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Histomorphometric evaluation of the association between bioglass and lyophilized bovine bone in the treatment of critical bone defects created on rat calvaria: a pilot study

Avaliação histomorfométrica da associação entre biovidro e osso bovino liofilizado no tratamento de defeitos ósseos críticos criados em calvárias de ratos. Estudo piloto

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Resumo

Objetivo: Avaliar histomorfometricamente o efeito de biovidro (B), osso bovino liofilizado (OB) ou da mistura desses dois biomateriais (B/OB - 1:1) no reparo de defeitos ósseos críticos em calvária de ratos. **Material e método:** Defeitos ósseos (8 mm Ø) foram criados cirurgicamente na calvária de 24 ratos, distribuídos em 4 grupos com 6 animais, de acordo com o tipo de biomaterial: coágulo sanguíneo (GC), biovidro (GB), osso bovino liofilizado (GOB) e a mistura desses dois biomateriais (GB/OB). Os animais foram eutanasiados após 15 e 60 dias do procedimento cirúrgico (3 animais por período). A avaliação histológica foi baseada na descrição da morfologia dos tecidos neoformados, enquanto para a avaliação histomorfométrica foi realizada quantificação da porcentagem de tecido ósseo, de tecido conjuntivo fibroso neoformados e de biomaterial remanescente no defeito ósseo. **Resultado:** Nos dois períodos experimentais, a análise histológica apresentou neoformação óssea, principalmente nas bordas dos defeitos, e ao redor de partículas de biomateriais remanescentes. A avaliação histomorfométrica demonstrou que no período de 15 dias o grupo GC apresentou maior percentagem de tecido ósseo em relação aos demais grupos estudados, enquanto que aos 60 dias o grupo GOB apresentou maior porcentagem de tecido ósseo em relação ao grupo GB. **Conclusão:** O osso bovino liofilizado apresentou maior formação óssea em relação ao biovidro, mas nenhum dos biomateriais foi superior ao coágulo. A associação do biovidro e osso bovino liofilizado não adicionou vantagem à formação óssea.

Descritores: Histologia; osso; reparo ósseo.

Abstract

Objective: This study sought to histomorphometrically evaluate the effect of bioglass (B), lyophilized bovine bone (BB) or the 1:1 mixture of these two biomaterials on the repair of critical bone defects in rat calvaria. **Material and method:** Bone defects (8 mm Ø) were surgically created in the calvaria of 24 rats, which were divided into the following 4 groups of 6 animals each according to the type of biomaterial used: blood clot / coagulum (control) group (CG), bioglass group (BG), lyophilized bovine bone group (BBG) and a group receiving a mixture of these two biomaterials (BG/BB). The animals were euthanized at 15 or 60 days after surgery (3 animals per period). Histological evaluation was based on the morphological description of the newly formed tissues, and a quantification of the percentage of bone tissue with newly formed fibrous connective tissue and the percentage of biomaterial remaining in the bone defect was performed for the histomorphometric evaluation. **Result:** In both experimental periods, the histological analysis showed new bone formation, especially at the edges of the defects and around remaining biomaterial particles. Histomorphometric analysis showed that the CG contained a higher percentage of bone tissue over the 15-day period compared to that of the other groups. At 60 days, the BBG showed a higher percentage of bone tissue compared to that of the BG ($p < 0.01$). **Conclusion:** Lyophilized bovine bone led to greater bone formation compared to bioglass, but none of the biomaterials was superior to blood clot. Moreover, the combination of bioglass and lyophilized bovine bone did not provide an advantage for bone formation.

Descriptors: Histology; bone; bone repair.

INTRODUCTION

The use of dental implants for the treatment of edentulism has become increasingly popular, showing high rates of clinical success[1,2]. However, some factors limit the installation of dental implants, with bone availability being the most important local factor[3,4].

Several biomaterials have been suggested to overcome the lack of available bone[3-5]. Among these biomaterials, autologous bone is considered the gold standard for bone regeneration[3,6]. However, factors such as donor site morbidity, limited bone availability and high bone resorption rates are some of the limiting factors to the clinical application of these grafts[3,6,7]. The mixture of autogenous bone with biomaterials has been proposed as an alternative to increase graft availability and reduce resorption rates; however, this strategy does not eliminate problems associated with donor site morbidity[7].

