Journal of the International Academy of Periodontology (2023) **25/1**: 31-44

DOI: https://doi.org/10.14436/1466-2094.25.1.031-044.oar

Influence of mineralized and demineralized xenograft bone on the repair of intraoral bone defect in rats

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Abstract

Introduction: Mineralized bovine bone grafts have been widely used as substitutes for bone losses in dental clinics. Studies have shown that exposing the organic components of the bone matrix at surgical sites accelerates bone deposition.

Objective: The present study evaluated the bone repair progress of an intraoral bone defect in rats after grafting with mineralized (MBB) and demineralized bovine bone (DBB).

Materials and Methods: An intraoral bone defect was created after extraction of the right maxillary first molar, drilling the area of the alveoli of the four distal roots using a diamond tip. The defect was filled with the MBB or DBB graft. Grafting effects were evaluated after 1, 7, 14, 21 and 49 days, using radiographic and histological data.

Results: After 14 days, all groups showed full mucosa epithelialization at the surgical site. Radiographic data showed an improvement in bone deposition in defects grafted with the organic matrix. This data was confirmed by the histological analysis. A higher level of bone maturation of the neoformed trabeculae and a faster reabsorption rate were also observed to be a feature of the DBB graft.

Conclusion: The present in vivo data revealed that the DBB graft may represent an alternative to mineralized biomaterials.

Keywords: Xenograft. Bone repair. Demineralized bone matrix. Intraoral bone defect.

Introduction

Extensive bone loss caused by fractures, tumor excision and metabolic or degenerative diseases usually require additional therapeutic resources for the restoration of the bone function (Watanabe *et al*., 2016). In dentistry, in the case of absence or deficiency of a support, bone grafts have been indicated as substitutes or as transient filling biomaterials (Shirmohammadi *et al*., 2014). In the latter case, grafts tend to be partially or totally reabsorbed over time, while they are replaced by neoformed bone tissue (Oryan *et al*., 2014).

Bone grafts have different origins and presentations. The xenografts, obtained from animal sources such as porcine-derived and bovine-derived (Yaghobee *et al*., 2018), are commercially available as fragmented (bone particulates) or as three-dimensional blocks, similar to the natural bone structure, with its porous and trabecular structure (Dasmah *et al*., 2012).

Properties such as osteoconduction and osteoinduction should be considered as criteria for the selection of biomaterials used in bone grafts (Palachur *et al*., 2014; Jo *et al*., 2018). Furthermore, the osseointegration of biomaterials to the remaining bone walls can provide stability to dental implants, which is a basic condition that supports all the functional requirements of the tissue (Oryan *et al*., 2014; Lei *et al*., 2015).

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Bovine-derived biomaterials used in bone grafting are mostly osteoconductive, whereas the autogenous grafts are recognized as osteoinductive. However, autogenous fragments are obtained from surgical procedures and the amount of tissue that can be harvested from the donor area can result in increased morbidity and does not always meet the requirements to increase bone loss recovery. These limitations have encouraged the development of research seeking alternative materials or improvements to grafts already available in a clinical setting for bone replacement therapies, which are available as mineralized blocks or granulates (Khoshzaban *et al*., 2011; Bahammam, 2016; de Assis Gonzaga *et al*., 2017; Thorwarth *et al*., 2006). They have been shown to have suitable biocompatibility, osseointegration, mechanical resistance, and also present a good bone deposition inductor potential (Palachur *et al*., 2014; Berglundh *et al*., 1997; Carmagnola *et al*., 2003; Fickl *et al*., 2008; Pichotano *et al*., 2019; Naros *et al*., 2019).

Although most commercial grafts are available as mineralized fragments, new research has now revealed the positive influences of the organic components of the bone matrix in the process of osteogenesis during bone repair. Different growth and differentiation factors are among these organic components, such as transforming growth factor–beta (TGF-β), insulin-like growth factors I and II, platelet derived growth factor (PDGF), fibroblast growth factor and bone morphogenetic proteins, BMPs (Palachur *et al*., 2014; Drosos *et al*., 2015; Fernandez de Grado *et al*., 2018). In this way, the exposure of the organic matrix present in bone grafts may represent a strategy for optimizing bone repair at recipient sites. Therefore, the chemical treatment used to expose the organic phase of the biomaterials should maintain the integrity of the components associated with the osteogenic and osteoinductive properties. EDTA (ethylenediamine tetraacetic acid) with neutral pH can be an alternative to minimize damage to the bone structure during the demineralization process. According to Hülsmann *et al*. (2003), EDTA is an organic compound that acts as a chelating agent, forming very stable complexes with several metallic ions (such as calcium), removing them from the tissue with minimal histological changes.

Thus, the aim of the present study was to evaluate the *in-vivo* influence of mineralized and demineralized xenografts during the bone repair of a jaw defect in a rat. The morphometric results (macroscopic, radiographic and histological analysis) reiterated the main hypothesis that the organic bone matrices exert an osteoinductive influence in the grafted sites.

Methods

Biomaterials

Bovine bone (LuminaBone® - Critéria, Brazil) was used as biomaterial in this study, in both mineralized and demineralized forms.

Mineralized bovine bone (MBB) was used in granules of approximately 3 mm. Demineralized bovine bone (DBB) was obtained by immersion of the mineralized blocks in 10% EDTA for 72 hours.

Bio-Oss® granulated bone (Geistlich, Switzerland) was used as a positive control exclusively in its mineralized version, for comparisons with MBB, due to its history of good results in the literature and in clinical practice.

Validation of the demineralization protocol – organic matrix preservation analysis after EDTA treatment

A stereomicroscope (EZ4D – Leica; and LAS EZ 2.1.0 software) with 35x magnification was used to assess the maintenance of the three-dimensional structure of the fragments selected for grafting. This analysis considered the trabeculae and pores preservation of biomaterials after the EDTA demineralization protocol. The preservation of bone matrix content was assessed by immunohistochemistry and immunofluorescence analysis.

DBB was fixed in neutral buffered formalin and embedded in paraffin. Sections of 5μm were stained with hematoxylin and eosin (H&E) according to routine histology. The presence of BMP4 was confirmed by immunohistochemistry and osteopontin and collagen type I, by immunofluorescence. For this, 5-μm sections were deparaffinized in xylol, rehydrated in a gradual series of ethanol and washed in PBS. For immunohistochemistry, the endogenous peroxidase was blocked by hydrogen peroxide. Non-specific binding sites were blocked with 2% BSA in Tris-HCl (pH 7.4) for 1 h for both techniques. Sections were incubated overnight at 4°C with anti-BMP4 (Santa Cruz Biotechnology anti-mouse), anti-osteopontin (Abcam anti-mouse) and anti-collagen type I (Abcam anti-rabbit), all polyclonal primary antibodies, and diluted 1:200 in PBS. After washing with PBS, sections were incubated in room temperature for 1 h with the specific secondary antibody for immunofluorescence (488 goat anti-rabbit and 488 goat anti-mouse, Alexa Fluor-Molecular Probes), diluted 1:700 in 2% BSA in PBS. For immunohistochemistry, the secondary antibody used was EnVision Dual Link System-HRP (DAKO). The DAB precipitation showed the presence of BMP4 protein. Hematoxylin was used to counterstain. In both techniques, the sections were washed with PBS and mounted with 80% glycerol. Images were captured using the Olympus BX-50 microscope (Olympus, Hamburg, Germany). Negative controls were performed by the omission of the primary antibodies.

