Ultrasonics - Sonochemistry 56 (2019) 437–446

Contents lists available at ScienceDirect

Ultrasonics - Sonochemistry

journal homepage: www.elsevier.com/locate/ultson

Sonochemical time standardization for bioactive materials used in periimplantar defects filling

Pedro Henrique Silva Gomes-Ferreira[a,*], Paulo Noronha Lisboa-Filho[b], Ana Carolina da Silva[b], Odair Bim-júnior[b], Fábio Roberto de Souza Batista[a], Ana Cláudia Ervolino-Silva[c], Idelmo Rangel Garcia-Junior[a], Roberta Okamoto[c,1]

[a] Department of Surgery and Integrated Clinic, São Paulo State University, Araçatuba Dental School, Araçatuba, SP, Brazil
[b] Department of Physics, São Paulo State University, School of Sciences, Bauru, SP, Brazil
[c] Department of Basic Sciences, São Paulo State University, Araçatuba Dental School, Araçatuba, SP, Brazil

ARTICLE INFO

Keywords:
Dental implants
Bioglass
Biomaterial
Bone regeneration

ABSTRACT

The aim of this study was determinate the best sonochemical time in order to obtain better bone characteristics when a bioactive material (Biogran) is used in the filling periimplantar defects. In this study, 32 rats were submitted to surgical proceedings to create a periimplantar defect that was filled with Biogran receiving different sonochemical times: 15 (G1), 30 (G2), 45 (G3) or 90 min (G4). The biomaterial was characterized through X-ray diffraction and scanning electron microscopy (SEM). In vivo analysis was performed through micro CT, laser confocal microscopy, immunohistochemistry and evaluation of bone cytoarchitecture through hematoxylin and eosin (HE) staining. The data were submitted to statistical testing, considering a significance level of $p < 0.05$. Rx diffraction of pure bioglass showed that it is predominantly amorphous; otherwise, there are small peaks at 23° and 31°. SEM shows that the longer the sonochemical time, the less edges the biomaterial will present. Within the groups, G1 and G2 showed the best quantity and quality by micro CT ($p > 0.05$). The best bone turnover result was found in G1 and G2, otherwise the better results were related to neoformed bone area, bone mineral apposition rate and bone implant contact to G1 ($p < 0.05$). G1 had the best results in terms of bone cytoarchitectural evaluation and immunohistochemistry. It is possible to conclude that Biogran that received 15 min of sonochemical treatment (G1) presented periimplantar bone repair with the best extracellular matrix properties, including the best quality and quantity of vital bone.

1. Introduction

The search for rehabilitation with osseointegrative implants has resulted in many cases with low quantities of support bone, which leads to the need to search for alternative approach like bone grafts before or during the implant's installation [1–4]. Machined titanium implants have been a significant discovery for edentulous patients' rehabilitation, presenting success through osseointegration phenomena [5]. Osseointegration has been studied for and improves surface treatment by accelerating this process and decreasing the wait time for rehabilitations after an implant's installation [6–10].

Considering the increase in biomaterials' use as bone substitutes to decrease the morbidity of patients during and after surgery [11], research has begun to improve the surfaces of these biomaterials. The theory behind this improvement is related to the functionalization of these materials with bioactive particles that act in bone turnover through the ultrasound sonochemical technique [12]. This consists of assisting synthesis though ultrasound, which makes viable a variety of materials nanostructured from ultrasonic waves in liquid medium, causing chemical and physical transformations through interactions between the radiation and material [13,14]. The effects of this method appear after the acoustic capitation phenomena, or the formation, growing and implosive collapse of bubbles in liquids in the order of microseconds, before obtaining the mixture and homogenizing two or more distinct substances [13].

Studies in the literature show the use of this technique for functionalizing a biomaterial in a silica base (Biogran® – Biomet 3i, Palm Beach Gardens, Florida, USA) with a selective modulator of estrogen receptors (Raloxifeno – Evista®, Lilly, USA), with a sonochemical time of 30 min [12]. This is the only trial in the literature to improve

* Corresponding author at: Rua José Bonifácio, 1193, Zip code: 16015-050, Vila Mendonça, Aracatuba, Sao Paulo, Brazil.
E-mail address: pedroferreirabmf@gmail.com (P.H.S. Gomes-Ferreira).
[1] Roberta Okamoto is affiliated with Research productivity scholarship (process: 308126/2014-9).

https://doi.org/10.1016/j.ultsonch.2019.04.032
Received 22 February 2019; Received in revised form 4 April 2019; Accepted 20 April 2019
Available online 22 April 2019
1350-4177/

synthetic biomaterials designated for bone grafts.

Biogran is an alloplastic material composed of bioactive glass with a particle size of approximately of 300–355 μm (Biomet 3i, Palm Beach Gardens, Florida, USA). Studies have shown good results for this biomaterial when used in conjunction with implants, promoting osseointegration and peri-implant bone gain [15]. The peculiar characteristic of silica in its composition makes this material bioactive. It becomes relatively structured when in contact with body fluids, promoting the exchange of sodium, calcium and hydrogen ions until the formation of an external layer of silica dioxide and hydroxyapatite, which favors the resorption and formation of new bone tissue [16,17]. Besides this, this biomaterial presents facility in reduction and homogenization through its physical and structural characteristics in the sonochemical method, which will be used in this study [12].

An interface implant/bone graft is a complex repair situation that involves revascularization, graft incorporation and the implant's integration [18]. The association of biomaterials with implants has been studied and used frequently in cases of periimplantar defects after extractions or bone fenestrations [19]. In this context, the use of grafts and biomaterials in implant dentistry becomes important, especially when the receiver bed of implants presents inappropriate quantities and quality of bone. Considering the potential in this research area, it is necessary to evaluate the best pattern for the sonochemical method when using these biomaterials, for posterior utilization in treating periimplantar defects and in maxillofacial reconstructions. After standardization of times when using the sonochemical method, researchers will be able to test other biomaterials and medications following this same protocol. Considering this, the present study's aim is to standardize the amount of time used to perform the sonochemical technique on a bioactive glass (Biogran®) used to fill periimplantar defects.

2. Material and methods

2.1. Animals

Initially, the power test was performed to determine the number of samples for each group. The minimum number was 6 animals (power test = 0.8). After approval of the Ethics Committee on Animal Research of the Araçatuba Dental School – UNESP (Process FOA n° 199-2017), 32 male rats (*Rattus novergicus albinus*, Wistar) weighing around 500 g were randomly divided into four experimental groups (G1, G2, G3 and G4) (Table 1), according to times of the sonochemical exposure and surgery of the bone defect and installation of the implants to which they were submitted. For this research was performed of according to the ARRIVE guidelines [20].

2.2. Preparation and characterization of the biomaterial (Biogran)

To standardize the best sonochemical time for Biogran, the experiment evaluated this technique by processing 1 g pure biomaterial samples of Biogran using an ultrasonic processor for processing/sonication times of 15, 30, 45 or 90 min. The sonication parameters were defined as follows: power of 750 W; frequency of 20 kHz; and 40% of the equipment's nominal amplitude (450 W/cm^2). Ultra-pure water was used as a means to obtain homogeneous systems and to reduce the particle size. After processing, the samples were left in a stove at 60 °C to dry for 8 h. After this process, the biomaterial was sterilized in ultraviolet (UV).

Table 1
Experimental groups according to sonochemical time.

G1–15 min, Filling of periimplantar defect with Biogran® sonicated for 15 min
G2–30 min, Filling of periimplantar defect with Biogran® sonicated for 30 min
G3–45 min, Filling of periimplantar defect with Biogran® sonicated for 45 min
G4–90 min, Filling of periimplantar defect with Biogran® sonicated for 90 min

X-ray diffraction studies were performed on a Rigaku diffractometer, model D/MAX 2100 PC, using CuKα radiation, voltage of 40 kV, 20 mA current, 1° divergence slit, 0.3 mm slot, nickel filter in a range of angular sweep of 20°–100° and step of 0.02°, with fixed time of 1.6 s/step. Morphological and dimensional analyses were performed by scanning electron microscopy using a JEOL microscope model 7500F with a theoretical resolution of 1 nm, using 2 kV of acceleration voltage. In this way, it was possible to characterize the sonicated biomaterial at different times.

