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Histological evaluation of experimental bone grafting *in vivo* of lyophilized deproteinated bovine bone

Avaliação histológica do enxerto ósseo experimental *in vivo* de osso bovino liofilizado desproteinado

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Keywords:
biomaterials; bone grafting; bovine bone.

Abstract

Bone grafting biomaterials are important substances in current dentistry. Thus, the objective of this study was to observe in an experimental animal model, the clinical and histological response in the effectiveness of defect filling and osteopromotion of a deproteinated lyophilized bovine bone, in the search for dental applications. It was concluded that the application of the biomaterial demonstrated maintenance of oral tissue volume; histological characteristics observed were favorable to the repair and neoformation of oral bone, with high vascularization, fusiform-like cell proliferation, and a foreign-body type cell inflammatory infiltrate.

Palavras-chave:
biomateriais; enxerto ósseo; osso bovino.

Resumo

Biomateriais de enxerto ósseo são substâncias importantes na odontologia atual. Assim, o objetivo do estudo proposto foi observar em um modelo animal experimental a resposta clínica e histológica na efetividade de preenchimento e osteopromoção de um osso bovino liofilizado desproteinado, visando às suas aplicações odontológicas. Ao final, constatou-se que a aplicação do biomaterial demonstrou uma melhor manutenção de volume tecidual bucal, e histologicamente observaram-se características favoráveis ao reparo e à neoformação bucal óssea, grande vascularização, proliferação celular mais fusiforme e infiltrado inflamatório com presença de células do tipo corpo estranho.

Introduction

The biomaterials of bone grafting are biologically active substances that assist in the formation of bone in living organisms, result of the activity of osteoblasts, osteoclasts and osteocytes [21]. In the clinical application, many of these biomaterials are used for bone filling, in defects where bone neoformation would be incomplete or impossible without the use of grafting. Cases of trauma, severe bone resorption, increase of alveolar ridges, and also in the need to obtain a physical barrier that prevents the soft tissue migration for the interior of the injury are indications for bone grafting. The traditional methods of treatment include the use of autogenous bone grafting (bone from the own organism), due to osteogenic properties and readily incorporation to the tissue. However, the acquisition of autogenous bone can add risks to the patient, including additional surgical injury, increase of the postoperative morbidity and weakening of the local donor site.

Pretending to substitute total or partially, or even to improve the performance of the autogenous bone grafting, tests with dry biomaterials designed to the grafting have been carried for a long time [22]. In the decades of 80s and 90s a multiplication of the studies with biomaterials occurred: xenogenic grafts (from different species) [5, 16] and alloplastic grafts (synthetic biomaterials), also associated to dental implantology [7, 20]. Xenogenic biomaterials are alternative composites to the use of autogenous bone grafting, produced with potentially similar properties of the receptor tissue, and when used in the defects can form an obstacle to the invasion of the adjacent soft tissue.

Gendler (1986) [12] affirms that the decalcified bone matrix serves for orthopedic and surgical-reconstructive clinical situations, through the filling of bone defects and stimulation of regeneration in fractures. Other authors [18] study bone regeneration in rabbits (cranial region), in the use of endochondral bone grafting and demineralized bone matrix. Positive results are found in the use of demineralized bovine bone applied to defects of rat tibia with three millimeters size [19].

Before the clinical application, studies in animals can clarify the bone response to the insertion of these biomaterials, besides assisting in the collection of information on the mechanisms and sequences of the process of repair in grafting [5]. Thus, the objective of this study was to observe, in an experimental animal model, the clinical and histological response in the effectiveness of defect filling and osteopromotion of a deproteinated lyophilized bovine bone, in the search for dental applications.

Material and methods

These procedures were approved by the Committee of Ethics of Positivo University Center (UnicenP), Curitiba, Brazil. This research consisted of a surgical stage (Surgical Center, Bioterium, UnicenP), a laboratorial stage (Laboratory of Histopathology, Faculty of Dentistry, UnicenP) and of an image analysis stage. All the necessary care for infection control was taken: sterilization of instruments in autoclave, disinfection of auxiliary equipment and use of PPEs (personal protection equipments) by the operator.