The demineralization protocol was not used in the Bio-Oss biomaterial because it has 100% of its inorganic composition, without having proteins and compounds of the organic matrix.

Animals, surgical procedure and experimental groups

In this study, 120 adult male Wistar rats (Rattus norvegicus; 3-4 month old) with body weight < 280 g were used. The management of the animals and surgery protocols were previously approved by the Commission of Ethics in the Use of Animals of the Universidade Federal de Minas Gerais (Protocol 7/2015).

For the surgical procedures, the animals were anesthetized with intramuscular injection of 2% xylazine hydrochloride associated with 10% ketamine hydrochloride, both at 0.1ml/100g. The bone defect was created by the extraction of the right maxillary first molar. The remaining area of the alveoli of the four distal roots was drilled under irrigation using a cylindrical diamond tip KGS-2094, coupled to the dental micromotor (Driller). The osteotomy generated a bone defect with approximate dimensions of 2.5 mm in diameter, and 2.5 mm in depth in all animals.

The bone defects were filled as follows: Group I (MBB), filled with mineralized bovine bone in granules (LuminaBone®); Group II (DBB), filled with demineralized bovine bone fragments (LuminaBone®); Group III (BO), filled with Bio-Oss® granulated bone (Geistlich, Switzerland); and Group IV (NC), filled with blood clot. Bio-Oss® particulated bone (Geistlich, Switzerland) was used in its commercial version (mineralized granulate). As it is widely studied in the literature, Bio-Oss® was used in this study as a positive control for comparisons between the MBB group. Blood clot was used as a negative control for comparisons with the MBB and DBB groups. All biomaterials were transplanted until the volume of the defect was completely filled. After filling, the mucosa on each defect was repositioned and sutured. Animals were kept under heating until recovery from anesthesia, and Tramal (4mg/kg) and antibiotic oxytetracycline (Terramycin Injection Solution, 10mg/kg) were administered during the post-surgical stage every 24 h. Animals were kept on a paste diet for 5 days. Surgical stitches were removed on the 7th postoperative day. The animals of the four groups were euthanized at 1, 7, 14, 21 and 49 days after the surgical procedure (n= 6 animals per period, 30 animals in total for each group).

Macroscopic analysis

The effects of each biomaterial on wound closure (on gingival healing) were evaluated using macroscopic photographs obtained with macro lenses and a Panasonic DMC- TZ3, Lumix photographic camera. Mucosal epithelization level was measured by morphometric analysis of the granulation tissue area, using Adobe Photoshop. For this, the selected area was obtained in standardized macrophotographs and positioned under a grid with 117 equally distributed points (Figure 1). The points on tissue granulation were counted and converted into percentages, related to the total number of points (total area selected). High numbers of spots on tissue granulation indicated a low level of gingival healing, and vice-versa.

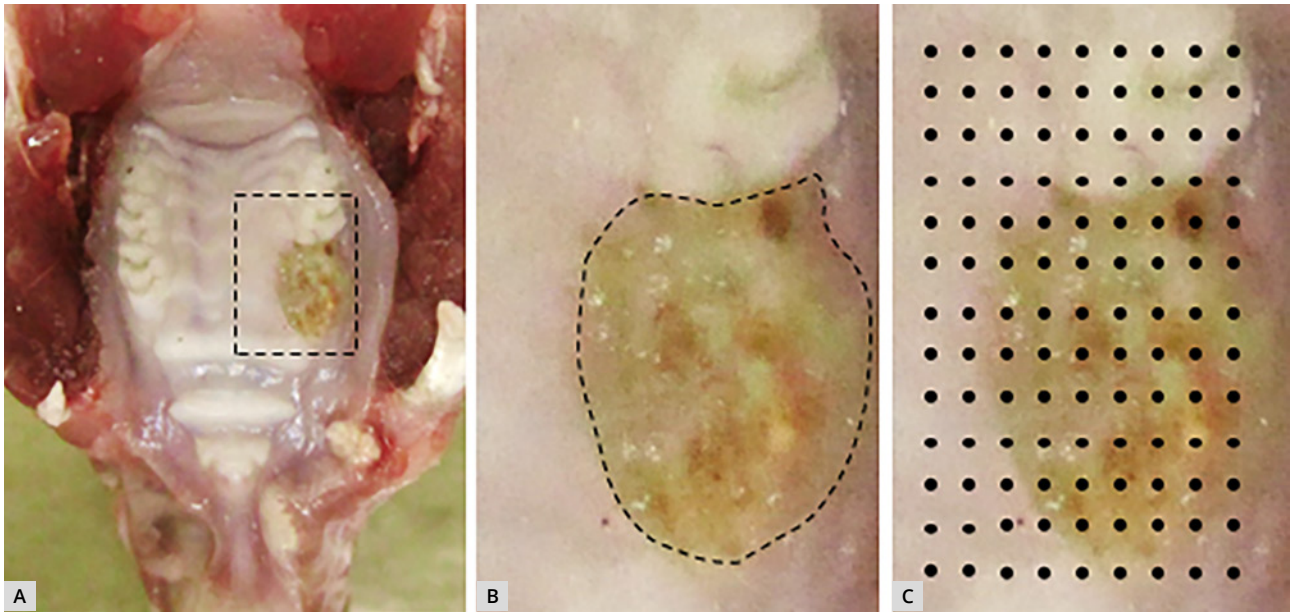

Figure 1. Gingival healing granulation analysis. (A) Standardized macrophotography. The black grid region represents the evaluation area for gingival healing, depicted in B. The black dotted line in B delimits the tissue granulation. (C) Equally distributed points over the evaluated area. The points on the tissue granulation were converted into percentages in relation to the total number of points in the area under consideration.

The area selected for this methodology took into account that the extraction procedures, creation of the defect and access to the surgical site, regardless of the type of biomaterial used, require manipulation and mechanical removal of the surrounding soft tissues. Therefore, the mucosal evaluation area extends beyond the access area to the bone defect. Anatomical structures such as the palatine raphe, in addition to the last (most posterior) fold of mucosa that covers the anterior palate and the limits of the crown of the second molar, were taken as references for the delimitation of the dotted area and analysis of the same region in all animals.

Radiopacity index evaluation

For radiographic evaluation, the right hemi-jaws containing the defects were fixed in neutral buffered formalin for 72 h, and then transferred to 70% ethanol. Pieces were placed on the Durr Dental 3x4 match plates (Bietigheim, Bissingen, Germany), always in the same position. The Gendex 756DC (Pennsylvania, USA) radiographic device was used with an exposure time of 0.125 dm/s, 65 kV and 7 mA, and fixed focus/film distance of 10cm. Plates were then scanned by the Durr Dental VistaScanPerio Plus processor. Using the Adobe Photoshop CS5 software, three points of 5x5 pixels each were defined within the defect area. These points, called regions of interest (ROI), were positioned on the digital radiographs: (I) at the apical, (II) medial and (III) cervical regions of the bone defect, at 1-mm distance from the mesial root of the upper second molar. The radiopacity index was calculated as the mean of the gray tones recorded in each of the three points for each animal. Radiopacity index obtained 24 h after the surgical procedure and graft filling was used as reference of the natural radiopacity of the biomaterials, and discounted as background.

