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Histological Comparison between Biphasic Calcium Phosphate and Deproteinized Bovine Bone on Critical-Size Bone Defects

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The limited options for bone repair have led to an extensive research of the field and the development of alloplastic and xenogeneic grafts. The purpose of this study was to evaluate bone repair with two bone substitutes: deproteinized bovine bone (DBB) and biphasic calcium phosphate ceramic (BCP) in critical-size defect. A total of 8-mm defects were made in the parietal bones of rabbits (n=12). The animals were divided into three experimental groups: sham (defect filled with a blood clot), DBB (defect filled with DBB), and BCP (defect filled with BCP). After the experimental periods of 15 and 45 days, the animals were euthanized and submitted to histomorphometric analysis. The total defect area, mineralized tissue area, biomaterial area, and soft tissue area were evaluated. A greater amount of immature bone tissue and biomaterial particles were observed in the BCP group compared to DBB and sham at 45 days ($p<0.05$). There was no difference in the qualitative pattern of bone deposition between DBB and BCP. However, the sham group did not show osteoid islands along with the defect, presenting a greater amount of collagen fibers as well in relation to the DBB and BCP groups. There was a greater number of inflammatory cells in the DBB at 45 days compared to BCP and sham groups. In conclusion, BCP and DBB are options for optimizing the use of bone grafts for maxillofacial rehabilitation. Bone defects treated with BCP showed greater deposition of bone tissue at 45 days.

Key Words: bone formation, biomaterials, deproteinized bovine bone, hydroxyapatite, bone substitutes.

Introduction

Biomaterials have been widely used for the rehabilitation of large bone defects due to trauma, tumor resection, or long-term tooth loss. According to their origin, biomaterials can be classified as autogenous, allogeneic, xenogenous, and alloplastic (1). Autogenous bone grafts, known as the gold standard, allow the maintenance of cell viability and osteogenic capacity (1,2). However, the need for an additional surgical area, graft resorption, and increased risk of morbidity are disadvantages inherent to this type of graft (3,4).

Allogeneic grafts are harvested from donors of the same species and have osteoconductive and osteoinductive properties due to the presence of bone morphogenetic proteins (BMPs). Xenogenous grafts are harvested from other species, mainly bovine, being deproteinized and lyophilized before use (1,5,6). Xenogenous grafts are widely used biomaterials with high success rates (7-9). These biomaterials do not cause an immune response and are considered biocompatible. One of these materials is the Bio-Oss® (Geistlich-Pharma, Wolhusen, Switzerland), consisting of calcium carbonate apatite. It is osteoconductive, has a porosity between 75 and 80%, and can be used for a maxillary sinus lift, resulting in adequate osseointegration of the dental implant (7). The material is slowly reabsorbed, and its residues can be found nine years after grafting (6,10).

Alloplastic grafts are a large group of synthetic calcium-based biomaterials of different compositions, including calcium phosphate, calcium sulfate, bioactive glasses, and polymers (5). They are widely used in the preservation of the alveolar ridge and maxillary sinus floor augmentation, with high success rates (2,7). In addition, allografts are available without quantity restriction and there is no risk of disease transmission.

Hydroxyapatite is an insoluble biphasic alloplastic biomaterial produced by the sintering of hydroxyapatite (HA) and beta-tricalcium phosphate (β-TCP). Several HA/(β-TCP) mixtures have been tested as graft materials with favorable clinical results (2,8,10,11). Studies suggest that each alloplastic material has a different resorption rate, and they can be used alone or associated with autogenous bone without altering the osteoconductive properties (1,12).

The bone deposition on the surface of different types of biomaterials has been widely studied. Cordaro et al. (9) found no difference in new-formed bone with deproteinized bovine bone (DBB) graft and β-TCP in maxillary sinus lift procedures (7,9,13). Recent results of

our group demonstrated that biphasic calcium phosphate (BCP) associated with autogenous bone resulted in a greater amount of new-formed bone than DBB in initial evaluations (12). In fact, each biomaterial has peculiarities regarding characteristics and biological responses (14). In addition, the experimental model has a relevant influence on these responses (15). In this context, the evaluation of DBB and BCP in experimental models with larger animals such as rabbits is interesting and allows the creation of critical-size defects in the same animal. Regarding bone metabolism, aspects such as bone composition and healing process describe the rabbit as a more suitable model (16,17). Therefore, the encouraging findings of BCP in early bone repair associated with autogenous bone (12) led us to investigate if BCP would have similar outcomes in critical-size bone defects by itself (not associated with autogenous bone). Thus, the primary objective of this study was to evaluate qualitatively the repair process of critical bone defects in rabbit calvaria using biphasic calcium phosphate and deproteinized bovine bone. Secondary objectives were to quantitatively analyze soft and mineralized tissues after 15 and 45 days of grafting procedure using DBB and BCP.