2.3. Periimplant defect surgery and implant installation

The animals were fasted for 8 h prior to the surgical procedure and sedated with a combination of 50 mg/kg intramuscular ketamine (Vetaset – Fort Dodge Saúde Animal Ltda, Campinas, São Paulo, Brazil) and 5 mg/kg xylazine hydrochloride (Dopaser – Laboratório Calier do Brasil Ltda – Osasco, São Paulo, Brazil), and they received mepivacaine hydrochloride (0.3 ml/kg, Scandicaine 2% with epinephrine 1:100,000, Septodont, France) as local anesthesia and for hemostasis of the operative field. After sedation of the animals, tricotomy was performed in the medial portion of the right and left tibia, and the region to be incised received antisepsis with polyvinylpyrrolidone degermant iodine (PVPI 10%, Riodeine Degermante, Rioquímica, São José do Rio Preto), associated with topical PVPI. With a blade number 15 (Feather Industries Ltda, Tokyo, Japan) in a scalpel handle, an incision approximately 1.5 cm long was made in the left and right tibial metaphysis regions. Then, the soft tissue was removed with the aid of periosteal detachment, exposing the bone to the surgical procedure.

Sixty-four commercial pure-grade IV titanium implants with surface treated by double acid attack (nitric acid, hydrofluoric acid and sulfuric acid) were installed, with a diameter of 2 mm and a height of 4 mm, and sterilized by gamma ray. Milling was performed with a 1.6 mm diameter spiral drill in the two cortices, and a 2 mm milling cutter, pilot (2/3) and 3.0 mm in the superior and medullary cortices of the tibiae (Fig. 1), which was assembled with an electric motor (BLM 600®;

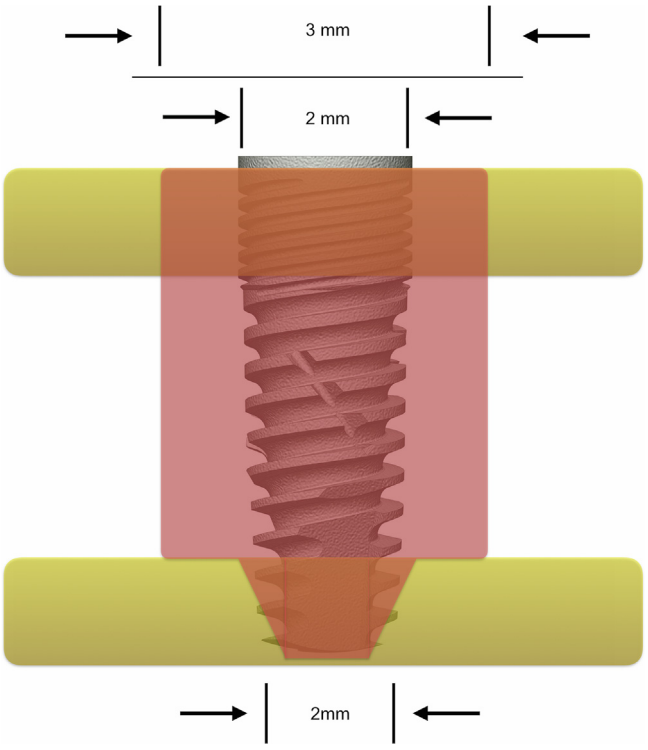

Fig. 1. Schematic demonstrating the installation of the implants in the experimental groups G1, G2, G3 and G4.

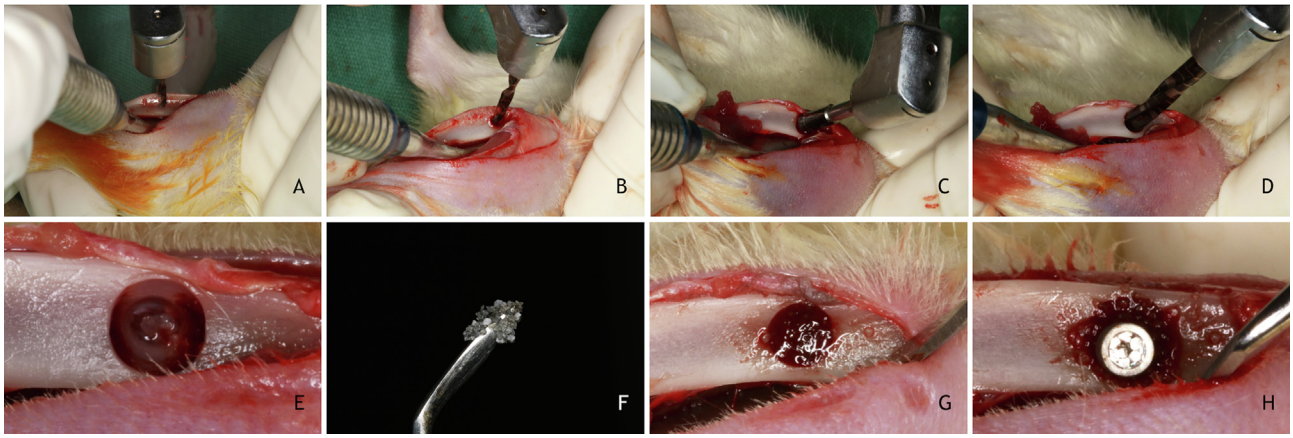

Fig. 2. (A) To make the defect, a 1.6 mm drill is used in the upper and lower cortical; (B) 2 mm cutter (upper cortical); (C) Pilot cutter 2/3 (upper cortical); (D) 3 mm cutter (upper cortical); (E) defect created in the tibial metaphysis with 3 mm in the upper cortical and 1,6 mm in the inferior cortical; (F) sonicated biomaterial; (G) Biomaterial filling the defect; (H) implant installed in the center of the defect.

Driller, São Paulo, SP, Brazil) at a speed of 1000 rpm, under irrigation with isotonic solution of 0.9% sodium chloride (Fisiológico®, Laboratórios Biosintética Ltda®, Ribeirão Preto, SP, Brazil), and contra-angle with 20:1 reduction (angle piece 3624N 1:4, head 67RIC 1:4, KaVo®, Kaltenbach & Voigt GmbH & Co., Biberach, Germany). After the creation of the defect, the biomaterial was introduced into the defect in the different experimental groups (G1, G2, G3, G4). Then, the implant was installed in the most central portion of the defect free of bone contact in the medullary portion and upper cork (Fig. 2A–H).

Each animal received an implant in each tibial metaphysis. The tissues were sutured in planes using absorbable wire (Polygalactin 910 – Vycril 4.0. Ethicon, Johnson Prod., São José dos Campos, Brazil) with continuous stitches in the deep plane and with monofilamentary wire (Nylon 5.0. Ethicon, Johnson, São José dos Campos, Brazil), with interrupted sutures in the outermost plane. In the immediate postoperative period, each animal received a single intramuscular dose of 0.2 ml Penicillin G-benzathine (Pentabiotic Veterinary, Small Animals, Fort Dodge Saúde Animal Ltda., Campinas, SP). The animals were kept in cages throughout the experiment with food and water ad libitum.

Day zero was considered the accomplishment of the bone defect and installation of the implants and grafts in the rats' tibiae. Fourteen days after, the implants were installed intramuscularly with 20 mg/kg of the fluorochrome calcein [21,22]. After another 28 days (42 days after implant installation), alizarin red fluorochrome was administered at a dose of 20 mg/kg for each animal [21,22]. The animals were euthanized 60 days after the installation of the implants (18 days after the administration of alizarin). After the reduction and achievement of the pieces, the samples were submitted to microtomographic analysis, confocal laser microscopy and immunohistochemical and histological staining in hematoxylin and eosin (HE).