Histomorphometric analysis

Bone repair evolution was evaluated in histological sections of the hemi-jaws at 7, 14, 21 and 49 days after the surgical procedure and graft filling. Histological sections allowed evaluating aspects of bone maturation and graft osseointegration and reabsorption over time. For this, jaws were fixed in neutral buffered formalin for 72 h and demineralized in 10% EDTA, pH 7.2. After washing in water, samples were dehydrated in increasing baths of ethyl alcohol, diaphonized in three xylol baths and embedded in paraffin. Sections of 5µm obtained in the frontal plane were stained with Masson's Trichrome and H&E, for morphometric analysis; and with Picrosirius Red, for analysis of collagen fiber maturation by polarized microscopy. In order to facilitate the description and interpretation of the results, the bone defect area, which is delineated by the lateral walls —vestibular and palatine— and the bottom wall, was virtually divided into three equal parts: the apical third, middle third and cervical third (Figure 2).

Bone deposition was determined by histomorphometric analysis performed in Masson's Trichrome stained sections. Images were captured under Olympus BX-50 light microscope, coupled to Q-color 3 camera and evaluated using the ImageJ software. Morphometry data was expressed as the mean of three sections, taken at different points of the bone defect. The percentage of the area occupied by bone neoformation, identified by the deposition of trabeculae stained by Masson's Trichrome, was determined using the mean of three measurements obtained in the three different depths, trained in a blind analysis.

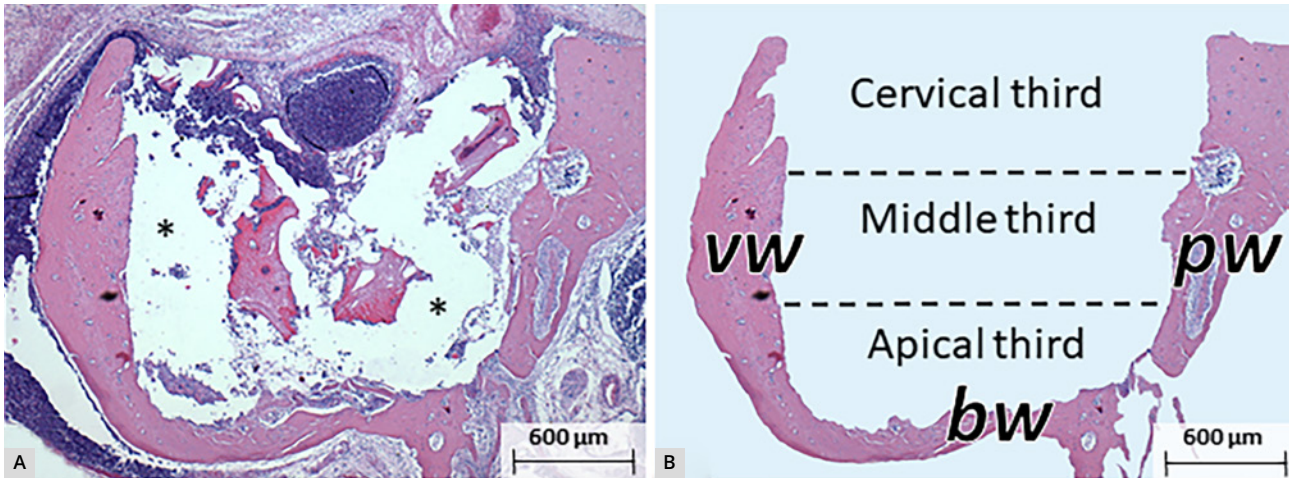

Figure 2. Schematic representation of the bone defect and the method used to the histomorphometric analysis. In (A), an original histological image of a section in the area of the bone defect. Blank areas (*) represent the area filled with the biomaterial. In (B), a representative image of the bone defect, showing the presence of the remaining bone walls (vw = vestibular wall, pw = palatal wall; bw = bottom wall/floor of the bone cavity).

Bone maturation analysis

The maturation of the neoformed bone trabeculae in the defect was determined in sections stained with Picrosirius Red. We used 5-μm histological sections selected at three different depths of serial cuts of the bone defect. Briefly, sections were dewaxed in two xylol baths (20 min each), hydrated with three baths of decreasing series of alcohols (100%, 90% and 70% for 5 min each), and immersed in water for another 5 min. Sections were stained with Picrosirius Red for 45 min in an oven at 60°C, and submerged in acidic solution (hydrochloric acid 0,01N) for 5 min. Sections were subsequently stained with Harris Hematoxylin for 5 min, washed for an additional 5 min in running water and dehydrated in an increasing sequence of alcohol for one minute in each solution (90%, 100% and 100%). Finally, the slides were mounted using Entellan (Merck, Germany). In the microscopic evaluation, polarized light emission on the sirius red dye allows distinguishing the collagen quality by the birefringence and organization of its bundles. Collagen fibers from neoformed bone matrix (immature collagen) are thin, with poor green birefringence; while organized fibers appear in yellow and red (type I fibers). Red fibers represent the maximum maturation of the matrix. Images were obtained using the Olympus BX-50 polarized light microscope. Data was collected only on neoformed bone trabeculae and quantified using ImageJ software.

Biomaterials resorption evaluation

Biomaterial resorption rate was estimated by calculating the area occupied by the biomaterial on the 49th day, related to the area observed after 24 h of the grafting procedure, by histomorphometry of H&E stained sections.

Statistical analysis

All results were analyzed using PrismStatiscal software (Graphpad, San Diego, CA). Data was represented as mean standard deviation and statistically compared with confidence levels > 95% ($p<0.05$), using One-way ANOVA and Tukey's test. The direct comparison between two groups was performed by Student *t*-test.

Results

Effects of the demineralization protocol on the bone graft

The EDTA demineralization process shows the potential to preserve the three-dimensional bone structure of xenografts. Stereomicroscope image revealed trabeculae and interconnected pores similar to mineralized graft, demonstrating the preservation of bone structures (Figure 3A and 3B).

Three non-collagenous bone proteins (BMP4, Collagen type I and osteopontin) were evaluated for immunohistochemistry and immunofluorescence analysis as references for monitoring the preservation of bone structures and organic matrix. BMP4 and collagen type I showed a more diffuse expression pattern (Figures 4A and 4D, respectively); while osteopontin revealed a more localized expression at the edges of the trabeculae (Figure 4C).