Material and Methods

A split-body experimental study was conducted using sixteen female adults New Zealand rabbits (*Oryctolagus cuniculus*), with mean age of 4 months, weighing approximately 3 kg. During the experimental period, the animals were kept with food and water ad libitum, at 20 °C and with 8 to 10 h of light exposure daily. Animals underwent a 12-hour fast prior to the surgical procedure. Animals were purchased from the Central Bioterium from Pontifical Catholic University and were quarantined in pens for 14 days prior to surgical procedures for ambiance and adaptation to the surroundings.

The experimental protocol was in accordance with the World Society for the Protection of Animals, with approval by the animal ethics committee of the institution (protocol 309/2015). During the experimental study 4 animals were lost, 2 during anesthesia and 2 in post-operative period.

The animals were allocated into three groups, according to the biomaterial used: DBB, BCP, and sham. An assistant research performed the randomization using a table of random numbers assigning each experimental unit (defect) to a treatment. In the DBB group, the defect was filled with deproteinized bovine bone (Bio-Oss®, Geistlich Pharma AG, Wolhusen, Switzerland). In the BCP group, the defect was filled by BCP alloplastic material (Maxresorb® (60% HA / 40% β-TCP) (Botiss Dental GmbH, Berlin, Germany). In groups BCP and DBB, the biomaterial was inserted until the entire defect was filled. Whereas in the sham group, no material was added, and the defect was filled with blood clot only. The groups were evaluated at 15- and 45-days post-intervention. Five experimental units were used from each group at each experimental time (n=5). Assuming an average standard deviation of 5%, a two-sided test, the sample size (n=5) would provide a 90% power.

Surgical Procedures

The rabbits were sedated by isoflurane inhalation and anesthetized with 10% ketamine hydrochloride (50 mg/kg) associated with 2% xylazine hydrochloride (5 mg/kg) by intramuscular injection.

After anesthesia, depilatory cream was used for fur removal of the frontoparietal region followed by vigorous asepsis using povidone-iodine. To assure trans-operative hemostasis and post-operative comfort for the animal, 0.9 mL of 2% mepivacaine with 1:200,000 norepinephrine was infiltrated in the calvaria. With a No. 10 scalpel blade, a longitudinal mucoperiosteal straight incision was performed following the sagittal suture. With the aid of a periosteal elevator, full-thickness flaps were detached, completely exposing the cortical bone of the parietal region (Fig. 1A). Two bone defects were performed on the parietal bones of all rabbits, laterally to the sagittal suture, with a surgical motor and a 16:1 reduction contra angle using an 8-mm diameter surgical drill (Neodent®, Curitiba, Brazil) (Fig. 1B) under abundant and continuous irrigation with saline and

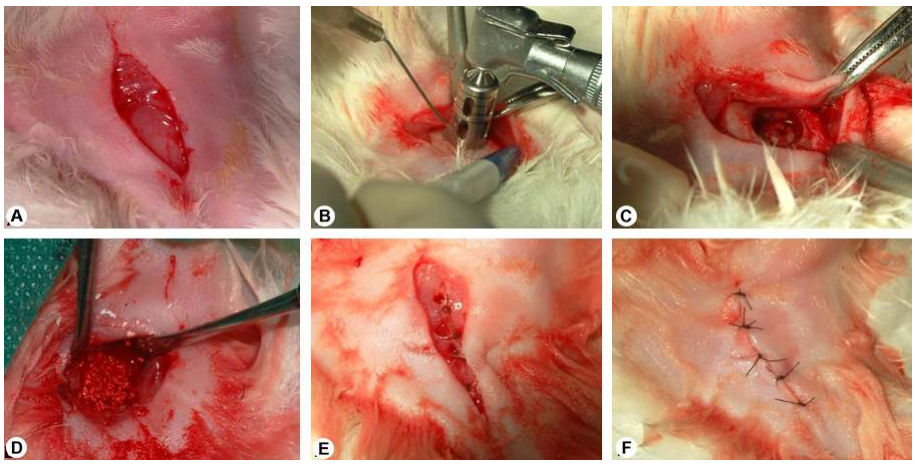

Figure 1. Schematic representation of experimental surgical procedures. The anteroposterior incision in the skullcap (A). Use of an 8mm trephine drill in the parietal bone to obtain the critical defect (B, C, and D). Suture of the periosteum (E) and suture of aponeurotic galea fascia (F).