2.4. Micro-CT analysis

After the animals were euthanized, the right tibiae of the animals in the four experimental groups (G1, G2, G3, G4) were reduced and fixed in 10% buffered formalin solution (Reagentes Analíticos, Dinâmica Odonto-Hospitalar Ltda, Catanduva, SP, Brazil) for 48 h and washed in running water for 24 h. After fixation, the pieces were left in 70% alcohol for the microtomographic analysis to be performed. Through a Skyscan microtomograph (SkyScan 1272 Bruker MicroCT, Aatselaar, Belgium, 2003), the pieces were scanned using 8 μm slices (70 kV, 142 μA), with a 0.5 mm Al filter and rotation step of 0.6 mm, 2016 × 1344 columns by lines, exposure of 1600 ms and acquisition time of 1 h and 1 min (Fig. 3). The images obtained were reconstituted, and the area of interest was determined by the software NRecon

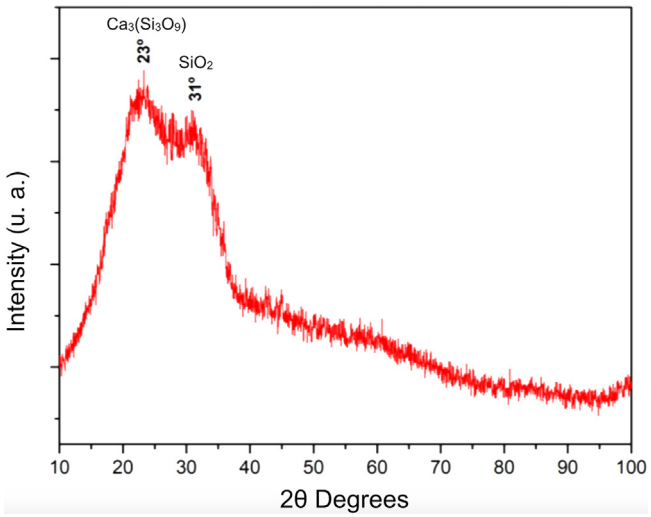

Fig. 3. Biogran® Diffractogram. Two peaks referring to silicon dioxide – SiO_2 and calcium silicate – Ca_3 (Si_3O_9) are identified.

(SkyScan, 2011; Version 1.6.6.0), with smoothing of 5, correction of artifact rings of 7, beam hardening correction of 40% and an image conversion range of 0.017–0.280. In the Data Viewer software (SkyScan, Version 1.4.4 64-bit), the images were lined up, and a new dataset was saved. This dataset was then used in the CTAnalyser software (CTAn; 2003-11, SkyScan, 2012 BrukerMicroCT Version 1.12.4.0) to evaluate the region between the third and fifth implant turn in 100 slices, counted from its most central portion [23,24]. The percentage patterns of bone volume (BV/TV), bone trabecular thickness (Tb.Th) and separation and number of trabeculae (TB.Sp and Tb.N) were then defined [25] to perform the 3D reconstruction with the CTvox software (SkyScan, Version 2.7).

2.5. Confocal laser microscopy

After the microtomographic analysis, the same pieces (eight right tibia per group) were dehydrated from an increasing sequence of alcohols: 70%, 90% and 100%. The pieces were then immersed in a solution of methyl methacrylate (MMAL) (Classical, Classic Dental Articles, São Paulo, SP, Brazil), followed by three baths of MMAL. The benzolium peroxide catalyst (1%, Riedel-de Haën AG, Seelze-Hannover, Germany) was added to the latter bath. The pieces were placed into a test tube filled with the solution and kept in an oven at 37 °C for 5 days,

until the final polymerization of the resin.

After the polymerization, the test tubes were broken, and the resin was longitudinally cut with the aid of a MaxiCut mounted on a bench motor (Kota – São Paulo – SP, Brazil). The pieces were then bilaterally scrubbed with 120, 300, 400, 600, 800, and 1200 crescent granulations mounted on an automatic polishing machine (ECOMET 250PRO/AUTOMET 250. Buehler, Lake Bluff, Illinois) until the cuts reached a thickness of 80 µm. A digital caliper was used for measurement (Mitutoyo, Pompeia, SP, Brazil).

The slices were then mounted on glass slides with mineral oil (liquid petroleum, Mantecor, Taquara, RJ, Brazil) and sealed with a glass cover and enamel to prevent oil leakage and possible dehydration of the piece. They were evaluated longitudinally in the region of the bone/implant interface corresponding to the third, fourth and fifth turns of the implants. These slices were captured with a Leica CTR 4000 CS SPE (Leica Microsystems, Heidelberg, Germany) confocal laser microscope using a 10× objective (original increase of 100) at the Bauru Dental School – USP. Thus, images of the calcein and alizarin red fluorochromes separately (old bone/new bone) were used to evaluate bone turnover and the overlap of the two images in terms of the mineral apposition rate (MAR), linear contact extension bone/implant (ELCOI) and neoformation bone area (AON).

The images were analyzed in the Image J (Image Processing and Analysis Software, Bethesda, Maryland, USA) program. Through the Free Hands tool, the areas of fluorochrome (calcein/alizarin) precipitation as well as AON were measured. With the Straight tool, the MAR was found through five measurements extending from the outer margin of the calcein toward the outer margin of the alizarin. The value obtained was divided by 28, which represents the interval of days between the injections of the two fluorochromes analyzed [26]. For ELCOI, using the same tool, the total bone in contact with the implants was delineated.

2.6. Immunohistochemical analysis

The left tibiae (n = 8) of the animals were removed, reduced and fixed in 10% formaldehyde (Analytical Reagents, Dinâmica Odonto-Hospitalar Ltda, Catanduva, SP, Brazil) for 48 h, washed in running water for 24 h and demineralized in 10% EDTA. Then, the samples were dehydrated with an increasing sequence of alcohols and diaphanized in xylol for subsequent inclusion in paraffin. Cuts with thickness of 5 µm were obtained and mounted on glass slides.

The reaction was performed on four pieces (left tibias) per group, using the indirect immunoperoxidase method with an amplifier. To inhibit endogenous peroxidase, 3% hydrogen peroxide (Merck Laboratories) was used. Antigen was retrieved by immersing the slices in citrate phosphate buffer, pH = 6, maintained in warm humidity, for 20 min. The non-specific reaction was blocked with bovine albumin (Sigma). The primary antibodies used were against WNT, alkaline phosphatase (ALP), osteopontin (OPN) and osteocalcin (OC), whereas these polyclonal antibodies are produced in goats (Santa Crua Biotechnology). As the secondary antibody, rabbit anti-goat (Pierce Biotechnology) was used, and the amplifiers were avidin and biotin (Vector Laboratories) and chromogen to diaminobenzidine (Dako). Immunolabeling analysis was performed through scores attribution, according to the area of positive presence of the proteins in the repairing tissue (interest area). For 25% of positive immunolabeling, it will be attributed the score 1; for 50% score 2; and for 75% score 3.

The aim of the immunolabeling analysis was to characterize bone remodeling through the WNT protein and provide evidence of osteoblast differentiation, activation and recruitment. Alkaline phosphatase indicated the beginning of mineralization, with phosphate ion precipitation. Osteopontin showed the ratio of mature osteoblasts, and osteocalcin, a late protein characterized by the deposition of calcium in the extracellular matrix.

2.7. Histological analysis

The pieces submitted to immunohistochemical analysis received the same treatment as the pieces submitted to histological analysis (the remaining four left tibias of each group). After obtaining the 5 µm slices and mounting them on slides, they were stained with hematoxylin and eosin (HE) to evaluate peri-implant bone cytoarchitecture in the different groups.

2.8. Statistical analysis

The GraphPad Prism 7.01 software was used for the statistical analysis. Homoscedasticity was analyzed with the Shapiro–Wilk test. The one-way ANOVA test was used to evaluate all parameters of the Micro CT, besides MAR, ELCOI and AON, regarding overlapping fluorochromes, as well as the Tukey post-test, when necessary. Two-way ANOVA was used to analyze the data concerning the area of the fluorochromes. The level of significance was set at $p < 0.05$.

3. Results

3.1. Characterization of the biomaterial

3.1.1. X-ray diffraction

The X-ray diffraction of the bioglass showed that the material is predominantly amorphous, but it was possible to identify small peaks at 23° and 31°, corresponding to calcium silicate and silicon dioxide, respectively. These peaks indicate that crystals grew during the material manufacturing process (Fig. 3).

3.1.2. Scanning electron microscopy

The SEM results show that sonication time did not appear to produce notable differences in Biogran particle size. The processed samples had smoother edges and relatively smaller sizes in a growing shape (Fig. 4d–m), as compared to Biogran particles that were not sonicated (Fig. 4a), showing that the longer the sonochemistry time, the smoother and smaller the particles.