Effects of biomaterials on mucosal healing

Gingival epithelialization at the surgical site was measured by the amount of granulation tissue visible over the operated area, after 7 and 14 days of grafting. It was not possible to observe any interference of the MBB, DBB and the positive control at the wound closure; the evolution of the process was similar to that

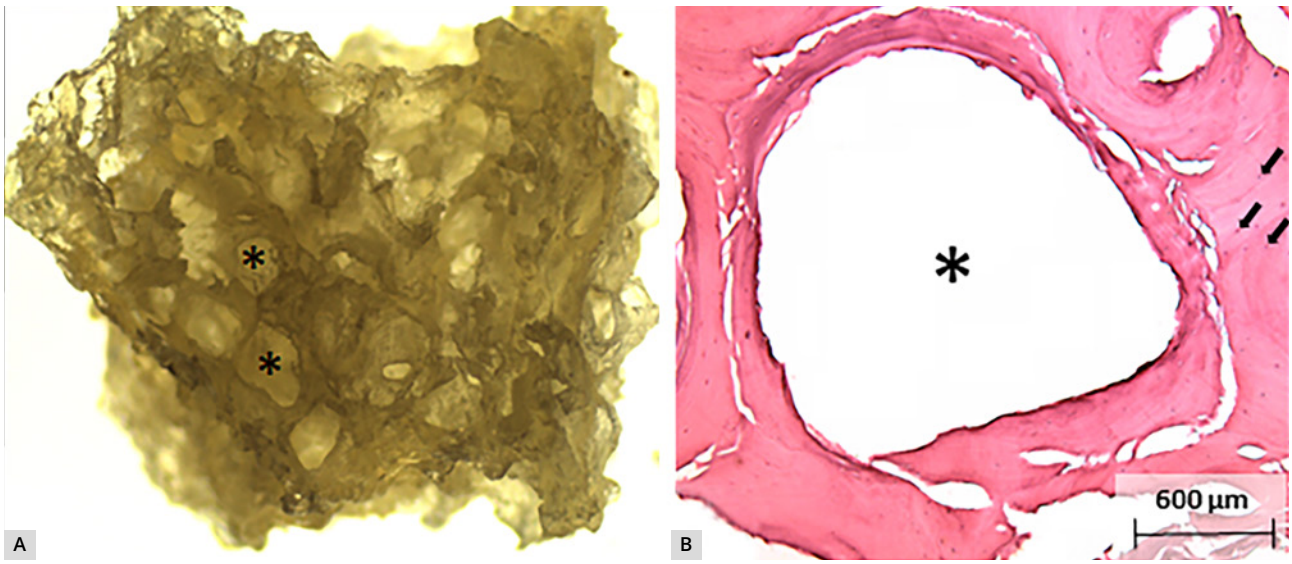

Figure 3. Aspect of the demineralized bovine bone in EDTA, by stereomicroscope image and histologial analysis. (A) Medullary space (*) surrounded by bony trabeculae; (B) histological image of the medullary space (*) shown in A. The black arrows indicate the presence of cellular debris (cell nuclei).

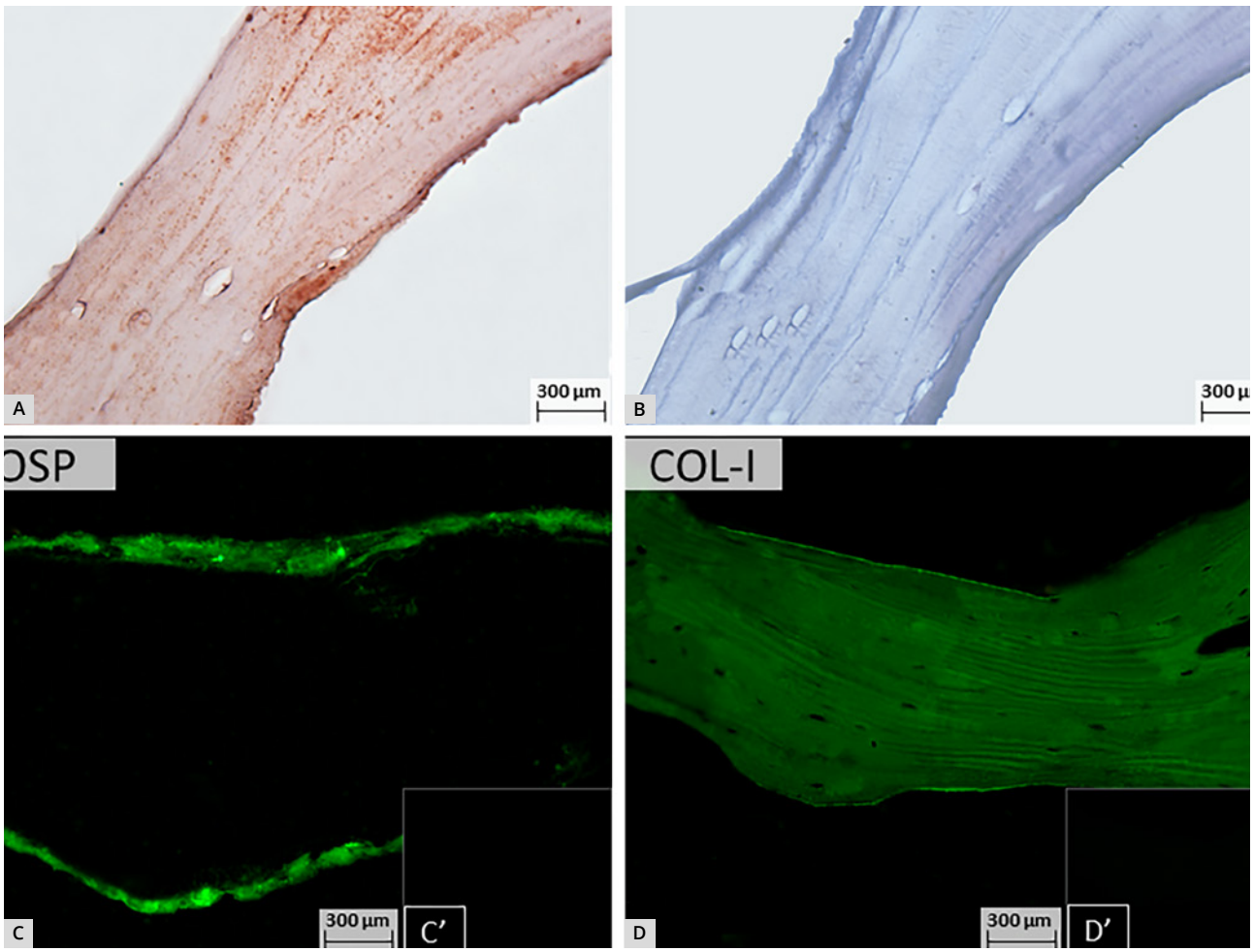

Figure 4. Bone proteins staining in the DBB organic matrix. In (A), BMP4 staining; in (C), osteopontin; and in (D), collagen type I. (B), (C′) and (D′) are the negative controls.

observed in the control group. After 7 days, the mucosa was under repair, with granulation tissue present in all groups. The DBB group showed a lower percentage of granulation tissue (31.2%), followed by MBB (34%); NC control (35.6%) and BO (41.2%) (Figure 5A-D, I). Although the DBB group presented the highest epithelial area and the BO group, the smallest one, it was possible to observe any significant difference between groups. All groups showed full mucosa epithelialization after 14 days of grafting (Figure 5E-H).

