Brazilian Dental Science
Ciência Odontológica Brasileira
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UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
Instituto de Ciência e Tecnologia
Campus de São José dos Campos

ORIGINAL ARTICLE
doi: 10.14295/bds.2016.v19i3.1291

Bovine and equine biomaterials in mandibular alveolar dog model: Split-mouth study

Biomateriais de origem bovina e equina em alvéolos mandibulares de cães: Estudo boca-dividida

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ABSTRACT

A predictable rehabilitation of severely atrophied alveolar ridge defects still remains a matter of concern in implant dentistry. **Objective:** The aim of this study was to compare histologically biomaterials of bovine (Genox®) and equine (Bio-Gen®) origin associated or not to platelet-rich plasma (PRP), analyzing bone maturity, amount of mature (type I) and immature (type III) collagen present at sixty and ninety days in post-extraction alveolus. **Materials and Methods:** Four Beagle dogs were used from which the six mandibular premolars were extracted. The alveolus contained respectively: right side, Bio-Gen ® (group B), preserved the clot (group C), Genox ® (group G); left side, Bio-Gen ® + PRP group (BP), Clot + PRP (group CP) and Genox ® + PRP (group GP). After sixty and ninety days, two dogs were sacrificed at each date and the pieces were histologically processed and stained with picrosirius red, a specific stain for analysis of different types of collagen. **Results:** Comparisons of the averages of mature collagen at 60 days indicated significant differences between Group G when compared to Groups C and B. The biomaterial of bovine origin demonstrated higher amounts of mature collagen in 90 days while the biomaterial of equine origin had a higher average of immature collagen in 90 days. **Conclusion:** Groups associated to PRP were effective in the production of mature and immature collagen. Genox® associated with PRP demonstrated a more advanced stage of bone regeneration presenting as an alternative to fill post-extraction alveolus.

KEYWORDS

Biocompatible materials; Bone regeneration; Bio-Gen; Genox; Platelet-Rich plasma.

RESUMO

Reabilitar defeitos ósseos alveolares severamente atrofiados de forma previsível continua a ser um motivo de preocupação em implantodontia. **Objetivo:** O objetivo deste estudo foi comparar, histologicamente, biomateriais de origem bovina (GenOx®) e equina (Bio-Gen®) associados ou não ao plasma rico em plaquetas (PRP), analisando a maturidade óssea pela quantidade de colágeno maduro (tipo I) e imaturo (tipo III) presentes em alvéolos pós-exodontia no acompanhamento de 60 e 90 dias. **Materiais e Métodos:** Foram utilizados quatro cães da raça Beagle, dos quais foram extraídos os seis pré-molares da mandíbula. Os alvéolos continham, respectivamente: lado direito - Bio-Gen® (grupo B), coágulo (grupo C), GenOx® (grupo G); lado esquerdo - Bio-Gen® associado ao PRP (grupo BP), coágulo associado ao PRP (grupo CP) e GenOx® associado ao PRP (grupo GP). Depois de 60 e 90 dias, dois cães foram sacrificados em cada data e as peças foram histologicamente processadas e coradas por picrosirius red, um corante específico para análise de diferentes tipos de colágeno. **Resultados:** Comparações entre as médias de colágeno maduro em 60 dias indicaram diferenças significantes entre o Grupo G quando comparado aos Grupos C e B. O biomaterial de origem bovina apresentou maior quantidade de colágeno maduro em 90 dias, ao passo que o biomaterial de origem equina apresentou alto índice de colágeno imaturo em 90 dias. **Conclusão:** Grupos associados ao PRP se mostraram eficazes para a maior produção de colágeno maduro e imaturo. O GenOx® associado ao PRP apresentou estágio mais avançado de regeneração óssea, sendo uma alternativa para preenchimentos de alvéolos pós-exodontia.

PALAVRAS-CHAVE

Materiais biocompatíveis; Regeneração óssea; Bio-Gen; Genox; Plasma rico em plaquetas.