3.2. Micro-Ct

The 45-min group (G3) presented the lowest results for percentage of bone volume (67.7% ± 7.7%), trabecular thickness (0.083 mm ± 0.008) and number of trabeculae (9.1 l/mm ± 0.59) and a higher result for the separation of trabeculae (0.041 mm ± 0.021). The 15- and 30-min times presented the best bone volume percentage (G1 and G2: 81.5% ± 4.8 and 82.06% ± 7.9), trabecular thickness (G1 and G2: 0.09 mm ± 0.017 and 0.08 mm ± 0.016) and number of trabeculae (G1 and G2: 9.8 l/mm ± 1.64 and 9.9 l/mm ± 0.63). Regarding the separation of trabeculae, the 30-min group (G2) presented the lowest value (0.027 mm ± 0.005), followed by the 15-min group (G1) (0.030 mm ± 0.002). The bone volume and quality results for sonicated bioglass at 90 min (G4) were better than those of G3 and worse than those of G1 and G2, presenting bone volume percentage of 72.8% (± 7.3), trabecular thickness of 0.073 mm (± 0.02), 9.71 1/mm (± 1.05) trabeculae and separation between trabeculae of 0.038 mm (± 0.009). The effect of sonication time of the bioglass on the 3-D parameters of the bone morphology did not present statistically significant results in the comparison between the different groups ($p > 0.05$, one-way ANOVA) (Fig. 5).

3.3. Confocal laser microscopy

The markings of old bone (green—calcein) and new bone (red—Alizarin) represent the peri-implant bone turnover present in each experimental group (Fig. 6). Both the intragroup and intergroup evaluations of fluorochrome (calcein vs. alizarin) precipitation

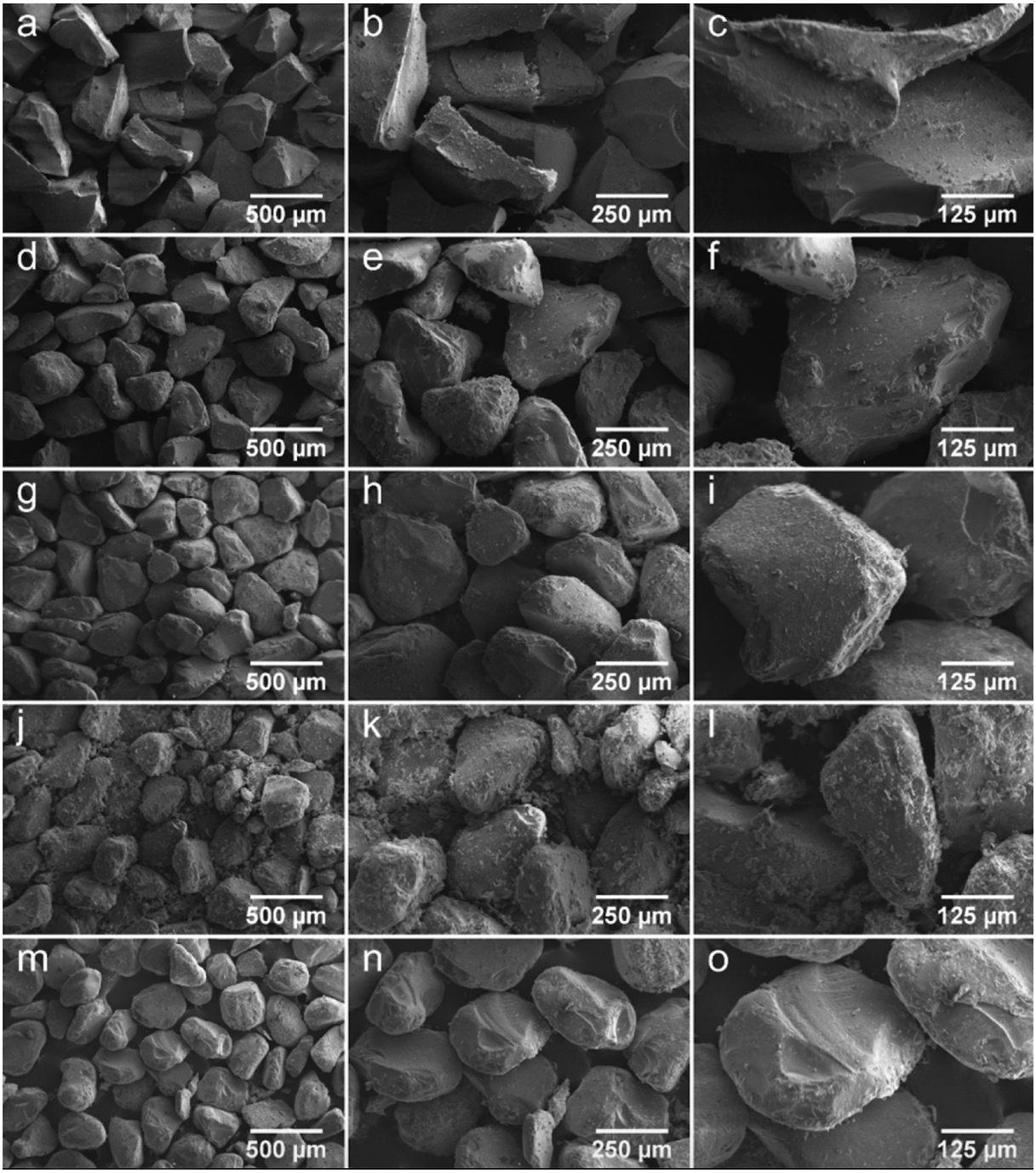

Fig. 4. SEM photomicrographs representative of pure BioGran (a–c); Sonicated at (d–f) 15 min; (g–i) 30 min; (j–1) 45 min; (m–o) 90 min. Magnifications of ×50, ×100, and ×200 from left to right in each sequence.

presented insignificant statistical results in the comparisons between the four groups ($p > 0.05$, two-way ANOVA). However, G1 (15 min) had the highest numerical values, followed by G2 (30 min), and the worst results were for G3 (45 min) followed by G4 (90 min), with the latter having the lowest calcium precipitation in the analysis (Fig. 7).

Through the green and red overlap, the area of neoformed bone (AON) was evaluated, with the best results for sonication time coming from the 15-min group (G1) at 18,409.74 μm^2 (± 1287), which was statistically significant when compared with G3 (12211.15 μm^2 ± 5937.04) and G4 (13282.52 μm^2 ± 5937.04) ($p < 0.05$, Tukey) (Fig. 8). In relation to the MAR during the peri-implant repair of the defects, the highest values were found in G1, with mineral deposition of 3.7 µm/day (± 0.26), which was statistically significant when compared to the other groups ($p < 0.05$, Tukey). The daily mineral deposition of G2, G3 and G4 was 2.4 (± 0.02), 2.06 (± 0.36) and 2.25 (± 0.11), respectively (Fig. 9). The linear extension of bone/implant contact (ELCOI) showed that G1 presented the best results, with 372.92 µm, being statistically significant in comparison with G3 (280.93 µm ± 24.17) and G4 (290.19 µm ± 52.24) ($p < 0.05$, Tukey) (Fig. 10).

3.4. Immunohistochemical analysis

The immunohistochemical response to peri-implant defects filled with Biogran at 15, 30, 45 and 90 min can be assessed in Fig. 11 and Table 2. The WNT protein, which is important to the transduction