Radiographic analysis of bone deposition

The BO group showed the highest natural radiopacity after 1 day of grafting, followed by MBB, DBB and NC (Figure 6A). A decrease in the radiopacity index was observed after 7 and 14 days of MBB and DBB grafting and also in the NC group (Figure 6A); while no significant decrease was observed when the BO was used. After 49 days, the DBB graft showed the highest radiopacity index, compared to the others and to the background (obtained after 1 day; Figure 6A).

Histological analysis showed the presence of biomaterial granules after 49 days in both MBB and BO grafting (Figure 6B and C), resulting in a smaller area destined for new bone formation. On the 49th day, the bone defects filled with DBB showed 100% of biomaterial reabsorption (Figure 6D and E).

Histomorphometric analysis of bone deposition

Compared to the NC group (control), the DBB graft revealed a higher percentage of neoformed bone trabeculae after 14 and 21 days of grafting (Figure 7A). Comparisons between DBB and MBB groups showed higher bone deposition for the group filled with demineralized biomaterial after 14 and 49 days of grafting (Figure 7A). Compared to the BO group, the DBB group revealed a higher bone formation in the period of 49 days. In the 14 days after grafting, the BO group presented better results when compared to the negative control (Figure 7A).

The DBB group showed an ascending line in the bone deposition curve at 7 days after grafting (Figure 7B), suggesting an acceleration of the repair process. Although the quantitative analysis has revealed no statistical difference in the final period (49 days), it is worth mentioning that it was possible to observe the concave bone surface only in the NC group, suggesting a small loss of bone volume in the cervical third of

the ungrafted defects (Figure 8A). A more regular bone deposition in the DBB group could be observed in the final stage, with a flat aspect, recomposing the entire upper limit of the bone cavity (Figure 8B).

In the comparison between MBB and BO, a higher bone deposition was observed in the BO group after 14 days of grafting, compared to the MBB one (Figure 7A). Both grafts showed an ascending line of bone deposition over time, but no significant difference was observed between MBB and BO (Figure 7B).

Maturation level of neoformed trabeculae

The collagen fiber maturation was one of the criteria used as an indicator of bone maturation. After 49 days, the percentage of mature collagen in the neoformed trabeculae was higher in the DBB group, followed by the control, MBB and BO groups (Figure 9). The mineralized groups (BO and MBB) showed less than 30% of bone maturation in the neoformed bone, with no statistical difference between them.

Biomaterial reabsorption

The DBB graft showed a rapid rate of reabsorption, compared to the mineralized biomaterials (Figure 10A). After 14 days, few residues of the DBB organic matrix could be histologically detected (Figures 10B and C); and after 21 days, the material had already been completely resorbed (Figure 10D). The mineralized biomaterials, on the other hand, could still be observed in the defect site after 49 days, integrated to the neoformed bone matrix (Figures 6B and 6C). The BO group showed a greater resistance to resorption than MBB (Figure 10A).

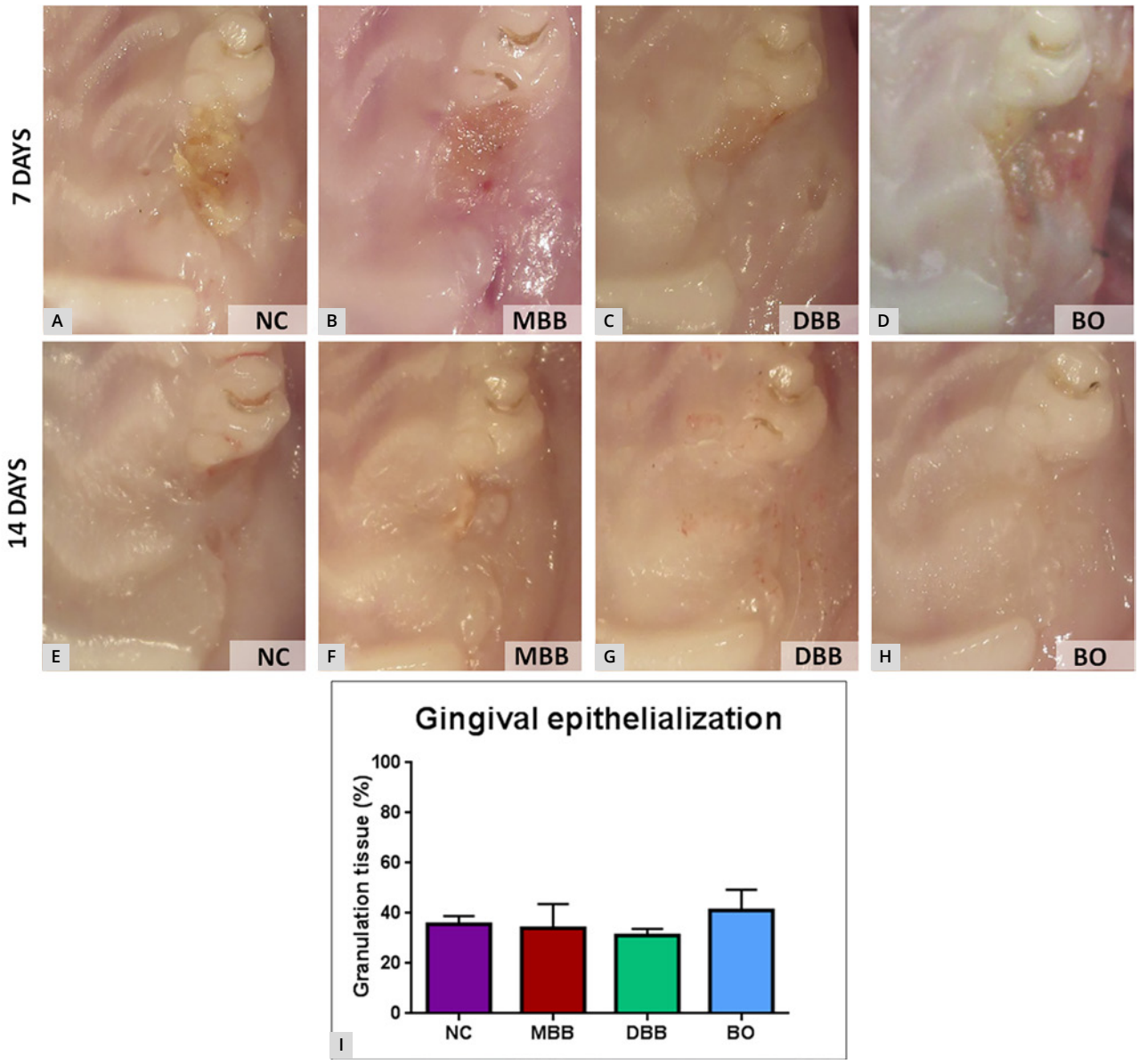

Figure 5. Macroscopic aspects and quantitative granulation tissue data. A to D) Illustrates the presence of granulation tissue at the surgical site at 7 days. E to H) At 14 days, all groups showed an absence of granulation tissue. In (I), the graph shows similarity in gingival epithelization among all groups, with no statistical difference.