Some bone substitutes, such as biomaterials from a heterogeneous origin, have been indicated for mixing with autogenous bone[7-10]. Some studies have reported clinical success with the combination of xenogenous and alloplastic bone with autogenous bone in procedures aimed to elevate the maxillary sinus membrane[8,9]. These biomaterials possess osteoconductive biological functions and serve as a scaffold for bone formation[3,8,9,11]; however, the different chemical characteristics of these biomaterials activate different biological mechanisms of bone formation[12].

According to the literature, the mixture of two biomaterials with similar biological properties that induce distinct biological reactions has not yet been investigated. Thus, the present study aimed to perform a histologic and histomorphometric evaluation of the use of bioglass, lyophilized bovine bone or a combination of these two biomaterials in critical bone defects in rat calvaria.

MATERIAL AND METHOD

The protocol used in the present study was approved by the Ethics Committee on Animal Research from FOAR-UNESP (CEEA), under protocol No. 015/2008. In the present study, 24 adult male rats (*Rattus norvegicus*, Holtzman) of approximately 3 months of age and with a mean weight of 350 g were used. The animals were kept in the vivarium of the School of Dentistry, UNESP, Araraquara (UNESP-FOAR). All animals were provided with solid food and water *ad libitum* throughout the experimental period and were housed in an environment with controlled light and temperature.

The animals were randomly divided into 4 groups, which were evaluated in two experimental periods (15 and 60 days), with 3 animals in each group/period. A bone defect was made in the calvaria of each animal, and the animals were then divided into the following groups according to the type of biomaterial used: control group (CG) - defect filled by blood clot; bioglass group (BG) - defect filled with bioglass (Procell, São Carlos, Brazil); lyophilized bone group (BBG) - defect filled with lyophilized bovine bone (Procell, São Carlos, Brazil); and the mixture of biomaterials group (BG/BB) - defect filled with a 1:1 mixture of lyophilized bovine bone and bioglass (Procell, São Carlos, Brazil).

The animals were anesthetized with a combination of ketamine and xylazine at a ratio of 0.08 ml/100 g body weight (ketamine hydrochloride - Francotar - Virbac Brazil Inc.) and 0.04 ml/100 g body weight (xylazine hydrochloride - Virbaxyl 2% - Virbac Brazil Inc.). Subsequently, the animals were shaved in the calvaria region, and the surgical field was cleaned with sterile gauze soaked in a povidone-iodine solution, with the animal placed in a ventral decubitus position on the operating table.

The surgical technique used was previously described in the literature[13]. Briefly, surgical access to the posterior portion of the calvaria was obtained through a central skin and muscle incision in the anterior-posterior direction, approximately 3 cm in length. The tissues were then divulsioned until exposure of the bone tissue.

In the middle portion of the calvaria, immediately after the apex of the posterior cranial suture, circular bone defects (8 mm diameter by 1.5 mm thickness) were prepared. To generate these defects, a trephine mill (3i Brazil Implants) with the following specifications was used: 8 mm outer diameter; mounted on a contra-angle (Anthogyr - Inject - Diadema, Brazil) with a 16:1 reduction; coupled with a motor for implantation (BML 600 Plus Driller - CK Driller - Brazil) at 1,500 rpm; and constant saline irrigation.

Each biomaterial was implanted into the bone defect via placement on the dura to completely fill the defect. The distribution of the implantation of the biomaterials was standardized by group (BG, BBG and BG/BB) and experimental period (15 and 60), with the exception of the CG, which after correction of the bone defect, only the blood clot was present. Subsequently, all defects were covered with collagen membrane (Genius-Baumer, Brazil) and plane sutured with Vycril 4.0 suture thread (Ethicon, Johnson & Johnson, Brazil). The animals were treated with 1 ml pentabiotic (Fort-Dodge, Brazil) per 100 g of body weight.

After the proposed experimental periods, the animals were euthanized under deepening general anesthesia. Soon after, a bicoronal incision was made to remove the entire top portion of the calvaria, including soft and hard tissues. Biopsies were identified and fixed in 10% buffered formaldehyde for approximately 72 hours. The specimens were subjected to routine laboratory procedures for decalcification and paraffin embedding.