aspiration. The external and internal cortical bones were removed with an Ochsenbein no. 1 micro chisel, and the dura mater was kept intact with subsequent irrigation of the cavity with saline (Fig.1C). Then, the bone defects were completely filled with one of the biomaterials or blood clot (Fig. 1D). The periosteum, fascia, and aponeurosis wounds were closed with vicryl 4.0 (Ethicon, Johnson & Johnson, São Paulo, SP, Brazil) (Fig. 1E), and the skin wound with 3-0 nylon suture (Ethicon, Johnson & Johnson, São Paulo, SP, Brazil) (Fig. 1F).

During anesthetic induction, animals received long-acting tetracycline (1mg/kg) intramuscularly. For post-anesthetic recovery, the animals received 50 mL of glycated serum, 0.1 mg/kg of morphine for analgesia, and 1 mg/kg ketoprofen intramuscularly every 24 h for 3 days. All surgical procedures were performed by the same operator (oral surgeon) and the same assistant (oral surgeon).

During the entire postoperative period, the animals were kept in pens provided with food and water ad libitum, and evaluated daily for surgical wound condition and general health. Animals were housed with a maximum of 4 rabbits per pen before and after surgical procedures. Pre, trans and post-operative practices were developed under the supervision of a veterinary surgeon. During the immediate post-operative period, animals remained under supervision and returned to their pen when showed relatively normal physiological characteristics. Animals were carefully evaluated during the first 24 post-operative hours and daily until euthanasia according to each experimental period (15 and 45 days). Normal eating patterns and other physiological characteristics monitored.

After 15 and 45 days, the animals were euthanized with 1g of sodium thiopental anesthetic (2mL) intravenously. The skulls were dissected, divided in half with a diamond disc, and stored in 10% formaldehyde for 48 h, and subsequently subjected to decalcification in 10% EDTA solution (pH 7.4) for approximately 12 weeks. Then, gradual dehydration in ethanol was carried out, followed by xylene washing and paraffin embedding. Semi-serial sections 4- to 6-µm thick with a 10 µm interval were obtained and stained with hematoxylin and eosin. In all sections, a random numerical sequence was assigned to codify the experimental periods and groups throughout the analysis and reduce bias. Histological evaluations were performed by a calibrated examiner.

Histological and Histomorphometric Analysis

The histological analysis was subjective and qualitative in order to evaluate the histopathological features in the soft and mineralized tissues. Images were captured with a camera coupled to a microscope (SDC-310 Samsung, Korea), and histomorphometric analysis was performed using ImageJ® software (Version 1.5a Wayne Rasband, Bethesda, MA, USA). Initially, total defect area was measured, followed by mature and immature bone area, biomaterial particles area, and soft tissue area. The vitality of the mineralized tissue was assessed by the presence of osteocytes in the bone gaps.

In the sequence, the soft tissue was evaluated by the presence of collagen fibers, fibroblasts, blood vessels, inflammatory cells, osteoclasts, and blank spaces, which are possibly occupied by interstitial fluid and edema. In this evaluation, the plug-in "Grid" was used for the insertion of 80 equidistant points, which were counted using the plug-in "point tool". These results represented volumetric density of each parameter evaluated.

Statistical Analysis

Two-way ANOVA was employed to analyze histological data of all groups, followed by one-way ANOVA and Tukey's test to determine significant differences within the same group related to time. Significance level was set at $p<0.05$. Data was analyzed using GraphPad Prism 7.0 (Graphpad Software Inc., San Diego, CA, USA).