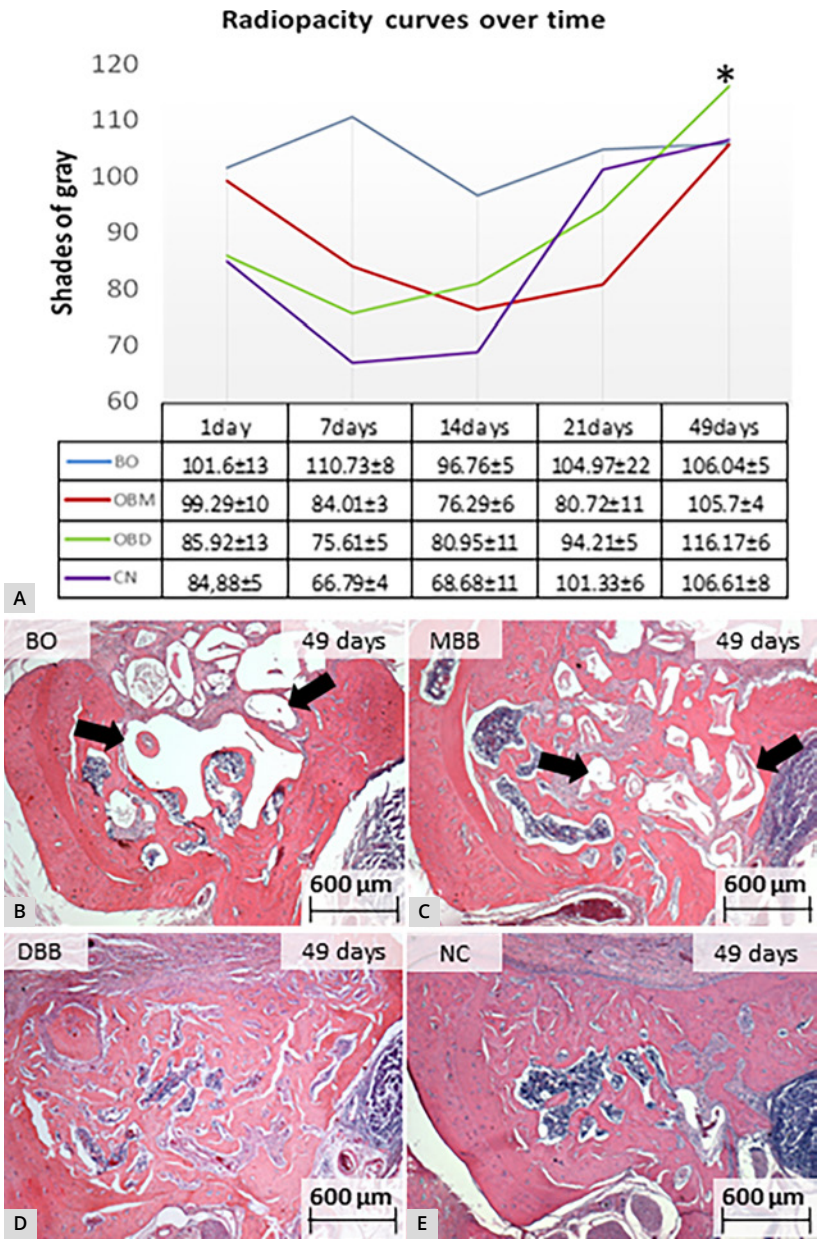

*Figure 6. Radiopacity index for each biomaterial over time. In (A), radiopacity index for each biomaterial over time. The BO biomaterial showed the highest radiopacity index after 1 day of grafting, while the DBB showed the highest index at 49 days. In (B) and (C) a representative image of the histological analysis performed in defects filled with BO and MBB grafts, respectively. Black arrows indicate the presence of remaining biomaterial granules even after 49 days of grafting. In (D), the absence of demineralized graft (totally reabsorbed). (E) Negative control. *p*

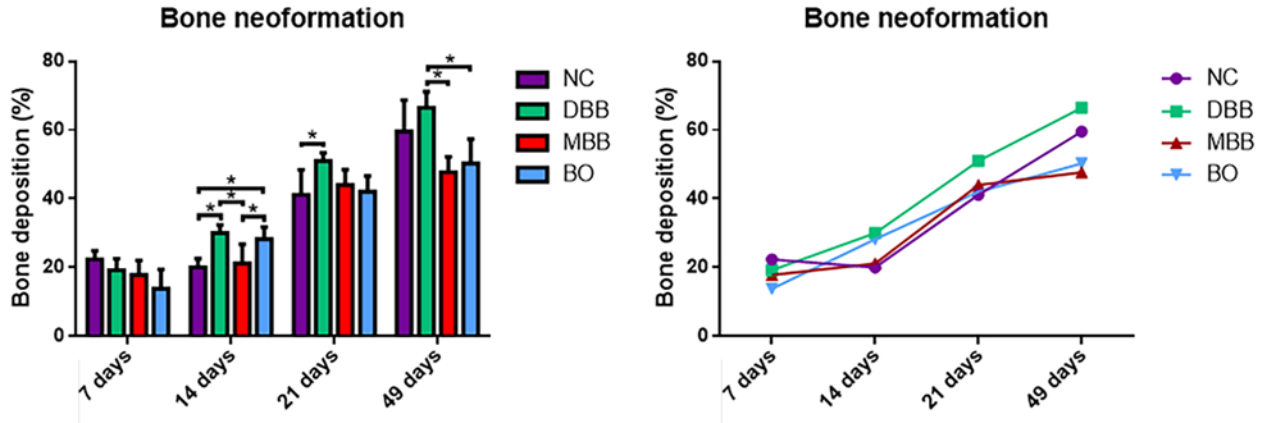

*Figure 7. Bone deposition curve throughout time in all groups. In (A), the percentage of bone deposition was higher when DBB graft was used, compared to the other groups, after 14 days of grafting. BO group showed a higher percentage of bone deposition at 14 and 49 days of grafting, compared to the MBB group. In (B), the bone deposition curve established over time showed an acceleration of the bone deposition process to the DBB group, when compared to the other groups. *p*