From each specimen, 25 serial sections were obtained with a 6 μm thickness, which were divided into slides with 5 sections each that were then stained with hematoxylin-eosin. Three slices per specimen were analyzed, with a standardized distance of 60 μm between them. The histological sections were analyzed by DIASTAR conventional light microscopy (Leica Reichert & Jung products, Germany) under 100X magnification. Representative images were scanned with a Leica Microsystems DFC-300-FX digital camera (Leica Microsystems, Germany) with a resolution of 1.3 megapixels, coupled to an optical microscope and analyzed using image analysis software (Image J, Jandel Scientific, San Rafael, CA, USA).

Histological and Histomorphometric Evaluation

For the descriptive histological evaluation, the morphological characteristics of the newly formed tissues were evaluated and data was collected regarding the formation and mineralization of the bone tissue and the presence of inflammation and osteoblastic and osteoclastic activity. For the histomorphometric evaluation, the original defect area was delimited, and the metric calibration of the microscope was used as a standard, demarcating an area of 8 × 1.5 mm corresponding to the original defect. Within this region, the percentage of bone tissue with newly formed fibrous connective tissue and the percentage of biomaterial remaining in the bone defect were delimited.

RESULT

Histologic Analysis

At 15 days, the CG presented an extensive area of the defect region occupied by disorganized fibrous connective tissue with a predominance of inflammatory cells (mainly macrophages) and blood vessels with cell stasis (Figure 1). The collagen membrane used to cover the defect was in the early stages of resorption, and the newly formed bone was limited to regions near the edges of the defect, with small areas of trabecular bone formation. For groups in which the critical defects were filled with biomaterials, the histological pattern was similar (Figures 2-4). No increase in inflammatory cells was observed. Around the biomaterial particles in the BBG and BG/BB, areas of newly formed bone were observed, which were not observed in the BG (Figures 2-4).

At 60 days, in all groups, evolution of the previous condition was observed. In particular, there was increased organization of the fibrous connective tissue, which was now dense, and decreased inflammatory infiltrate. The collagen membrane was in an advanced state of resorption. Discrete, newly formed bone was observed only in the regions near the edge of the defect (Figures 1-4). In the experimental groups, a decrease in the amount of biomaterial particulate and of newly formed bone areas around these particles was observed in the BBG and BG/BB (Figures 3 and 4) but not in the BG (Figure 2).

Histomorphometric Analysis

The overall analysis showed that all groups demonstrated an increase in the percentage of bone tissue and a reduction in the percentage of biomaterial at 60 days compared with at 15 days. However, the increase in bone percentage was lower in the BG compared with that in the other two groups.

At 15 days, the CG showed a higher percentage of bone tissue (3.94±0:32), followed by the BG/BB (3.33±0.82), BG (3.23±1.79) and BBG (1.55±0.49). At 60 days, the BBG showed a higher percentage of bone tissue (6.30±1.61), followed by the CG (5.32±2.04), BG/BB (4.55±1.02) and BG (3.26±0.98).

Regarding the percentage of fibrous connective tissue at 15 days, the CG showed a higher percentage of this tissue (96.05±0.32), followed by the BBG (79.28±3.98), BG (73.50±13.69) and BG/BB (68.49±16.70). At 60 days, the BG showed a lower percentage of fibrous connective tissue (73.87±7.84), followed by the BBG (80.58±4.72), BG/BB (80.76±5.59) and CG (94.67±2.04).

Regarding the percentage of biomaterial at 15 days, the BG/BB showed a higher percentage of biomaterial (68.49±16.70), followed by the BG (23.26±15.20) and BBG (19.24±3.96). At 60 days, the BG showed a higher percentage of biomaterial (22.86±8.48), followed by the BG/BB (14.68±6.22) and BBG