Results

After 15 days, sham group presented mineralized tissue only at the edges of the defects, and the entire extent of the defect was filled with soft tissue with areas of higher collagenization and the presence of blood vessels, within normal criteria. The same pattern was maintained at 45 days (Fig. 2). The defect of DBB and BCP animals was filled with biomaterial and soft tissue, with a slight presence of immature bone after 15 days. After 15 and 45 days, there was an increase in the amount of immature bone inside the defect in the BCP group compared to the DBB group. The presence of biomaterial was observed in the DBB and BCP groups in both experimental periods. In the DBB group, a significant reduction in the amount of biomaterial was observed after 45 days compared to 15 days. There was no significant difference in the amount of biomaterial between 15 and 45 days in the BCP group ($p=0.9998$), as illustrated in figures 3 and 4. The soft tissue of the DBB and BCP groups (15 and 45 days) presented well-organized fibrous connective tissue with deposition of collagen fibers in specific areas. The normal presence of blood vessels was also observed in all evaluated groups. Necrotic areas in soft or mineralized tissue, multinucleated giant cells, and foreign body reactions were not observed in any group. The percentages of bone tissue, soft tissue, and biomaterial obtained through histomorphometry are described in Figure 5.

The soft tissue inside the defect showed an increase in collagen fibers in the sham group after 45 days, compared to

15 days ($p<0.01$). After 45 days, DBB showed a reduction in collagen fibers in relation to the 15-day evaluation ($p<0.05$), while no variation was observed in BCP between 15 and 45 days ($p>0.05$) (Fig. 6). After 15 days, BCP showed an increase of fibroblasts in relation to sham and DBB ($p<0.05$). There was no difference in fibroblast percentage between DBB and BCP after 45 days ($p=0.9999$) (Fig. 6). An increase in blood vessels was observed in DBB compared to sham in all experimental periods ($p<0.05$) (Fig. 6). The sham group had higher inflammatory cell counts compared to DBB at 15 days ($p<0.001$). However, after 45 days, DBB inflammatory cell counts increased in relation to sham ($p<0.001$). There was no difference in inflammatory cell counts between BCP and DBB at 45 days ($p=0.5915$). A higher number of osteoclasts was found in BCP at 15 days when compared to the sham 15 ($p<0.01$), and the sham group presented small amounts of osteoclasts after 15 and 45 days (Fig. 6). Blank spaces, which are usually filled by the interstitial fluid and edema, were observed in greater quantity in the DBB when compared to sham and BCP at 45 days ($p<0.05$). All results regarding to histomorphometric evaluation are presented in figures 5 and 6.

Discussion

Several studies have evaluated bone deposition and tissue repair using biomaterials in different types of defects, both in humans (9,13,18,19) and in experimental models (6,10,12,20). The results of these previous studies indicate that the use of xenogenous and alloplastic biomaterials is preferred because of the reduced morbidity of grafting procedures in relation to autogenous bone. Despite extensive research, in the past 30 years, it is still needed to study the use of biomaterials that meets all gold-standard requirements and desirable properties (14).

Several studies within in this area were carried out in humans, aiming at providing results with clinical applicability (7,9,13,18,19). However, experimental models allow evaluations at shorter times, the control of external factors, and the selection of different experimental periods. For such reasons, the model used in the present study has been widely applied (6,10,21). The type of defect performed in rabbits' calvaria allows an adequate interface with the biomaterial and is similar to the maxillary bone in terms of embryological origin and morphology (22). Moreover, rabbits are larger than mice or rats and their bone regeneration process is more similar to that of humans (16,17,23).

In the present study, an increase in bone deposition in DBB and BCP was observed at 15 and 45 days in relation to sham, confirming the osteoconductive ability of both