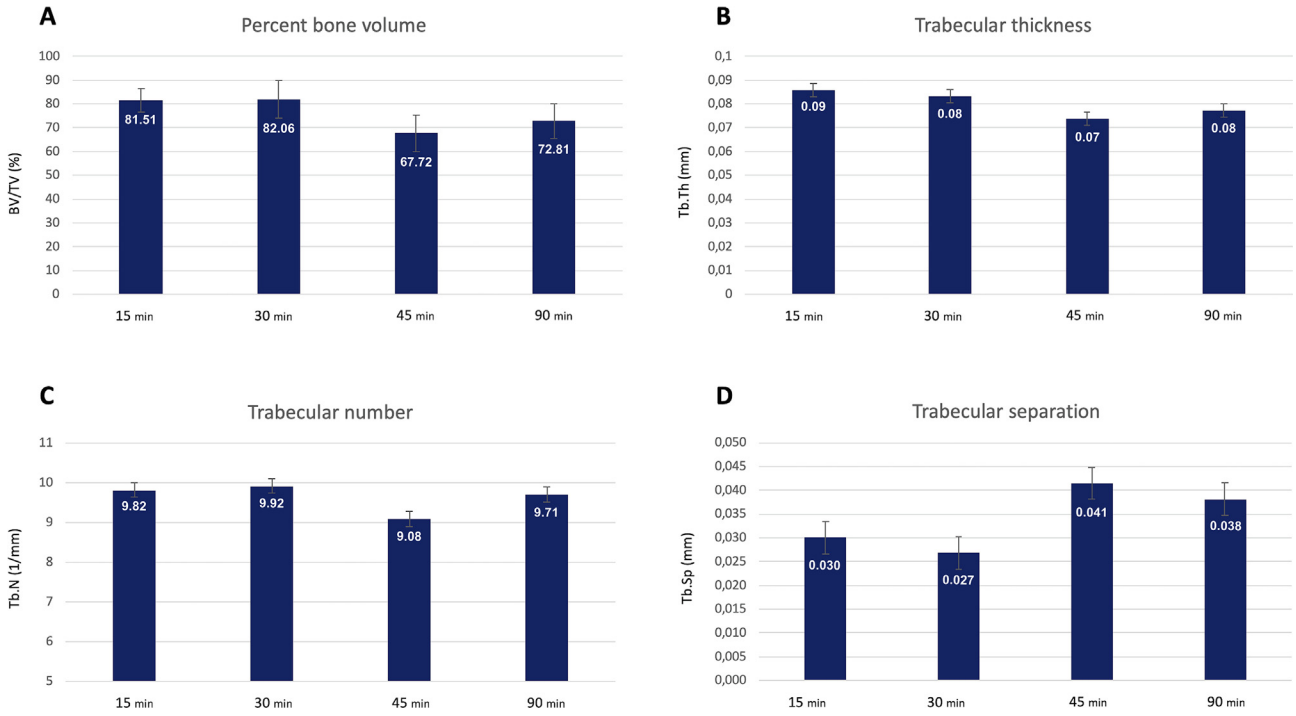

Fig. 5. Effect of BioGran® sonication time on different parameters: (A) percentage of bone volume, (B) trabecular thickness, (C) separation of trabeculae and (D) number of trabeculae. ($p > 0.05$, one-way ANOVA).

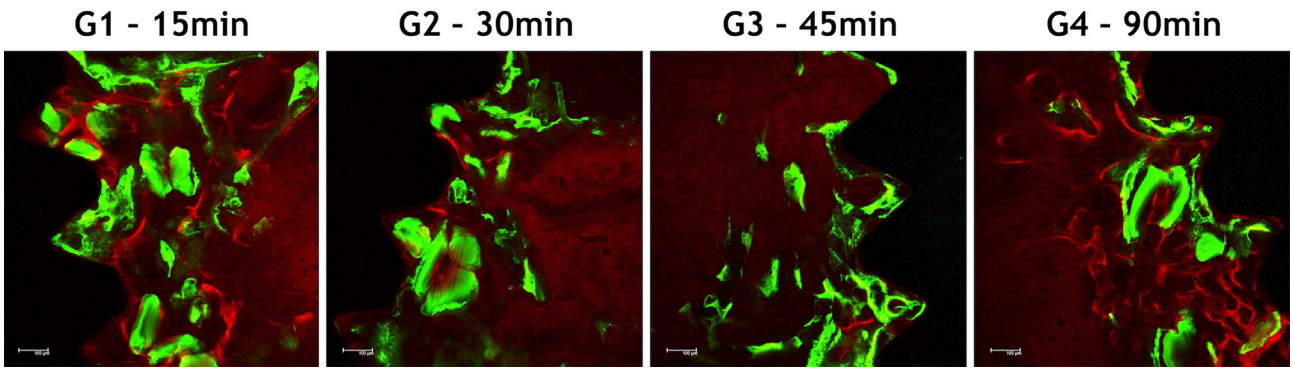

Fig. 6. Calcium (green) and alizarin (red) marking of the fluorescent lines in the calcium matrix, demonstrating the dynamics of the periimplantar bone of the experimental groups: G1, G2, G3 and G4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

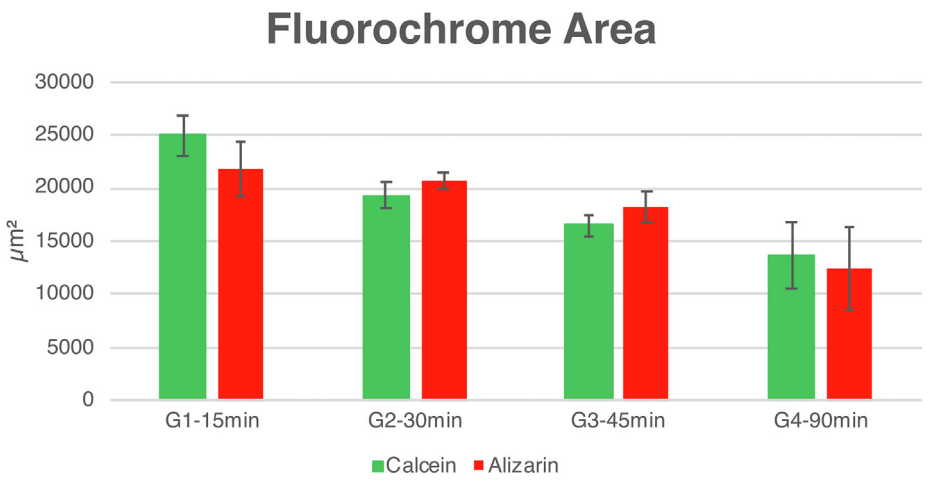

Fig. 7. Peri-implant bone area (μm^2) of the groups expressed by calcein and alizarin ($p > 0.05$, two-way ANOVA).

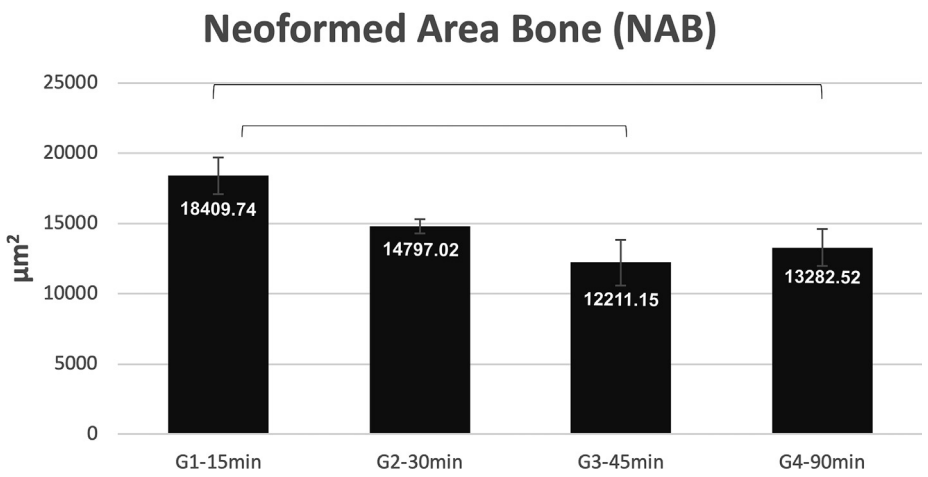

Fig. 8. Bone neoformation area (BNA) in µm2 of groups G1, G2, G3 and G4 ([−p < 0.05, Tukey).

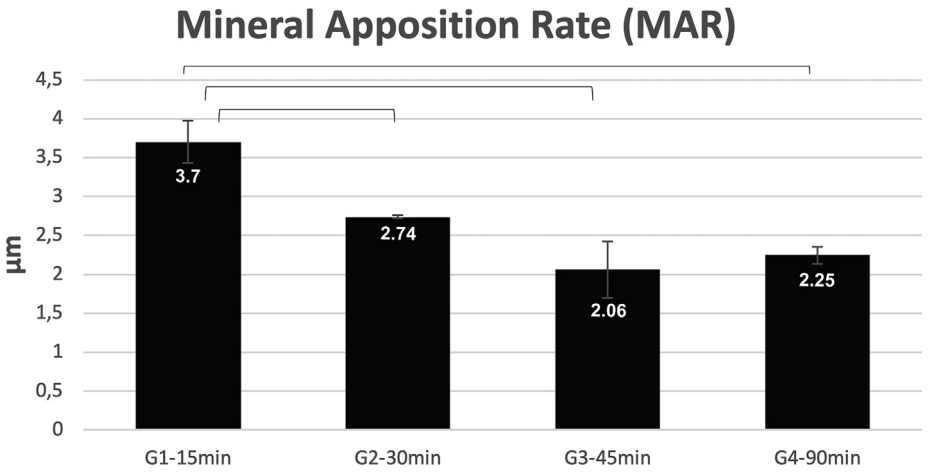

Fig. 9. Linear evaluation of the mineral apposition rate per day (µm) of groups G1, G2, G3 and G4 ([−p < 0.05, Tukey).