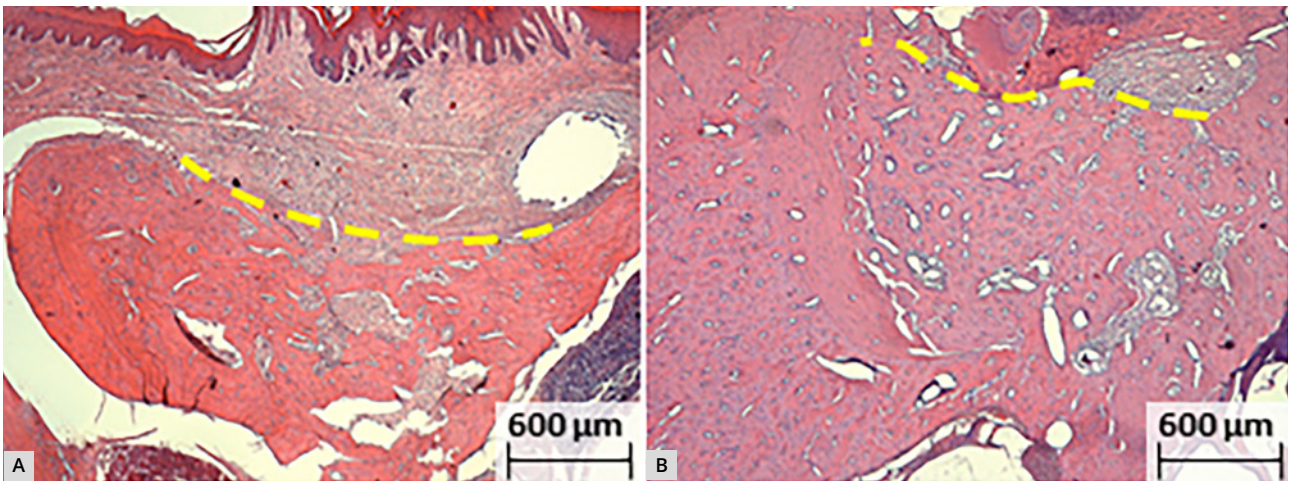

Figure 8. Histological images of the final repair stage. Limit of bone deposition in the cervical third. In (A), the control group (NC). Note the concave surface, dashed line in yellow. In (B), data obtained using the DBB group.

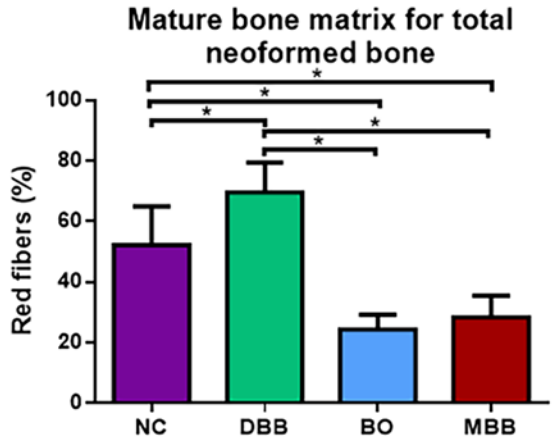

*Figure 9. Quantification of the red collagen fibers. Higher bone maturation was observed in the neoformed trabeculae of the DBB group, followed by the NC group. The bone trabeculae deposited in the mineralized groups presented lower bone maturation index, without statistical differences between them. * p*

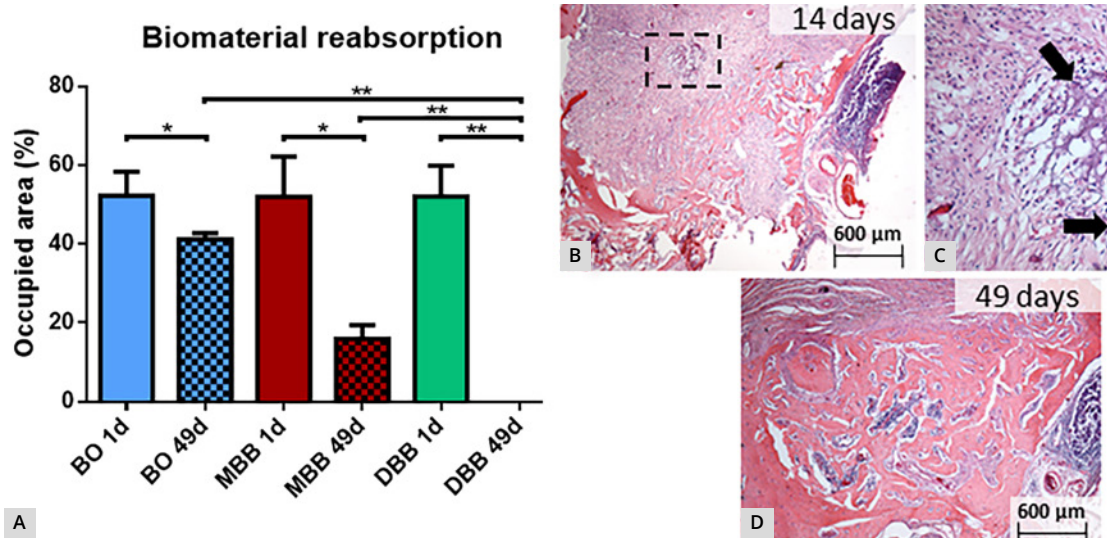

*Figure 10. Analysis of the biomaterial reabsorption. The DBB was almost completely reabsorbed at 49 days of grafting. In mineralized group (BO and MBB) the area occupied by the granules was significantly reduced. However, the area occupied by BO at 49 days was statistically larger than the area occupied by MBB, which indicates its relative resistance to resorption. In (B), few biomaterial residues could be observed in the defect area at 14 days. In (C), the magnified image of the black grid region observed in (B), with the evidence of organic matrix xenograft residues (black arrows). In (D), the area of the defect completely free of DBB biomaterial, filled with connective tissue and trabeculae in formation. *p*

Discussion

Bovine bone matrices available in different forms (mineralized, deproteinated, partially demineralized or completely demineralized) have been widely evaluated as biomaterials for bone grafts (Watanabe *et al*., 2016; Saima *et al*., 2016; Shirmohammadi *et al*., 2014; Lei *et al*., 2015; Carmagnola *et al*., 2003; Hulsmann *et al*., 2003; Tomasi *et al*., 2018). Studies show that the efficiency of xenografts in bone repair can be improved by exposing organic components of their bone matrix (Drosos *et al*., 2015; Hinsenkamp *et al*., 2015; Katz *et al*., 2009; Labutin *et al*., 2018). According to Huber *et al*. (2017), the demineralization of bone grafts allows the collagen matrix and other proteins to be exposed, such as BMP and growth factors, favoring the osteoinductive potential.

The osteoinductive capacity of demineralized bone matrices depends on the preparation technique. The demineralizing agent employed must maintain the osteoinductive effects (Roberts *et al*., 2012; Kumar *et al*., 2013; Zhao *et al*., 2021). Hydrochloric acid has been used in some studies in graft demineralization (Hammoudeh *et al*., 2017; Huber *et al*., 2017). However, a chelating solution (10% EDTA), in place of acidic pH solutions, facilitates the application of the technique with better time control and material preservation (Gomes *et al*., 2019; Bertassoli *et al*., 2020). Here, demineralized xenografts obtained after treatment by 10% EDTA solution demonstrated the preservation of the collagenous trabeculae bypassing regular medullary spaces, as well as the presence of BMP4, osteopontin and collagen type I.