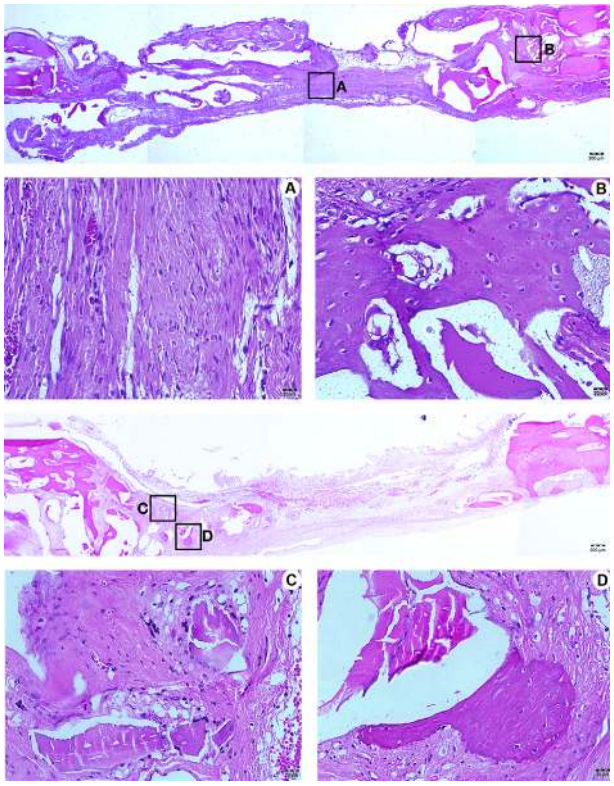

Figure 2. Bone deposition pattern of groups without the use of biomaterials (SHAM). Animals were evaluated for histological changes at 15 (A, B) and 45 (C, D) days after grafting procedures.

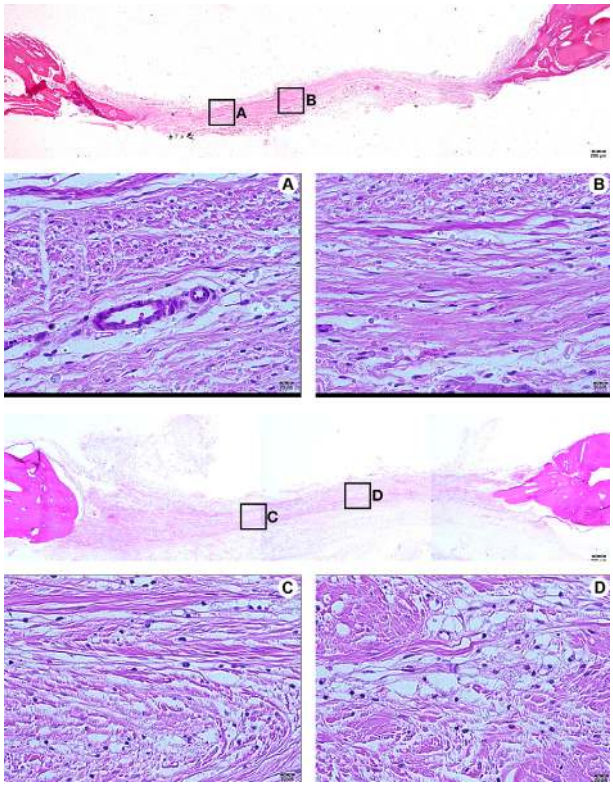

Figure 3. Bone deposition pattern of groups grafted with deproteinized bovine bone (DBB). Animals were evaluated for histological changes at 15 (A, B) and 45 (C, D) days after grafting procedures.

biomaterials. The sham animals showed no areas of mineralized tissue inside the defect. Bone deposition in the sham group probably occurred at the edges of the defect, considering that a critical-size defect was performed. Moreover, DBB and BCP had areas of mineralized tissue along with the defect, closely to biomaterial particles. BCP is osteoinductive and able to stimulate the new formation of bone suggesting that it may be an alternative to the treatment of large bone defect (24).

Different types of biomaterials promote different bone deposition. In our study, BCP resulted in higher bone deposition rates compared to DBB at 45 days, which corroborates the results obtained by other authors (6,10,12,25). However, similar amounts of newly formed bone with the same histological characteristics have also been reported for DBB and BCP (9,20).

Perhaps, the higher bone deposition observed in the BCP group is related to the role of ceramics in osteoinduction (21,24,26), as ectopic bone formation has been observed in muscle and new bone has been found in bone sites without any contribution from osteoconduction (1). Thus, it is possible that endogenous proteins such as BMPs are absorbed and get concentrated on the ceramic surface, contributing to the attraction of osteogenic cells. In addition, the topography of ceramics and the release of inorganic ions from ceramics could also trigger the process of osteogenic differentiation and bone formation (26). Previous authors observed a significantly greater amount of new-formed bone using BCP at 4 and 8 weeks when compared with other biomaterials, including DBB (10). Thus, our results together with previous ones, confirm the applicability of DBB and BCP as possible alternatives