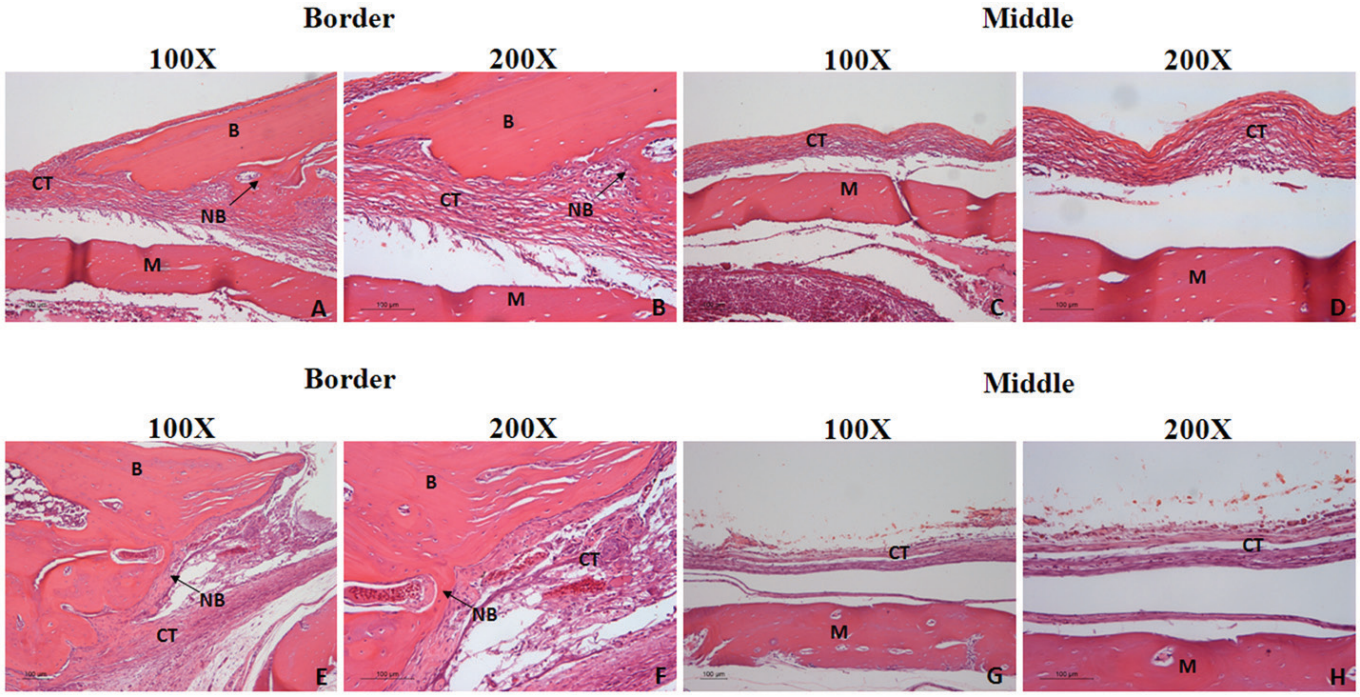

Figure 1. Representative images of histological descriptions of the CG at 15 days (A, B, C, D) and 60 days (E, F, G, H, I) (HE). M-Membrane; B-Bone; NB-Newly formed bone; CT-Connective tissue. (Original magnification 100x and 200x).