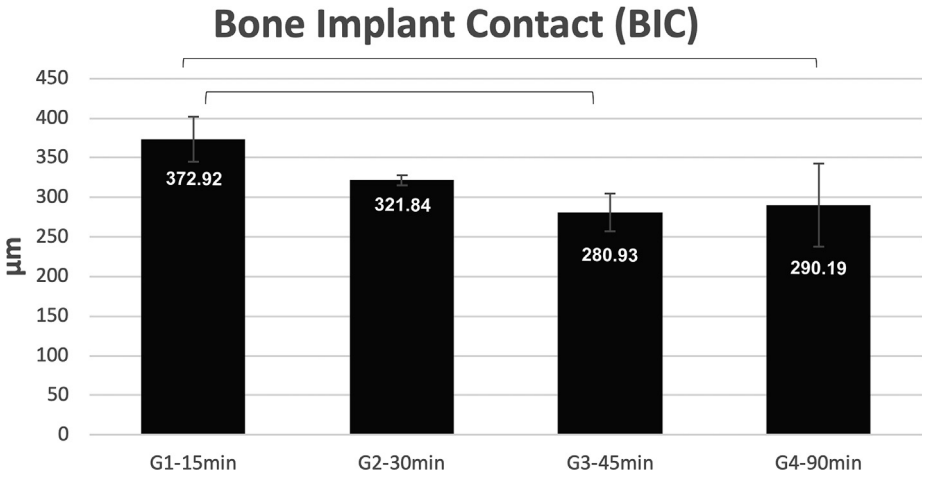

Fig. 10. Evaluation of the peri-implant perimeter through the linear extension of bone/implant contact (ELCOI) (µm) of groups G1, G2, G3 and G4 ([−p < 0.05, Tukey).

pathway and bone formation, showed moderate marking for G1 and G2 but was reduced in G3 and G4, with light markings. Alkaline phosphatase (ALP), an important protein that signals the deposition of phosphate in the extracellular matrix, showed intense labeling for G1, moderate immunolabeling for G2 and G3 and light immunolabeling for G4. When evaluating mature osteoblasts for the presence of osteopontin (OPN), light immunolabeling was observed for G1, G2 and G4, and moderate immunolabeling for G3. The protein osteocalcin (OC) expressed in the extracellular bone matrix related to calcium deposition showed moderate immunolabeling for G1, G2 and G4, but this protein was not yet mineralized in connective tissue for G2. For G3, the immunolabeling was mild.

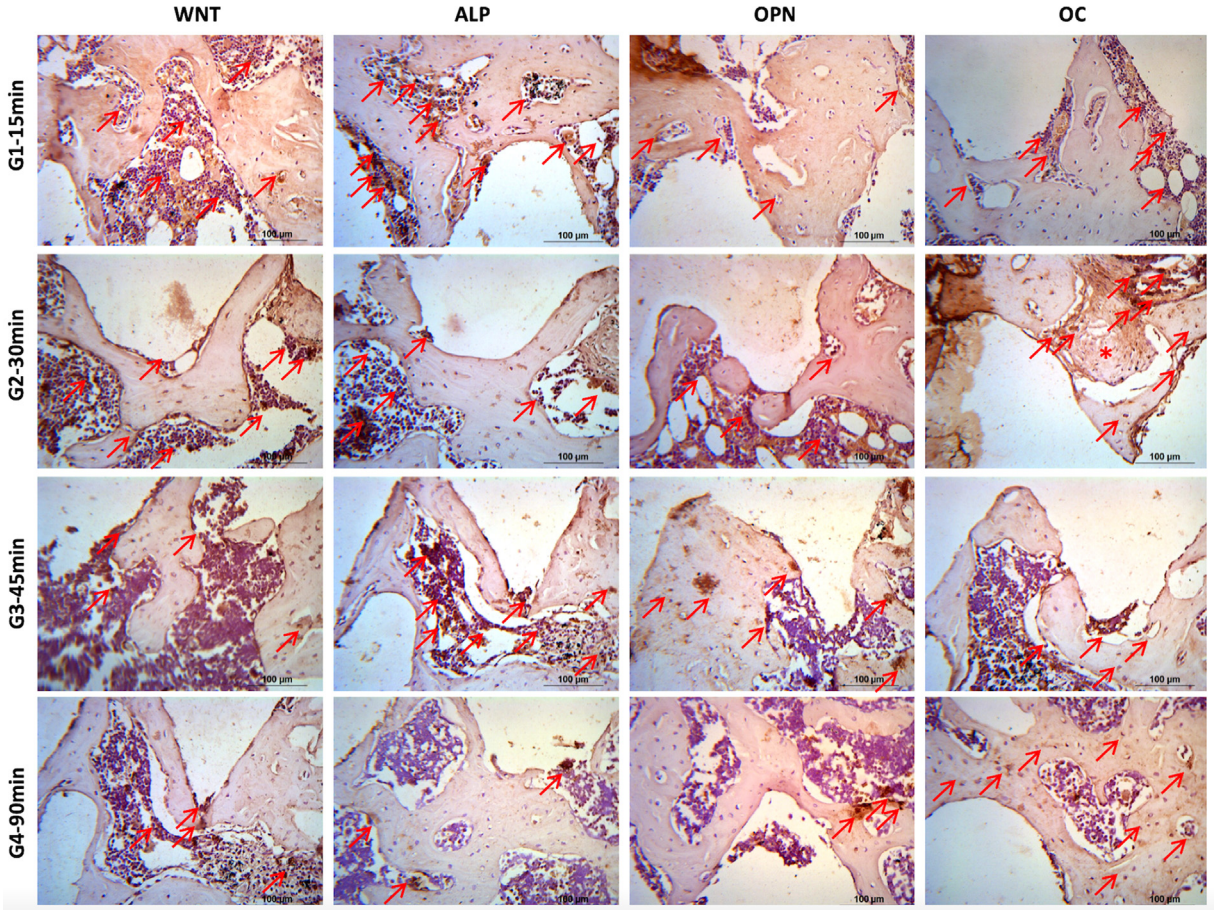

Fig. 11. Immunohistochemistry using proteins against Wnt, ALP, OPN and OC in the period of 60 days. Immunolabelling of these proteins in G1, G2, G3 and G4 groups. (Original, 25×). Immunolabelling was defined as: mild; moderate and intense. * Labeling of the protein in the extracellular matrix in connective tissue not yet mineralized. The red arrows show the immunolabelling of proteins in the extracellular matrix. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Immunolabelling scores for the WNT, ALP, OPN and OC proteins at the different sonochemical times 15, 30, 45 and 90 min (period of 60 days). * labeling of the protein in the extracellular matrix in connective tissue not yet mineralized.

	WNT	ALP	OPN	OC
G1–15 min	2	3	1	2
G2–30 min	2	2	1	2*
G3–45 min	1	2	2	1
G4–90 min	1	1	1	2

3.5. Histological analysis

Photomicrographs of the histological slides from the 60-day period were obtained panoramically with 10× and 25× objectives, making it possible to evaluate bone neoformation on a larger scale. G1 presented great bone formation in contact with the implant and less connective tissue and cellular viability with the presence of osteocytes, showing mature bone tissue. The quality of bone formation present in G2 was similar to that of G1, but with more connective tissue between the bone trabeculae formed. G3 and G4 presented lower bone formation when compared to the other groups, but the bone that was formed also showed good quality (Fig. 12).

4. Discussion

In this work, Biogran® biomaterial was chosen because it presents satisfactory osteoconductive properties and is already used as a bone graft option in dentistry [27,28]. The sonochemical method was employed because it is economically feasible and efficient in reducing and homogenizing particles due to its capacity to provide high temperature and pressure in a short time, inducing a rapid morphological alteration [14,29–32].

As confirmed by X-ray diffraction in the present work, Biogran® is amorphous; that is, it does not exhibit diffraction maxima, but it becomes crystalline when in contact with biological fluids due to ionic exchanges [31], initially forming apatite crystals. This has five steps, beginning with the adsorption of specific proteins in the SiO_2-HAp layer (hydroxyapatite), then action of macrophages, adhesion and cell differentiation; cell matrix formation, and matrix mineralization [33].

In the present study, SEM was able to evaluate the edges of the biomaterial, which was smoother and relatively smaller as the sonochemical time increased, in comparison to unsonicated biomaterial. However, the shape/size of the particles of the processed samples appeared similar, regardless of the sonication time. Therefore, it was possible to characterize the biomaterial with SEM and X-ray diffraction so that it did not lose its chemical and physical structure after the sonochemistry. While maintaining the chemical and physical structure, we can notice that with the process of sonochemistry, the greater time generates greater reduction of the edges of the particles. However, during the process of bone neoformation around the Biogran®, it is broken and there is a continuation of bone neoformation within these cracks [28,31,33]. We believe that very small structures make this process of bone formation difficult.