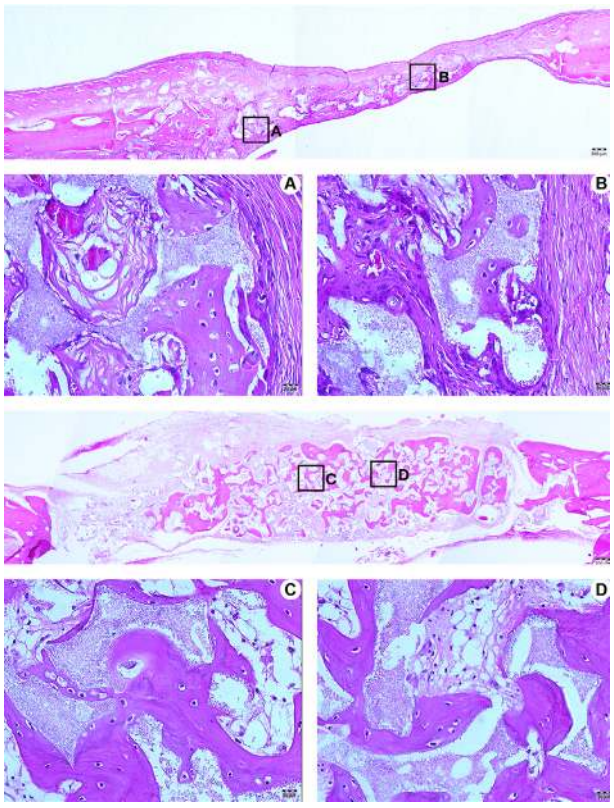

Figure 4. Bone deposition pattern of groups grafted with biphasic calcium phosphate (BCP). Animals were evaluated for histological changes at 15 (A, B) and 45 (C, D) days after grafting procedures.

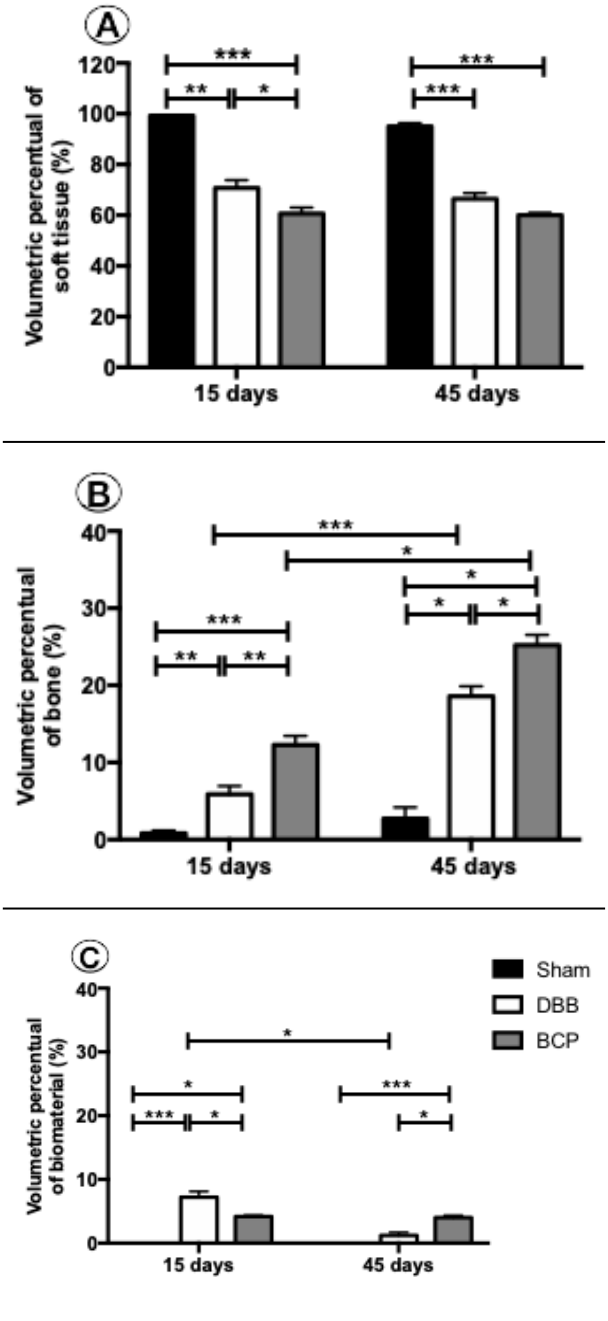

Figure 5 Histological evaluation of soft tissue (A), immature bone (B) and biomaterial (C) for Sham, DBB, and BCP groups after 15 and 45 days. * indicate p<0.05; ** indicate p<0.01; *** indicate p<0.001 (Twoway analysis of variance, followed by t-test).

to autogenous bone grafting (25,27). Otherwise, such materials could be used in association with autogenous bone grafts (12), minimizing surgical morbidity when greater amounts of bone are needed.