The surgical stage was performed in the anterior region of the mandible of rats (Rattus norvergicus, WISTAR), always by the same operator. In this phase, a surgical table especially elaborated was used [24], in order to facilitate the immobilization of the animal and visualization of the surgical field, with the possibility of the use of lenses and soft tissue retractors. The animals were divided in two groups, I and II, with monitoring of one and three weeks, respectively; each group with 6 animals, being 3 animals as control (C), and 3 animals with the

application of lyophilized bovine bone (L) (Biobone, Vianfarm, Brazil). The surgical steps followed: 1) Sedation with halotane; 2) Animals were properly weighed and anesthetized with ketamine (40mg/kg, Bayer, Germany) and xylazine (5mg/kg, Bayer, Germany) intraperitonially; 3) Animals were placed on the surgical table and the surgical area was cleaned with a povidine solution (LM Farma, Brazil); 4) A linear incision (0,9 ± 0,1mm) at the buccal area and previous to the mental foramen of the right mandible was made with a knife (n.15, BD, Brazil); 5) A bone defect was created by the action of a round carbide bur [3] with 3mm of diameter (Antilope, Switzerland) coupled in portable apparel in low rotation (Dentec 405N, Brazil) – this procedure was accomplished with abundant irrigation of sterile saline solution (Sandex, Brazil), in order to avoid the overheating of tissues and allowing a good visualization of the root surface of the inferior incisive, which was defined as the limit of the artificial lesion; 6) The lyophilized bovine bone was introduced in the bone defect agglutinated with local blood; 7) Afterwards, the soft tissues were sutured [4] with silk 4-0 (Johnson & Johnson, Brazil) – the same procedure was accomplished with the control animals (C), except the biomaterial application; 8) Animals were administered postsurgically (24h) with buprenorphine [10] (0.1-0.25 mg/kg PO, BID, Shering-Plough, USA); 9) Animals were kept in plastic boxes with food and water *ad libitum* [11]; 10) Euthanasia (gas chamber after general anesthesia, as described) happened at one week (GI) and three weeks (GII) postoperatively [3, 23].

At laboratory stage: a) Hemi-mandibles were harvested and fixed with a freshly 3% glutaraldehyde (Merck, Germany) in 0.1 mol/L sodium cacodylate buffer solution (Electron Microscopy Sciences, USA) for 48 hours; b) Removal of the superficial tissues (hair, skin and part of oral mucosa); c) Rinsing in running water and placing of samples in separate pipes of incubation with a quelant solution for decalcification [14]: EDTA (LABSYNTH, Brazil), pH 6.4 adjusted with solid NaOH (LABSYNTH, Brazil), at approximately 37ºC in agitating table (WCJ-802, PH CIENTIFICA, Brazil) connected to a pH meter (WAVE, Brazil) – decalcification time was approximately 5 days; d)After decalcification, samples were placed in a paraffin automated processor (893 ANCAP, Brazil) followed by embedding procedures; e)Sectioning on a microtome (CUT 5062 SLEE MAINZ, Germany), with 5-7μm of thickness, serially, transversally to the incisor at the region of grafting, from the beginning of the defect appearance to its end, establishing limits and medium region of the artificial injury; f)Sections were taken to a water bath (ANCAP, Brazil) and mounted onto glass slides (PERFECTA, Brazil); g)Slides were placed into an incubator/oven (ODONTOBRÁS, Brazil), at a 60ºC for two hours; h)After drying, slides were carried through a conventional protocol for Hematoxilin and Eosin staining (LABSYNTH, Brazil), assembled with mounting medium and covered with glass slip [13]. The acquisition of images was made with a camera (SDC-310, SAMSUNG, Korea) connected to the microscope (021/3 QUIMIS, Brazil).

In the analysis of images, the distances between the bone edges at the medium region of the surgical defects were compared, using the UTHSCSA Image Tool 2.00 (The University of Texas Health Science Center, the U.S.A). All the images were also qualitatively analyzed in relation to the type of inflammatory infiltrate, cellular proliferation, evaluation of the bone development and neovascularization.