In planning how to use this functionalized biomaterial in medications, analyses that can actually evaluate its action should be

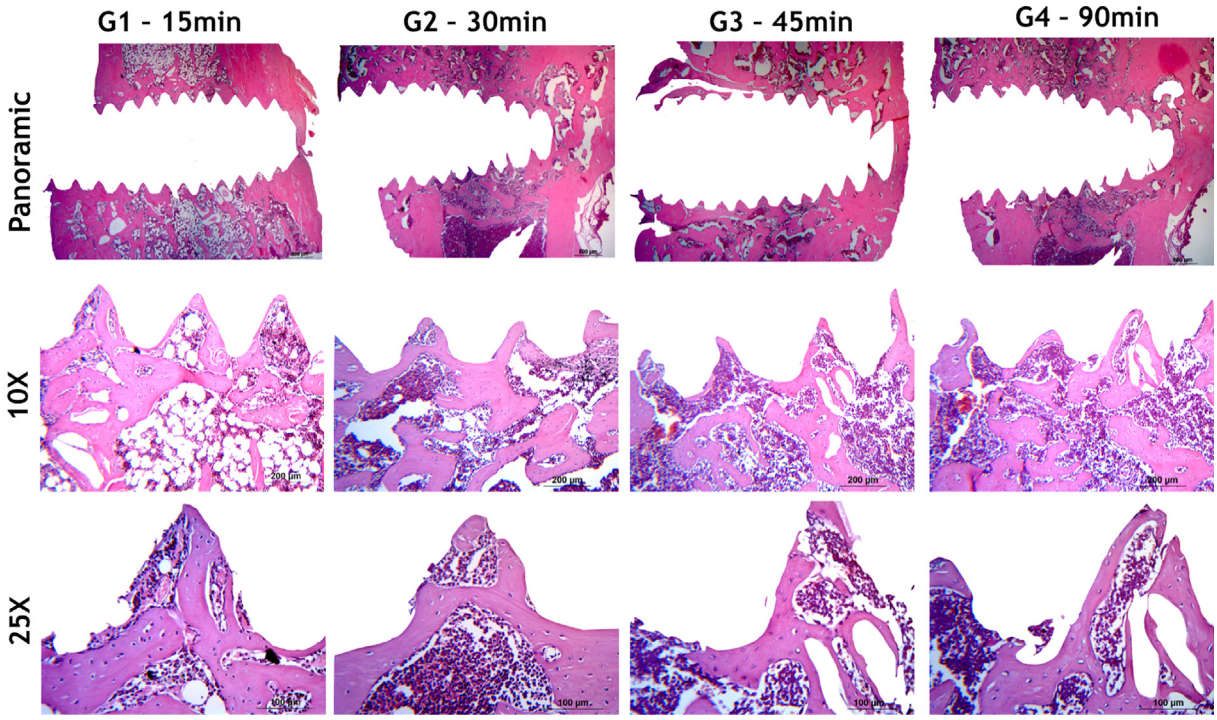

Fig. 12. Histological images of the experimental groups G1, G2, G3 and G4) (1.6× magnification – panoramic, 10× and 25×).

performed. With this in mind, this work brings in vivo results about different application times of bioglass sonochemistry. When performed this technique for 15 min it can be noticed a bone quality superior to the other groups, as evaluated by the periimplantar bone cytoarchitecture in HE, which also corroborates the best results for bone quality and volume in micro CT. In addition, the areas of bone neoformation and linear extension of bone/implant contact were significantly higher than in the groups treated with the sonicated biomaterial at 45 and 90 min, and its daily mineral apposition rate was significantly higher than those of all other groups.

The 30-min group showed no significant difference compared to the 15-min group in terms of volume and quality of bone trabeculate (micro CT), area of bone neoformation and linear extension of bone/implant contact, but always with lower numerical values. The 45- and 90-min times presented the worst results regarding volume, periimplantar bone characterization, mineral deposition, bone neoformation and bone in contact with the implant.

Therefore, sonochemistry for 15 min showed much better structural patterns than the other groups. At the cellular level, this time leads to better performance, with increased transduction pathway and bone formation expressed by WNT and higher deposition of phosphate and calcium in the cellular matrix. Close results were found at 30 min, but with a greater presence of connective tissue. The worst results found at the cellular level were found at 45 and 90 min.

The 30-min sonochemical time showed similar results to those of 15 min for some parameters, although the results were still better at 15 min. In addition, the pulses in the sonochemistry are given every 5 min with a cooling interval, which leads to an even longer end time. Thus, in addition to all the benefits found in the in vivo tests, the processing of the biomaterial becomes even more feasible when prepared in a shorter period of time. The only work to date that refers to the use of this technique to functionalize bioactive glass was that of Lisboa-Filho et al. in 2018 [12]. They used Biogran functionalized with raloxifene to fill defects in rats calvaria, with a 30-min sonochemistry time for the compound. The results showed that the biomaterial sonicated at a 20% concentration of raloxifene was similar to pure Biogran. Considering the findings of the present study, functionalizing (sonicating) the biomaterial in a 15-min sonochemistry time may lead to better results.

5. Conclusion

In view of the results, it is possible to conclude that Biogran® sonicated for 15 min (G1) presents bone repair of the peri-implant defects with better properties of the extracellular matrix, in addition to better turnover, bone quality and quantity, allowing the standardization of this timing (15 min) to other researches, which sonochemistry can be applied, added to some medication with or without bone anabolic character.

Acknowledgments

The authors would like to thank the foundation of amparo of the state of São Paulo Research Foundation – FAPESP (2017/08187-3) for the PhD scholarship of the first author (Gomes-Ferreira, PHS). To the Multiuser Laboratory of the School of Sciences of UNESP of Bauru for the processing of the biomaterial (sonochemistry) and analysis of X-ray Diffraction and Scanning Electron Microscopy. Emfils for providing the implants used in this research.

Conflict of interest

None.

References

[1] R.G. Lima, T.G. Lima, C.E. Francischone, C. Turssi, N.M. Souza Picorelli Assis, B.S. Sotto-Maior, Bone volume dynamics and implant placement torque in horizontal bone defects reconstructed with autologous or xenogeneic block bone: a randomized, controlled, split-mouth, prospective clinical trial, Int. J. Oral Maxillofac. Implants 33 (4) (2018) 888–894, https://doi.org/10.11607/jomi.6288 PubMed PMID: 30025006.

[2] A. Aloy-Prósper, D. Peñarrocha-Oltra, M. Peñarrocha-Diago, M. Peñarrocha-Diago, The outcome of intraoral onlay block bone grafts on alveolar ridge augmentations: a systematic review, Med. Oral Patol. Oral Cir. Bucal. 20 (2) (2015) e251–e258.

[3] S.A. Lee, C.T. Lee, M.M. Fu, W. Elmisalati, S.K. Chuang, Systematic review and meta-analysis of randomized controlled trials for the management of limited