The amount of biomaterial particles present in the defect was similar between 15 and 45 days for BCP and significantly reduced in DBB. However, both groups had biomaterial remnants after 45 days. Such finding is in accordance with previous studies, where no sign of DBB resorption was detected after 30 days (27,28) and DBB graft materials were not resorbed completely after 180 days (27). In fact, some authors have considered DBB a non-resorbable graft because it needs three to six years to resorb (28). Moreover, BCP reabsorption usually occurs earlier than DBB since β-TCP particles have a high resorption rate (12,21). However, some authors have not observed significant differences between DBB and BCP reabsorption after 180 days (27).

Bone formation did not occur in the defect of sham animals, while in the BCP and DBB animals bone deposition occurred in a centripetal manner, from the edges of the defect and throughout the defect, in close contact with the surface of the biomaterial. Our results corroborate those of other authors, in which BCP and DBB promoted similar bone formation (9,10,25).

Histological analysis showed a greater amount of

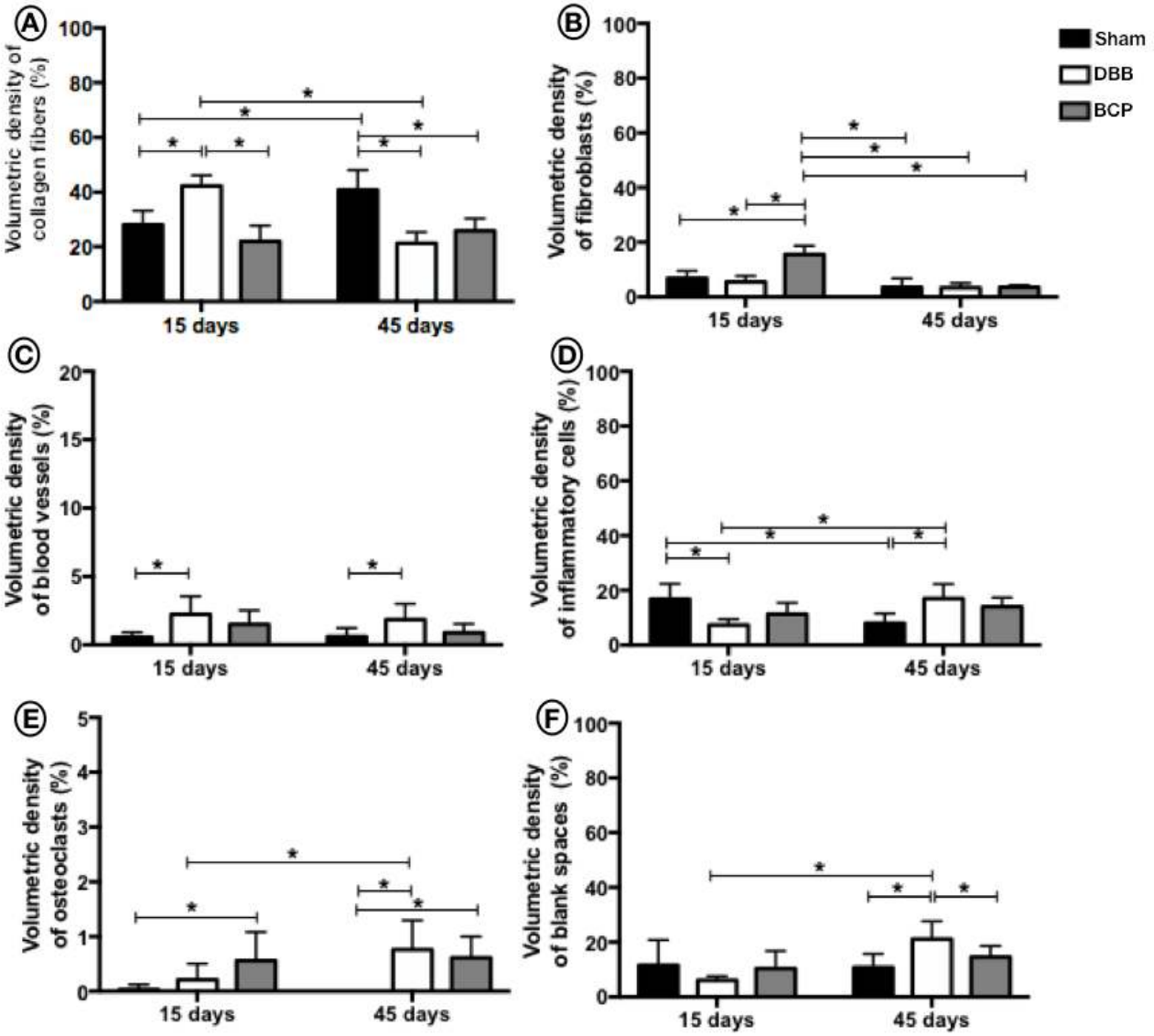

Figure 6: Volumetric density of collagen fibers (A), fibroblasts (B), blood vessels (C), inflammatory cells (D), osteoclasts (E), and blank spaces (F) in the grafted area of Sham, DBB, and BCP groups after 15 and 45 days. * indicate $p<0.05$; ** indicate $p<0.01$; *** indicate $p<0.001$ (Two-way analysis of variance, followed by t-test).

collagen fibers in the soft tissue present throughout the defect of the sham group after 15 and 45 days when compared to DBB and BCP. This finding is probably related to the absence of new-formed bone in those animals. On the other hand, the new-formed bone in the groups that received the biomaterials is related to the greater presence of osteoclasts compared to the sham groups. In addition, a higher number of blood vessels, inflammatory cells, and blank spaces was observed in the DBB. Some authors also demonstrated the presence of residual material, a large quantity of macrophages, presence of newly formed bone, blood vessels, and small amounts of collagen fibers (29) in groups treated with biomaterials. In addition, it has been demonstrated that particles of BCP used in bone repair are surrounded by connective tissue, osteoid islands, and new-formed bone (11).