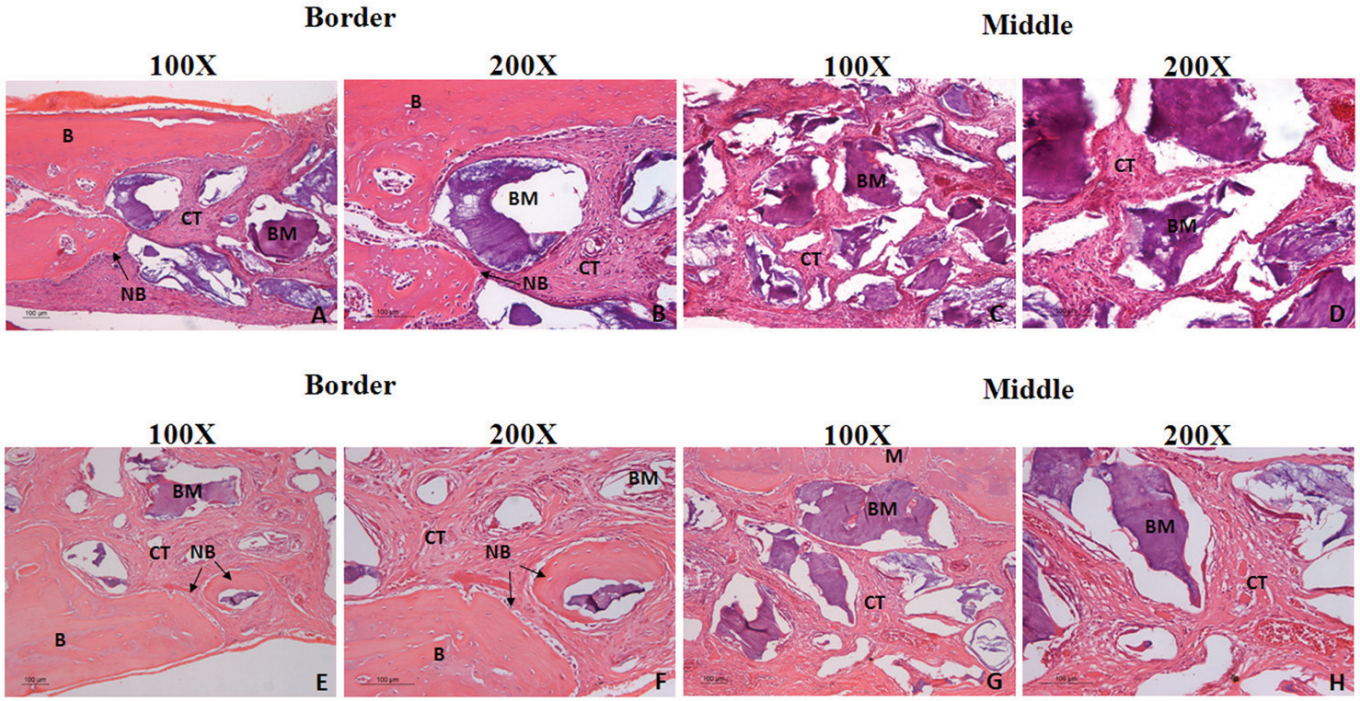

Figure 2. Representative images of histological descriptions of the BG at 15 days (A, B, C, D) and 60 days (E, F, G, H, I) (HE). BM-Biomaterial; M-Membrane; B-Bone-; NB-Newly formed bone; CT-Connective tissue. (Original magnification 100x and 200x).