vertical height in the posterior region: short implants (5 to 8 mm) vs longer implants (> 8 mm) in vertically augmented sites, Int. J. Oral Maxillofac. Implants 29 (2014) 1085–1097.
[4] N.U. Zitzmann, P. Schärer, Sinus elevation procedures in the resorbed posterior maxilla. Comparison of the crestal and lateral approaches, Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 85 (1) (1998) 8–17.
[5] P.I. Brånemark, B.O. Hansson, R. Adell, U. Breine, J. Lindström, O. Hallén, A. Ohman, Osseointegrated implants in the treatment of the edentulous jaw. Experience from a 10-year period, Scand. J. Plast. Reconstr. Surg. Suppl. 16 (1977) 1–132.
[6] G. Cannizzaro, G. Gastaldi, E. Gherlone, R. Vinci, I. Loi, A. Trullenque-Eriksson, M. Esposito, Two or three machined vs roughened surface dental implants loaded immediately supporting total fixed prostheses: 1-year results from a randomised controlled trial, Eur. J. Oral Implantol. 10 (3) (2017) 279–291.
[7] P. Felice, M.G. Grusovin, C. Barausse, G. Grandi, M. Esposito, Safety and effectiveness of early loaded maxillary titanium implants with a novel nanostructured calcium-incorporated surface (Xpeed): 3-year results from a pilot multicenter randomised controlled trial, Eur. J. Oral Implantol. 8 (3) (2015) 245–254..
[8] R.M. Hinkle, S.R. Rimer, M.H. Morgan, P. Zeman, Loading of titanium implants with hydrophilic endosteal surface 3 weeks after insertion: clinical and radiological outcome of a 12-month prospective clinical trial, J. Oral Maxillofac Surg. 72 (8) (2014) 1495–1502, https://doi.org/10.1016/j.joms.2014.04.016.
[9] V. Offermanns, O.Z. Andersen, G. Riede, M. Sillassen, C.S. Jeppesen, K.P. Almtoft, H. Talasz, C. Öhman-Mägi, B. Lethaus, R. Tolba, F. Kloss, M. Foss, Effect of strontium surface-functionalized implants on early and late osseointegration: a histological, spectrometric and tomographic evaluation, Acta Biomater. 15 (69) (2018) 385–394, https://doi.org/10.1016/j.actbio.2018.01.049.
[10] S. Kuroshima, T. Nakano, T. Ishimoto, M. Sasaki, M. Inoue, M. Yasutake, T. Sawase, Optimally oriented grooves on dental implants improve bone quality around implants under repetitive mechanical loading, Acta Biomater. 15 (48) (2017) 433–444, https://doi.org/10.1016/j.actbio.2016.11.021.
[11] C. Chan, I. Thompson, P. Robinson, J. Wilson, L. Hench, Evaluation of bioglass/dextran composite as a bone graft substitute, Int. J. Oral Maxillofac. Surg. 31 (1) (2002) 73–77.
[12] P.N. Lisboa-Filho, P.H.S. Gomes-Ferreira, F.R.S. Batista, G.A.C. Momesso, L.P. Faverani, R. Okamoto, Bone repair with raloxifene and bioglass nanoceramic composite in animal experiment, Connect. Tissue Res. 59 (Suppl. 1) (2018) 97–101, https://doi.org/10.1080/03008207.2018.1430143 PubMed PMID: 29745810.
[13] J.H. Bang, K.S. Suslick, Applications of ultrasound to the synthesis of nanostructured materials, Adv. Mater. 22 (10) (2010) 1039–1059, https://doi.org/10.1002/adma.200904093.
[14] L.B. Arruda, M.O. Orlandi, P.N. Lisboa-Filho, Morphological modifications and surface amorphization in ZnO sonochemically treated nanoparticles, Ultrason. Sonochem. 20 (3) (2013) 799–804, https://doi.org/10.1016/j.ultsonch.2012.11.013.
[15] A.A. Veis, N.N. Dabarakis, N.A. Parisis, A.T. Tsirlis, T.G. Karanikola, D.V. Printza, Bone regeneration around implants using spherical and granular forms of bioactive glass particles, Implant. Dent. 15 (4) (2006) 386–394.
[16] H.S. Oonishi, E. Kushitani, H. Yasukawa, L.L. Iwaki, J. Hench, E. Wilson, Tsuji T. Sugihara, Particulate bioglass compared with hydroxyapatite as a bone graft substitute, Clin. Orthop. Relat. Res. 334 (1997) 316–325.
[17] E. Schepers, M. De Clercq, P. Ducheyne, R. Kempeneers, Bioactive glass particulate material as a filler for bone lesions, J. Oral Rehabil. 18 (439–452) (1991) 49.
[18] M. Sjöström, S. Lundgren, H. Nilson, L. Sennerby, Monitoring of implant stability in grafted bone using ressonance frequency analysis. A clinical study from implant placement to 6 months of loading, Int. J. Oral Maxillofac. Surg. 34 (1) (2005) 45–51.
[19] R.E. Jung, G.A. Hälg, D.S. Thoma, et al., A randomized, controlled clinical trial to evaluate a new membrane for guided bone regeneration around dental implants, Clin. Oral Implants Res. 20 (2009) 162–168.
[20] C. Kilkenny, W.J. Browne, I.C. Cuthill, M. Emerson, D.G. Altman, Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research, PLoS Biol. 8 (6) (2010) e1000412.
[21] G. Ramalho-Ferreira, L.P. Faverani, F.B. Prado, I.R. Garcia Jr, R. Okamoto, Raloxifene enhances peri-implant bone healing in osteoporotic rats, Int. J. Oral Maxillofac. Surg. 44 (6) (2015) 798–805, https://doi.org/10.1016/j.ijom.2015.02.018.
[22] E.R. Luvizuto, S. Tangl, G. Zanoni, T. Okamoto, C.K. Sonoda, R. Gruber, R. Okamoto, The effect of BMP-2 on the osteoconductive properties of β-tricalcium phosphate in rat calvaria defects, Biomaterials 32 (15) (2011) 3855–3861.
[23] D. Oliveira, J.S. Hassumi, P.H. Gomes-Ferreira, T.O. Polo, G.R. Ferreira, L.P. Faverani, R. Okamoto, Short term sodium alendronate administration improves the peri-implant bone quality in osteoporotic animals, J. Appl Oral Sci. 25 (1) (2017) 42–52, https://doi.org/10.1590/1678-77572016-0165.
[24] B. Glösel, U. Kuchler, G. Watzek, Gruber R. Review of dental implant rat research models simulating osteoporosis or diabetes, Int. J. Oral Maxillofac. Implants 25 (3) (2010) 516–524.
[25] M.L. Bouxsein, S.K. Boyd, B.A. Christiansen, R.E. Guldberg, K.J. Jepsen, R. Müller, Guidelines for assessment of bone microstructure in rodents using micro-computed tomography, J. Bone Miner. Res. 25 (7) (2010) 1468–1486.
[26] D.W. Dempster, J.E. Compston, M.K. Drezner, F.H. Glorieux, J.A. Kanis, H. Malluche, P.J. Meunier, S.M. Ott, R.R. Recker, A.M. Parfitt, Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee, J. Bone Miner. Res. 28 (1) (2013) 2–17.
[27] F.A. Santos, M.T. Pochapski, M.C. Martins, E.G. Zenóbio, L.C. Spolidoro, E. Marcantonio Jr., Comparison of biomaterial implants in the dental socket: histological analysis in dogs, Clin. Implant. Dent. Relat. Res. 12 (1) (2010) 18–25.
[28] R.D.S. Pereira, J.D. Menezes, J.P. Bonardi, G.L. Griza, R. Okamoto, E. Hochuli-Vieira, Histomorphometric and immunohistochemical assessment of RUNX2 and VEGF of Biogran™ and autogenous bone graft in human maxillary sinus bone augmentation: a prospective and randomized study, Clin. Implant. Dent. Relat. Res. 19 (5) (2017) 867–875, https://doi.org/10.1111/cid.12507.
[29] T. Taghipour, G. Karimipour, M. Ghaedi, A. Asfaram, Mild synthesis of a Zn(II) metal organic polymer and its hybrid with activated carbon: application as antibacterial agent and in water treatment by using sonochemistry: optimization, kinetic and isotherm study, Ultrason. Sonochem. 41 (2018) 389–396, https://doi.org/10.1016/j.ultsonch.2017.09.056.
[30] Z. Li, Z. Wang, X. Du, C. Shi, X. Cui, Sonochemistry-assembled stimuli-responsive polymer microcapsules for drug delivery, Adv. Healthc Mater. 7 (11) (2018) e1701326, , https://doi.org/10.1002/adhm.201701326.
[31] S. Bagheri, H. Aghaei, M. Ghaedi, A. Asfaram, M. Monajemi, A.A. Bazrafshan, Synthesis of nanocomposites of iron oxide/gold (Fe(3)O(4)/Au) loaded on activated carbon and their application in water treatment by using sonochemistry: optimization study, Ultrason. Sonochem. 41 (2018) 279–287, https://doi.org/10.1016/j.ultsonch.2017.09.031.
[32] A.C. Cruz, M.T. Pochapski, R. Tramonti, J.C. da Silva, A.C. Antunes, G.L. Pilatti, F.A. Santos, Evaluation of physical-chemical properties and biocompatibility of a microrough and smooth bioactive glass particles, J. Mater. Sci. Mater. Med. 19 (8) (2008) 2809–2817, https://doi.org/10.1007/s10856-008-3407-4.
[33] L.L. Hench, Bioceramics: from concept to clinic, J. Am. Ceram. Soc. 74 (1991) 1487–1510.