Results

Clinically, it was observed that the presence of the lyophilized bovine bone hindered the loss of tissue volume at the surgical site, in contrast to control samples.

From microscopic fields, a diameter of defect, or distance between the bone edges was considered, at the medium portion of it: in serial sections the image that presented the largest distance between bone edges was selected (greater diameter) – see figure 1. Table 1 showed the mean values, as well as the normalization in relation to control sample of one week.

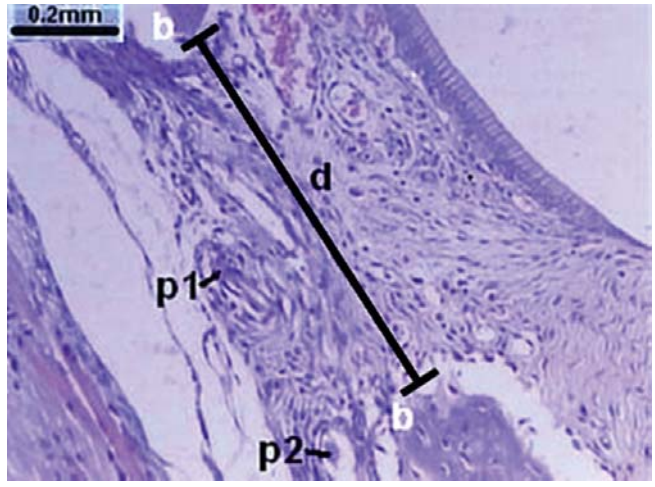


Figure 1 – Calculation of defect diameter at the medium region; three-week lyophilized bone sample
(d) Diameter of the defect; (b) Bone edges; (p1) Particle of lyophilized bovine bone in degradation; (p2) encapsulated lyophilized bovine bone particle

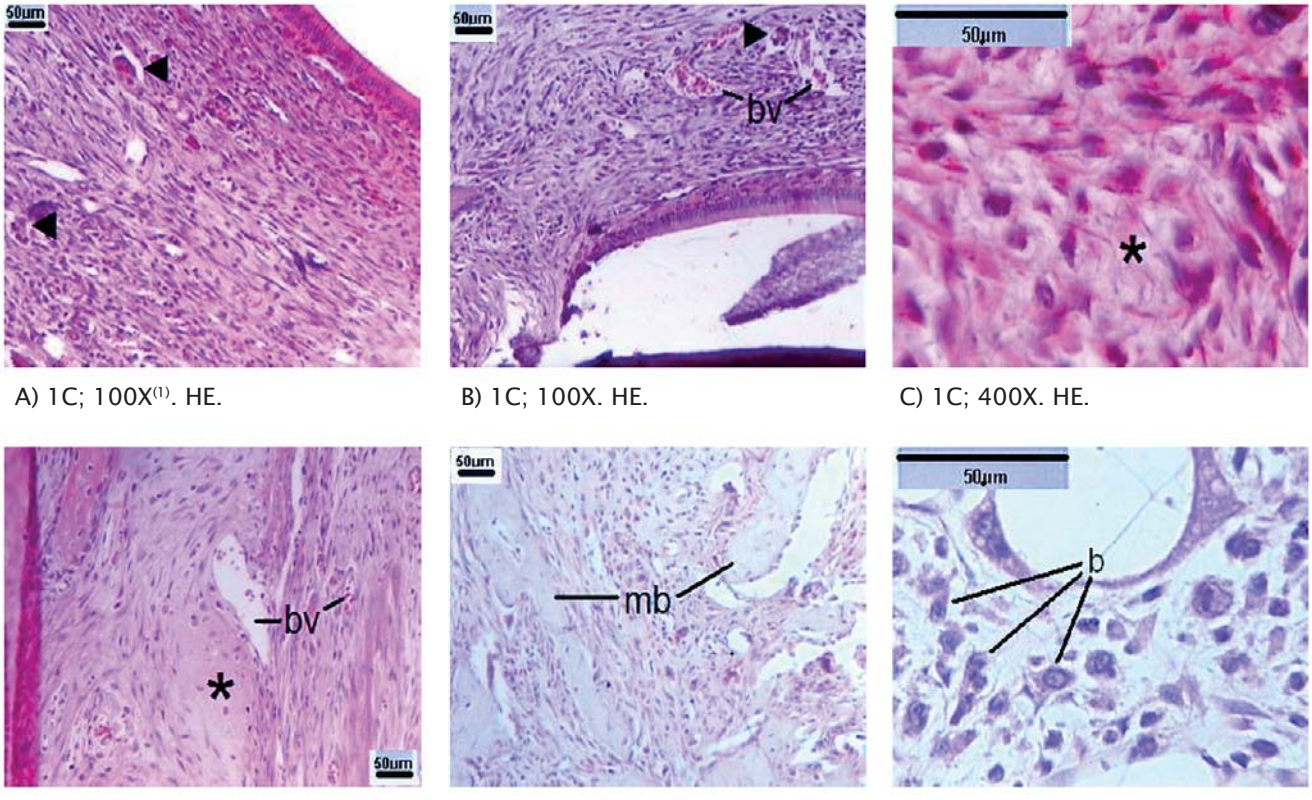
Table I – Mean values for defect diameters