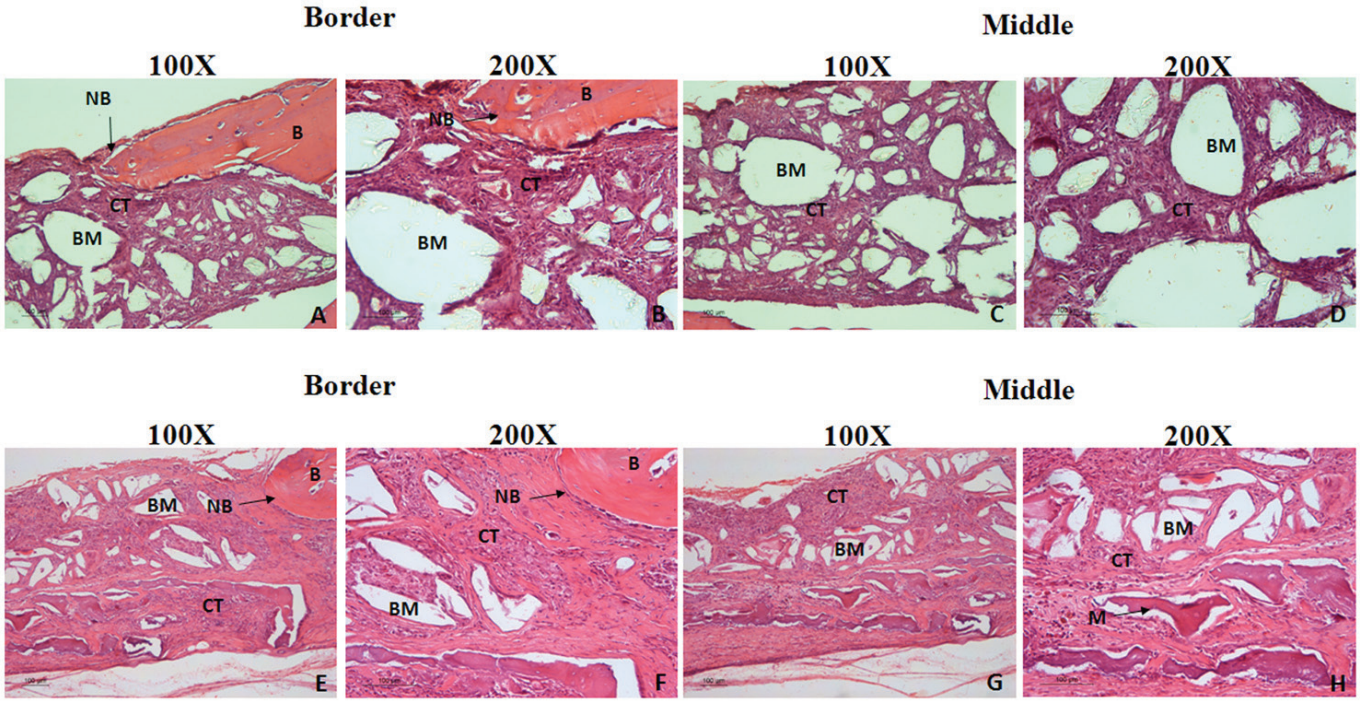

Figure 3. Representative images of histological descriptions of the BBG at 15 days (A, B, C, D) and 60 days (E, F, G, H, I) (HE). BM-Biomaterial; M-Membrane; B-Bone; NB-Newly formed bone; CT-Connective tissue. (Original magnification 100x and 200x).

(13.12±4.86). Figure 5 shows the mean and standard deviation of the percentage of different tissues present in the defects in all groups.

DISCUSSION

Regarding the results obtained in the present study, none of the biomaterials used induced persistent inflammatory reactions in the host, which demonstrates the biocompatibility of these materials. However, they induced different responses in relation to bone formation. Histologically, the BBG and BG/BB showed newly formed bone between the biomaterial granules.

Bone formation around bovine biomaterial particles has been demonstrated in other studies[8,9]. Although the lyophilized bovine bone is not completely resorbed[7,8], it shows excellent osteoconductive properties, allowing bone formation between its particles and between the particles and the recipient site and osseointegrated implants[8,9,14,15]. The histomorphometric data on