The data obtained in this study emphasize the importance of using biomaterials to optimize tissue repair, since DBB and BCP showed more favorable results than the control animals. Despite the differences observed between DBB and BCP, both biomaterials can be used as alternatives to autogenous bone graft (7,25,27). Moreover, no difference was found in marginal bone of areas treated with DBB and BCP that received osseointegrated implants, neither implant stability nor differences in the survival rate (4,7). The BCP treated defects showed greater bone deposition and a greater amount of biomaterial residual in the bone defect. Previous studies have reported more active bone deposition and better remodeling of bone defects that were grafted with BCP when compared to DBB (25).

The use of an animal model is a limitation of this study and the extrapolation of these data to clinical settings must be done with caution. Further studies with other methodologies such as microtomography and molecular evaluations should be carried out to optimize treatment strategies for the rehabilitation of patients in need of bone grafting procedures. In conclusion, both biomaterials demonstrated to be suitable to critical defect grafting, however BCP resulted in greater bone deposition especially an increase in the amount of immature bone in earlier analyses.

Resumo

As opções limitadas para reparo ósseo levaram ao desenvolvimento de abrangente pesquisa na área de enxertos aloplásticos e xenogênicos. O objetivo deste estudo foi avaliar o reparo ósseo com dois substitutos ósseos: osso bovino desproteinizado (DBB) e cerâmica fosfática de cálcio bifásica (BCP) em defeito de tamanho crítico. Material e métodos: defeitos críticos de 8 mm foram feitos nos ossos parietais de coelhos (n=12). Os animais foram divididos em três grupos experimentais: sham (defeito preenchido com coágulo sanguíneo), DBB (defeito preenchido com DBB) e BCP (defeito preenchido com BCP). Após os períodos experimentais de 15 e 45 dias, os animais foram sacrificados e submetidos à análise histomorfométrica. Foram avaliadas a área total de defeitos, área de tecidos mineralizados, área de biomateriais e área de tecidos moles. Resultados: maior quantidade de tecido ósseo imaturo e de partículas de biomaterial foram observados no grupo BCP em comparação aos grupos DBB e sham aos 45 dias ($p<0,05$). Não houve diferença no padrão qualitativo de deposição óssea entre DBB e BCP. Ainda, o grupo sham não apresentou ilhas osteóides ao longo do defeito, apresentando maior quantidade de fibras colágenas em relação aos grupos DBB e BCP. Houve maior quantidade de células inflamatórias no DBB aos 45 dias em comparação aos grupos BCP e sham. Conclusões: BCP e DBB são opções para otimizar o uso de enxertos ósseos na reabilitação de pacientes. Defeitos ósseos tratados com BCP mostraram maior deposição de tecido ósseo aos 45 dias.

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