Sample	Defect mean diameter (mm)	Normalization in relation to 1C (0,8mm)
1C	0,8	1,0
1L	0,7	0,9
3C	0,6	0,8
3L	0,6	0,8

(Nx) N = number corresponding to week period, x = type of sample; (C) Control; (L) Lyophilized bovine bone. Exemple: 1C = one-week control sample

Figure 2 showed the images A, B and C that corresponded to histological sections of one-week control samples. A chronic inflammatory infiltrate, typical granulation tissue, with the slight presence of large cells such as macrophages and multinuclear giant cells (MGCs) (figures 2A and 2B) could be seen. The neovascularization could be noted by the presence of blood vessels at the repair site in the figure 2B. In original magnification of 400X, a dense connective tissue was observed with a blastic type cell proliferation, probably osteo and fibroblastic, involved by extra cellular matrix, part of which could turn into bone matrix, or osteoyd tissue (figure 2C).

Histological sections D, E and F corresponding to three-week control samples also presented a chronic inflammatory infiltrate, apparently a little more discrete than one-week control samples, with a more advanced bone neoformation: immature bone (figure 2D) and mature bone – strong presence at the regions next to the bone edges of the defect (figure 2E). The aspect of the vascular neoformation and occurrence of multinuclear cells, similarly to one-week control (figure 2D) also could be observed, as well as the presence of mononuclear star-like blastic cells in proliferation (figure 2F).



A) 1C; 100X[1]. HE. B) 1C; 100X. HE. C) 1C; 400X. HE.