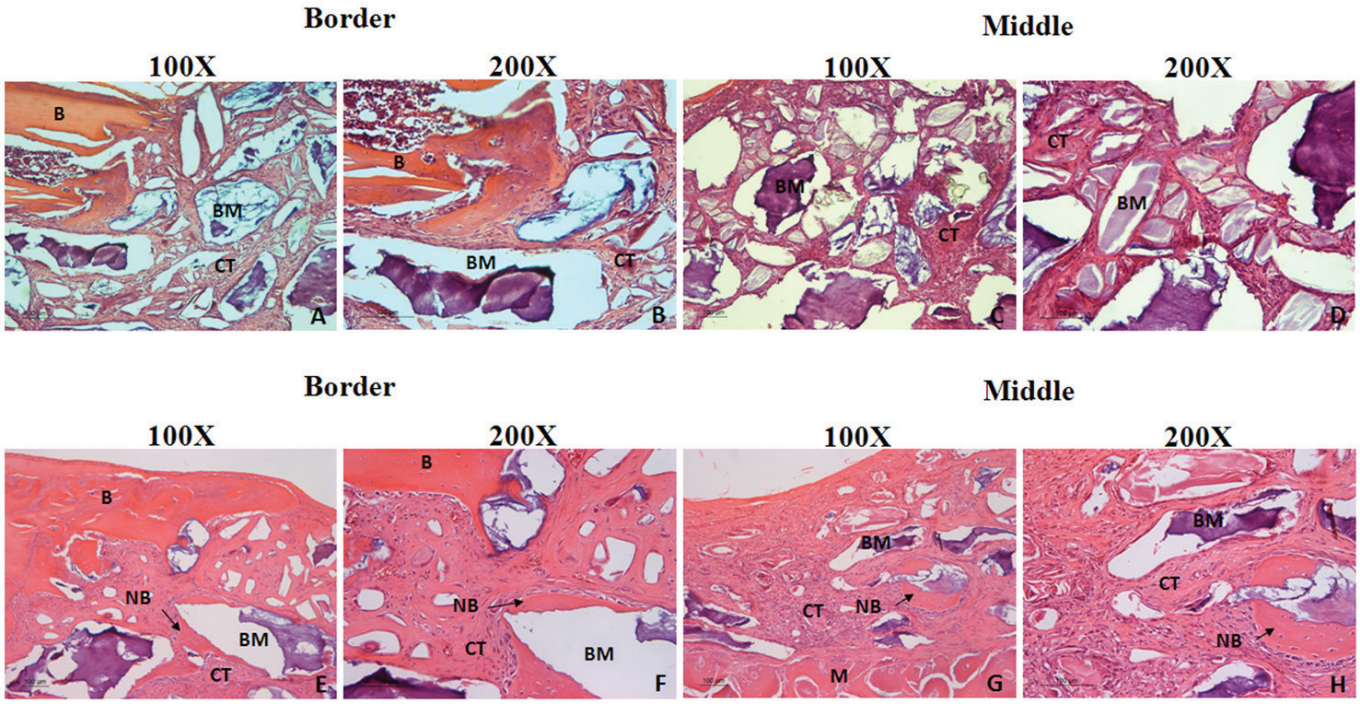

Figure 4. Representative images of histological descriptions of the BG/BB at 15 days (A, B, C, D) and 60 days (E, F, G, H, I) (HE). BM-Biomaterial; M-Membrane; B-Bone-; NB-Newly formed bone; CT-Connective tissue. (Original magnification 100x and 200x).

