adsorption is not. El-Ghannam et al. [35] investigation about the influence of crystallization of bioactive glasses on proteins adsorption, demonstrated that the exposure of amide I and unordered structure of adsorbed proteins were necessary to improve cell adhesion on bioactive glass surface, and that this process was inhibited by glass crystallization, limiting osteogenic cell adhesion. The behavior of host detection

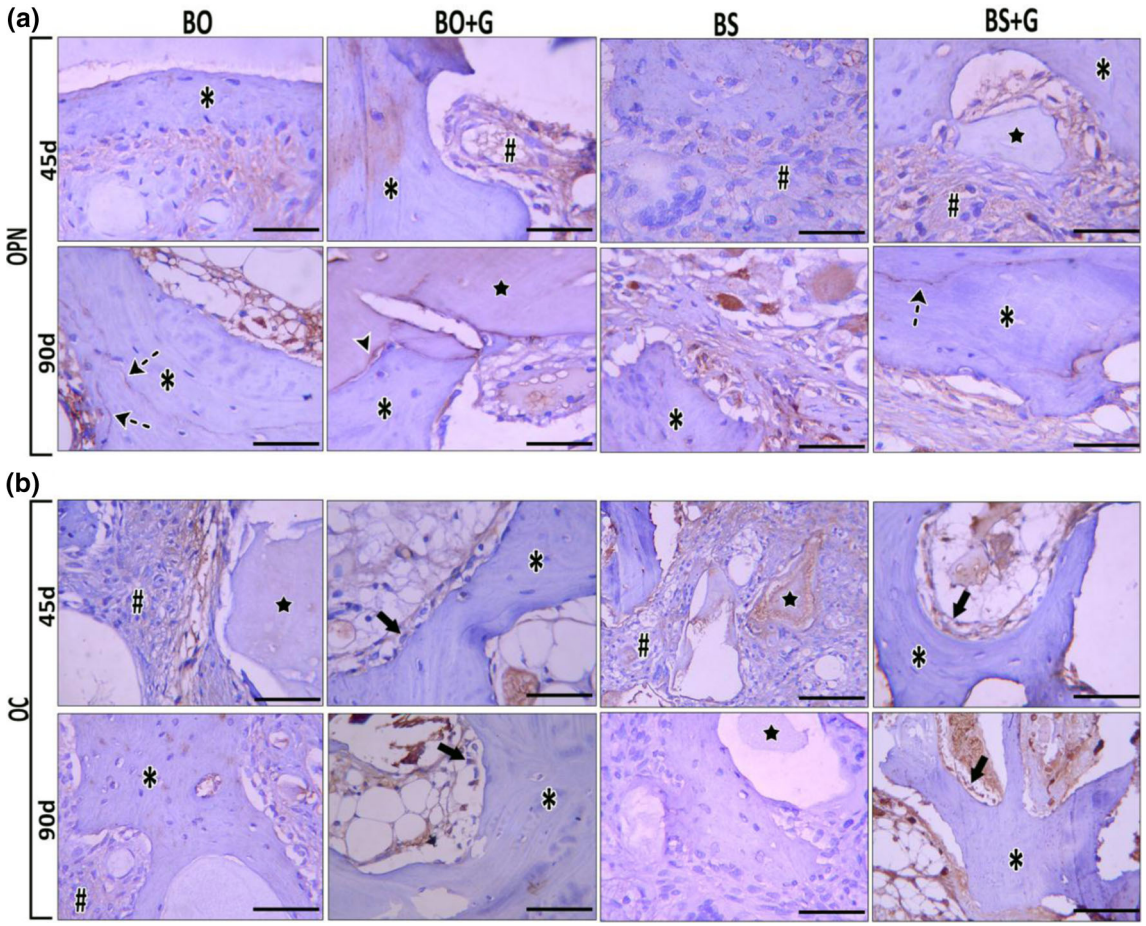

Fig. 5 **a** OPN immunolabeling—45 days) evident in connective tissue cells (*hash*) and intense in BO+G group; 90 days) intense labeling in reversal *lines* (interrupted *arrows*), and biomaterial/bone interface (*arrow head*) (*star* = biomaterial) (*bar* = 100 μm). **b** OC immunolabeling. 45 days) moderate labeling in bone matrix (*asterisk*) and osteoblastic cells (*thick arrows*); 90 days) osteoblasts (*thick arrows*) and osteocytes; mild labeling in bone matrix (*asterisk*) in BS group (*star* = biomaterial) (*bar* = 200 μm)

and response to inert and nontoxic biomaterials remain obscure, but, again, it seems that some adsorbed proteins act as antigens and directs immune and inflammatory response to foreign body reaction (FBR) [9].

Microarchitecture of the particles may also have contributed to difficult host tissue interaction, such as lack of porosity and rounded edges of the particles, as revealed by μCT morphological analysis. The same FBR was observed in bone calvaria repair filled with Biosilicate® [20]; however, in a much less intensity, not interfering in new bone formation and maturation. In addition, physiological particularities of the maxillary sinus must be considered, as the air pressure caused by the airflow inside it, which has been attributed to influence quantity and fate of newly formed bone [21].

The obtained results emphasize the necessity of testing new biomaterials in distinct anatomical sites in order to analyze its biological behavior considering its physico-chemical characteristics, along with the particular anatomical, physiological, and histological characteristics of the recipient site that may directly influence tissue response.

5 Conclusions

Sinus lift augmentation using the new glass ceramic presented significantly different healing process from the bovine HA with an intense FBR, resulting in a deficiency in bone formation, even when associated to autogenous bone graft. However, the association of the bone graft to both biomaterials improved collagenous matrix quality and mineralization. These results clearly indicate that further studies about Biosilicate® are necessary to identify the factors that resulted in an unfavorable healing response when used in maxillary sinus augmentation.

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