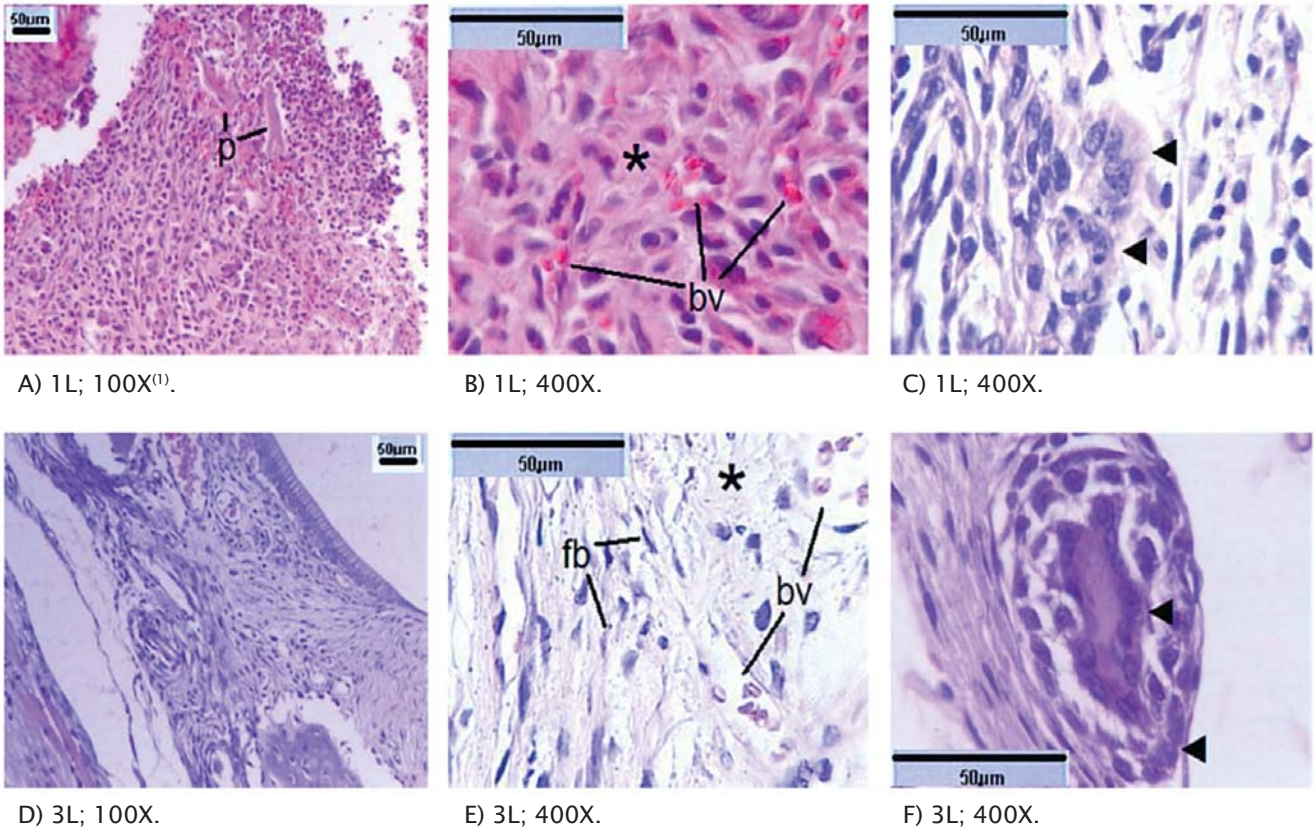
D) 3C; 100X. HE. E) 3C; 100X. HE. F) 3C; 400X. HE.

Figure 2 – Light microscopy of one- and three-week control samples
(1C) One-week control samples; (3C) Three-week control samples; (HE) Hematoxilin and eosin; (▸) Multinuclear giant cell; (bv) Blood vessel; (*) Osteoyd or immature bone; (mb) Mature bone; (b) Star-like blastic cells; ([1]) Value of original magnification – all photomicrographs of the figure

Figure 3 showed the slices from lyophilized bovine bone samples. The one-week samples demonstrated a chronic inflammatory infiltrate more intense than one-week control samples, also acute infiltrate, large number of cells surrounding the biomaterial particles (figure 3A), a similar blastic proliferation and consequent occurrence of matrix (figure 3B). Besides, an angiogenesis was noticed

(figure 3B) and occurrence of macrophages and MGCs was more intense than one-week control samples (figure 3C).

Samples of three-week lyophilized bone demonstrated a pattern of granulation tissue with chronic inflammatory infiltrate similar to three-week control (figura3 D), although with a more intense presence of MGCs and macrophagic cells (figure 3F). The vascular neoformation was also greater than the three-week control (figure 3E). The blastic proliferation was similar, surrounded by cells with the aspect of fibroblasts and others within the matrix, probably osteoyd (figure 3E).



A) 1L; 100X[(1)]. B) 1L; 400X. C) 1L; 400X.

D) 3L; 100X. E) 3L; 400X. F) 3L; 400X.