The hypothesis that these organic components exposed by the demineralization process can accelerate bone repair was confirmed by both the qualitative and quantitative results obtained in this study. Bone defects filled with the DBB revealed a higher percentage of bone deposition and neoformed trabeculae, with a higher level of bone maturation. Similar results had been observed in the study of Mahyudin *et al*. (2017), In which the authors compared the effectiveness of freeze-dried xenograft, freeze-dried allograft, hydroxyapatite xenograft, and demineralized bone matrix xenograft as bone graft to fill bone defects in femoral diaphysis of white rabbits. The authors concluded that the bone healing process in the demineralized matrix bovine bone group occurred faster when compared to other groups, with greater osteoinduction.

When considering the performance of bone grafts, their influence on wound closure/soft tissue healing should also be taken into consideration. Wounds left open for a prolonged period of time allow the access of physical or biological agents and may compromise the restoration of specialized epithelial and connective tissue function (Mittal *et al*., 2016; Al-Fotawei *et al*., 2014). Here, the complete epithelialization of the mucosa after 14 days of grafting was demonstrated, regardless of the type of grafted biomaterial, revealing that bovine xenografts do not compromise gingival healing. Additionally, the underlying epithelial and connective tissue can invade the defect area, leading to a loss of space for bone replacement. In fact, histological sections of ungrafted animals revealed a superficial concavity at the end of the bone repair process, suggesting a small loss of bone volume in the cervical region of the defect. Pippi (2017) claims that in spontaneous healing after flapless tooth extraction, the socket is filled by a blood clot. In the next step, the clot is replaced by an granulation tissue with increasing density in the first week. The top surface of the post-extraction socket remains concave due to soft tissue invasion, with general reduction in bone volume. After one year, the residual alveolar bone is triangular-shaped due to higher bone resorption. Socket grafting procedures with xenograft, allograft or autograft seem to reduce alveolar bone loss after tooth extraction (Pippi, 2017; Minetti *et al*., 2022). In line with Pippi's (2017) data, the present results reiterate the importance of filling the cavities with biomaterials that can guide the regeneration while inhibiting early epithelial invasion.

Digital radiographs are commonly used to assist the definition of the best strategy for the restoration of the bone functions. The level of bone neoformation can be obtained from digital radiography images and used as an estimate of the radiopacity gain observed by the end of the treatment, when compared to the baseline data (Yaghobee *et al*., 2018; Pichotano *et al*., 2019; Al-Fotawei *et al*., 2014; Dimitriou *et al*., 2012; Wang *et al*., 2017). The radiographic evaluation technique used in the present study was previously validated by Gomes *et al*. (2019), who used radiographic images to assess bone repair of intraoral bone defects in rats, both by fractal analysis (FA) and radiopacity analysis. The authors used the same type of bone defect of the presented study, grafted with mineralized and demineralized bovine bone. The comparative results showed that the radiopacity analysis method was considered the most appropriate, as the data generated by this method corresponded to the data from the histomorphometric analyses. Such results add credibility to the radiographic analysis method used in the present study.

The present study revealed that the DBB graft showed the best result in terms of radiopacity. Radiopacity only reflected bone deposition in the DBB group, since the biomaterial has an organic nature, with no significant impact on the radiopacity curve, unlike mineralized grafts. Additionally, histological analysis demonstrated that the organic graft was quickly reabsorbed, eliminating the possibility of its interference in the shades of gray that compose the

results from the analysis performed 49 days later. Both histological and radiographic analyses revealed a progressive gain of neoformed bone in the DBB groups at 7 days, with bone deposition peaks higher than in the control group (NC). After 21 days, the trabeculae were thicker, present in all thirds of the defect, suggesting a more advanced bone repair. After 49 days, however, the induction of bone deposition of the DBB was similar to the control in histological analyzes and higher in radiographic evaluation. For this reason, the quantitative histological data might be underestimated, as the region of interest used in this study was previously defined as below this boundary area with the oral mucosa, being restricted to the center of the bone cavity.

Concerning mineralized grafts (BO and MBB), a lower radiopacity gain after 49 days of grafting, compared to the control, was observed. The histological analysis performed in parallel revealed, however, that this result reflects a smaller free area for bone neoformation, compared to the control. BO was found to be resistant to reabsorption, meaning that the area of the defect remains largely occupied by the biomaterial granules. Similarly, MBB granules were also seen after 49 days of grafting. The literature is consistent with these results, by stating that Bio-Oss® becomes integral to the structure of the neoformed bone (Galindo-Moreno *et al*., 2010; Haas Junior *et al*., 2016).

The osseointegration of inorganic biomaterials, which means the structural connection between the remaining bone, the neoformed bone and the grafts, is of great relevance for the therapy success (Watanabe *et al*., 2016). Here, both MBB and BO showed osseointegrated granules in the apical and middle thirds, after 49 days of grafting. No osseointegrated granules were observed in the cervical third. Defects filled with BO or MBB have also revealed the absence of the bony coating on the remaining granules in the superficial third of the defect. A study claims that Bio-Oss® always requires a longer regeneration time before successful osseointegration (Liu *et al*., 2016), suggesting that 49 days were insufficient for the complete repair of the defect when filled with BO or MBB in the present study.

In a rehabilitated patient, not only the amount of bone formed is relevant, but the quality of the regenerated bone is also an important factor for bone-implant integration (Bracey *et al*., 2018). The present study revealed that the DBB group presented bone trabeculae with a higher level of bone maturation after 49 days of grafting, compared to the other groups. The presence of proteins such as BMP4 and osteopontin, available at the surgical site grafted with demineralized matrix (DBB), might have contributed, respectively, to the results of higher levels of bone deposition and greater bone maturation recorded with the histomorphometric analysis. BMP4 exposure favors cell differentiation and osteogenesis (Sawkins *et al*., 2013; Glowacki, 2015; Sierra-Garcia *et al*., 2016; Park *et al*., 2016; Durham *et al*., 2018), which might explain the accelerated bone deposition process observed after 21 days in the DBB group. The presence of osteopontin assists in mineral deposition during bone repair and might influence the maturation stage of the tissue (Nam *et al*., 2016; Lund *et al*., 2009), which could explain the better performance of the DBB group in terms of bone maturation. Molecules isolated from organic matrices, such as BMPs, have already been used in clinics (Zhang *et al*., 2018; Liu *et al*., 2013), but costs are still a limiting factor for their wide application. The natural bone matrix is a site that contains growth factors and osteogenic signaling molecules, such as transforming growth factor beta (TGF-beta), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and BMPs, which act together to improve cellular performance in the bone repair phases (Kumar *et al*., 2013).

In conclusion, in this experimental model in rats, the demineralized version of the graft, obtained by a simple EDTA demineralization protocol, induced faster bone deposition that culminated in a more mature bone tissue. MBB and BO biomaterials in their commercial mineralized versions presented similar performances regarding their influence on soft tissues, bone deposition level, osseointegration and quality of the neoformed bone matrix. These results open new perspectives for the development of alternative (demineralized) bovine grafts, indicated for the acceleration of the osteogenesis process in clinical conditions that do not demand the immediate mechanical resistance provided by the traditional mineralized grafts.

Acknowledgments

The present study was supported by the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq), *Fundação de Amparo à Pesquisa do Estado de Minas Gerais* (FAPEMIG) and *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES).

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