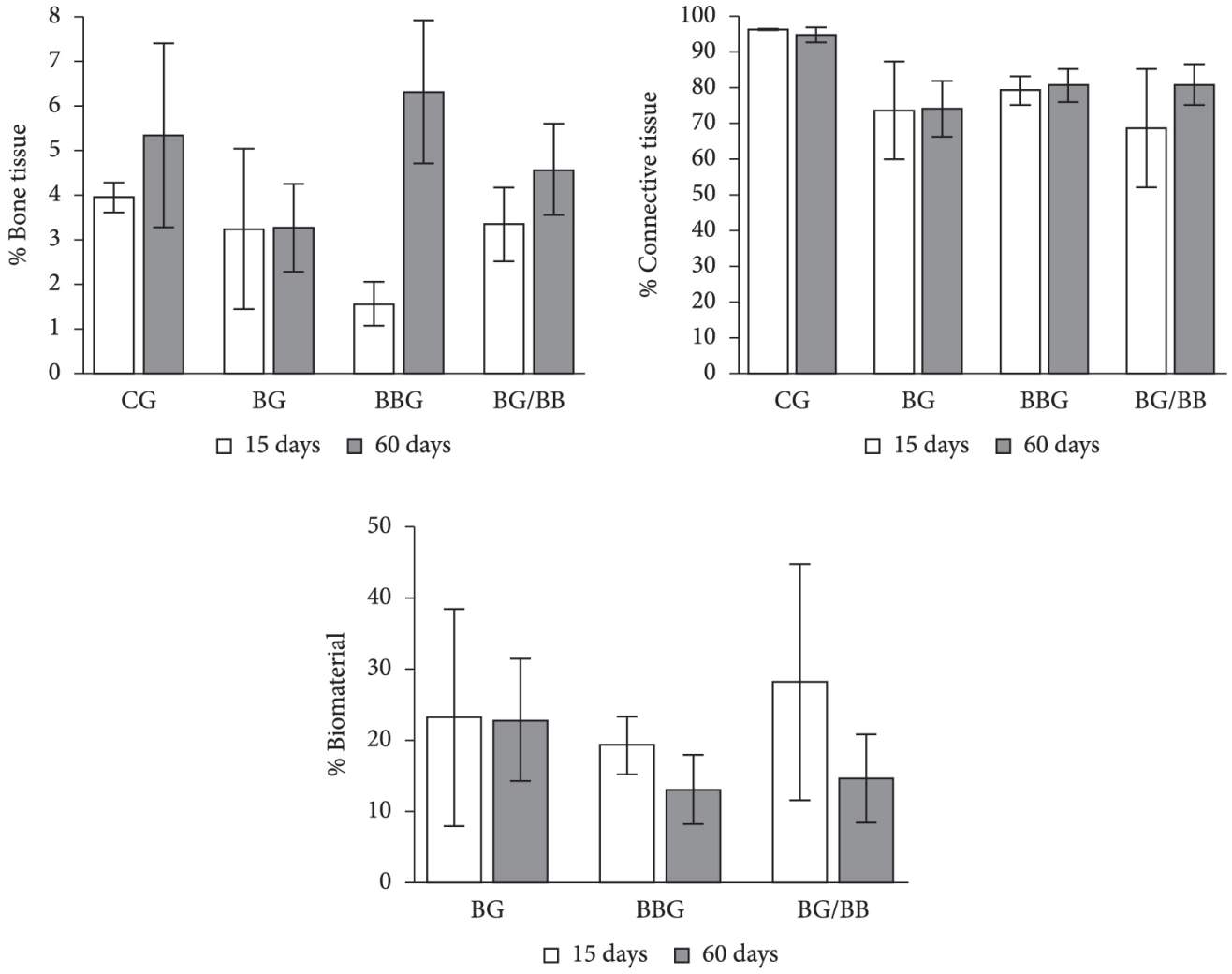

Figure 5. Evaluation of the percentage of different tissue types observed in all groups.

the percentage of bone tissue in our study demonstrated that at 60 days, the BBG showed a higher percentage of bone than that observed in the BG, reinforcing the greater osteoconductive potential of lyophilized bovine bone compared to bioglass[8,9]. These groups showed the same temporal hystometric patterns of occurrence of the different tissues that filled the critical defects, with a reduction in the percentage of biomaterial concomitant with an increase in the percentage of bone tissue. This is the ideal pattern of behavior for biomaterials expected to promote bone repair[3,7,10]. The use of biomaterials prevents the bone defect from being filled by fibrous connective tissue, as observed in the CG, which would be inappropriate for the restoration of bone tissue functions, precluding, for example, the installation of implants in the repaired region[16].

In the present study, we also observed a delay in the resorption and formation of connective tissue around the biomaterial particles in the BG. This histomorphometric aspect corroborates the results from other studies that observed an increased resorption of bioglass compared with bovine bone[8,9,11] as well as the formation of bone around the biomaterial particles[5,17]. The fact that these biomaterials were from different manufacturers and originated by different methods could explain the difference in the behavior of biomaterials used in the present study compared with previously published studies. In particular, different manufacturing processes can lead to completely different mineral contents, crystalline structures and topography characteristics that alter the materials' resorption properties[18]. None of the experimental groups showed greater bone formation than the CG, and this finding is in accordance with the results of a study that evaluated bone formation in critical defects in the jaws of dogs, which were filled with deproteinized bovine bone and bioglass associated with platelet-rich plasma[19]. Because these are biomaterials with strictly osteoconductive properties, there is no inducer stimulus of bone formation, and these biomaterials serve only as a scaffold for bone formation; in addition, these biomaterials need to be resorbed, which may delay or reduce the formation of bone tissue[8,9]. However, the bone tissue formed in the BBG and BG/BB was more homogeneous and was not confined to the edges of the defect, as observed in the CG.

Another point to be taken into consideration is the experimental periods used to evaluate the effect of these biomaterials in the present study. The 15-day period represents a premature analysis of biological reactions of the bone formation process, while the 60-day period represent a late assessment of the bone maturation process. These evaluation periods were previously used by our research group to evaluate the repair of bone tissue associated with the use of biomaterials in critical defects in the skulls of rats[13].

Because this was a pilot study, the results should be interpreted while taking into account some study limitations. For example, the small sample size evaluated in each period limited greater extrapolation of our results and did not permit statistical analysis. We selected a small sample size because the bioglass prototype was evaluated in vivo for the first time in the present study, and therefore, the initial intention was to gather basic evidence regarding the use of this biomaterial in more complex experimental designs. The preliminary results suggested the need to increase the sample number; however, due to the poor results obtained with this biomaterial, we opted not to increase the sample number.

In conclusion, none of the tested biomaterials was superior to the control treatment with respect to bone formation. Although lyophilized bovine bone led to greater bone formation than that formed using bioglass, the combination of bioglass with lyophilized bovine bone did not add any advantage with regards to bone formation.

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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