Figure 3 – Light microscopy of one- and three-week lyophilized bone samples
(1L) One-week lyophilized bone samples; (3L) Three-week lyophilized bone samples; (HE) Hematoxilin and eosin; (p) Biomaterial particle; (*) Osteoyd or immature bone; (bv) Blood vessel; (▸) Multinuclear giant cell; (fb) Fibroblast; ([1]) Value of original magnification – all photomicrographs of the figure

Discussion

In relation to the advantage of maintenance of tissue volume through filling, allowing the recovery of height and width of bones such as the maxilla and mandible, the use of a grafting biomaterial is justifiable, for the insertion of some biomaterial can be considered crucial, especially if this parameter can influence the face aesthetic, for example. The used of the lyophilized bovine bone in this work was favorable to the maintenance of tissue volume in the grafting.

Regarding the diameter at the medium region of the defect, the average for the lyophilized bovine bone was a little lower at one-week (0.7mm), in comparison with 0.8mm for the control. While at three-week the values equaled (0.8mm for both). The lyophilized deproteinated bovine bone used in this work is a xenogenic product (grafting biomaterial with origin of different species), with mechanism of osteoinduction [8], capable to induce the cellular activity, like osteoblast's, accelerating the bone neoformation. It can contain molecules that initially stimulate positively the remodeling, but can also contain strange molecules for the species tested, which can delay the tissue repair. Still, it must contain pores that facilitate cellular migration and its action.

The experimental model of Benhayoune *et al.* [2] demonstrates that the particle degradation of a biomaterial proportionally increases with the increase of the number of pores or irregularities in the surface of the particle itself – a physical characteristic to be considered in the release of ions.

In accordance with some authors [13], the proliferation of osteoblasts *in vitro* can be influenced by the presence of elements like calcium and phosphate, and this can be dependent of the amount of these ions. According to these same authors, such chemical elements are capable to activate the differentiation and expression of osteogenic cells that could lead to a faster bone neoformation. On the other hand, Knabe *et al.* [15] demonstrate *in vitro* that excessive amounts of calcium and phosphate can inhibit the cellular growth. Therefore, the amount of ions released by the lyophilized bovine bone at the grafting site can influence its osteoinduction property.

Concerning the process that happens in the control (blood clot only), there is also the ions of incentive to the bone production as calcium and phosphate, coming from the own organism, in a highly vascular tissue, thus facilitating the cellular and molecular diffusion. Thus, the control has the precursory cells of involved tissues (osteoblasts, fibroblasts and cementoblasts), the agents of incentive to regeneration (growth factors), ions to produce the inorganic portion of bone, and molecules for energy production and carbon source (oxygen and carbohydrates). The clinical problem of this system is its incapacity to maintain the remodeling space, which can cause a loss of tissue volume, as observed in this work.

Other reports [17] with different biomaterials and sites of those used in this present work, also disclose a chronic inflammatory infiltrate, typical granulation tissue, with the presence of multinuclear macrophagic and giant cells, that was lower in the control group, similarly to this research.

The cellular proliferation was intense in the control samples, being a little more star-like (more indifferentiated and/or with higher cellular activity) than fusiform-like (less active) (figure 2F). In the samples of three-week lyophilized bovine bone the proliferation was a little more fusiform-like, what can indicate a slightly higher occurrence of fibroblasts than osteoblasts, or either, a more mature period of cicatrization (figure 3E). Fibroblasts and osteoblasts have the same origin and can change into one another, depending on stimulation, or the surrounding micro-environment [1]. In the figure 3B (lyophilized bone samples) the occurrence of star-like cells was also noticed.

The results from similar studies can help in the search for the best conditions of clinical applications of the biomaterials [6]. Other studies such as X-Ray microanalysis and bone scintilography [9], besides electronic microscopy and the immunohistochemistry, also must provide with more information about the periodontal repair processes.

Conclusion

According to the aspects demonstrated in the application of the deproteinated lyophilized bovine bone, clinical evidences showed a better maintenance of oral tissue volume. In the histological analysis by light microscopy, favorable characteristics of repair and neoformation of oral bone were observed, such as great vascularization, more fusiform-like cellular proliferation and an inflammatory infiltrate with the presence of foreign-body giant cells – this type of inflammation suggests a more mature stage of cicatrization if compared with the control group.

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