INTRODUCTION

After tooth extraction the alveolus undergoes dimensional changes both in height and thickness particularly due to the absence of masticatory stimulation of the tooth on the alveolar bone. There are other associated factors such as diet, gender, age and hormone action, and these determine the degree and speed of alveolar bone loss. [1,2]

Preservation of the alveolar bone after tooth loss is essential particularly for the success of treatment with dental implants. When the bone dimensions of the alveolar ridge are insufficient bone defect reconstruction techniques using bone grafts and biomaterials may be considered in surgical planning. Science and technological development have progressed concomitantly and from this aspect the bone substitutes (grafts) have the properties necessary guarantee bone neoformation in bone defect reconstruction. [1,2]

Among the autogenous substances we may mention platelet-rich plasma (PRP) which is derived from repeated centrifugations of autogenous blood. [3] It is mainly constituted by seven proteins that are critical for growth. These growth factors include three isomers of platelet-derived growth factor (PDGFαα, PDGFββ and PDGFαβ), two transforming growth factors (TGFβ1 and TGFβ2), vascular growth factor and epithelial growth factor [4]. The aim of therapy using growth factors is to improve the tissue regeneration capacity which could lead to the increase in bone formation and mineralization, induce undifferentiated mesenchymal cells to differentiate into osteoblasts, diminish bone resorption, promote angiogenesis and produce collagen by the activation of fibroblasts [4,5]. PRP also contains fibrin, fibronectin and vitronectin, which are blood proteins that act as cell adhesion molecules for osteoconduction and as a bone matrix [6,7].

Xenografts are osteoconductive grafts and readily available and by means of different processing techniques these provide products that are biocompatible and structurally similar to human bone. Deproteinized bovine hydroxyapatite has been documented as being the most effective for promoting bone neoformation when compared with synthetic alloplastics [8]. Therefore, bovine bone mineral has been extensively studied and widely used in clinics [9,10].

In spite of bovine bone substitute undergoing the deproteinization process for the prevention of possible disease transmission there has been continual discussion with regard to the outbreak of bovine spongiform encephalopathy. [11] Therefore, an alternative type of donor which does not offer this risk is necessary. Considering the safety of xenogenic material bone derived from equines is proposed as an alternative xenogenic bone substitute which is hardly mentioned in the literature.

Collagen fiber proliferation and maturation within the dental alveolus after extraction serve as a structure for mineral deposition and the formation of bone trabeculae. [12,13] Collagen is the most numerous type of protein found in the body corresponding to 30% of the dry body mass. At present, over 20 genetically different types of collagen have been found in the skin, bone, cartilage, smooth muscle and basal lamina. Collagen type I, II and V participate to the structural formation of bone tissue with type I being the most abundant among them. [14]

In the present study we evaluated two types of biomaterials derived from equine (Bio-Gen®, Bioteck – Arcugnano Vicenza, Italy) and bovine (GenOx®, Baumer – Mogi Mirim, Brazil) bone, either associated with platelet-rich plasma (PRP) or not in the alveolar sockets of Beagle breed dogs in the period of 60 and 90 days post-extraction. Evaluation was made by analysis of the maturation of different types of collagen fibers present in the bone matrix.

MATERIAL AND METHODS

In order to conduct the experiment four dogs of the Beagle breed, female sex, from two

to three years of age with an approximate weight of 10 kg each were used. The animals were kept in the Central Bioterium of the State University of Maringá, Paraná, Brazil, under the care of a veterinarian. The study was authorized by the Ethics Committee on animal experimentation of the State University of Maringá, Protocol No.046/2010.

Surgical Protocol

To perform the surgical procedure the dogs first received a muscle relaxant 2% Xylazine (RompunR, Bayer, Mogi Mirim, Brazil) and sodium thiopental which is a sedative medication. After this, they were submitted to general anesthesia and the first, second and third pre-molars of each mandibular arch were extracted and the cavities were filled respectively with: right side - Bio-Gen® (Group B), blood clot (Group C), GenOx® (Group G); left side - Bio-Gen® associated with PRP (Group BP), blood clot associated with PRP (Group CP) and GenOx® associated with PRP (Group GP) Suturing was performed with Vicryl 4.0 thread (Ethicon, Johnson & Johnson. Somerville, NJ, USA.). During the post-operative period the dogs received a special patê-based diet and water ad *libitium*. Figure 1 illustrate the surgical protocol.

The animals were sacrificed with an overdose of 10 ml ketamine (10% ketamine hydrochloride, Agener União, Pouso Alegre, Minas Gerais, Brazil) 60 and 90 days after the experimental period began. They were then subjected to total sectioning of the jaw for microscopic analysis.

For manipulation of the PRP, 20 ml of blood was collected from each animal with 10% Sodium Citrate, which was used as anticoagulant agent. The blood was homogenized and centrifuged at 1200 rpm (revs per minute) for 10 min using the centrífugal appliance, Sin Centrifuga (Sin Sistema de Implantes, São Paulo, Brazil), to separate the portion of Platelet-Poor Plasma (PPP) from the portion that contained erythrocytes and the buffy coat which contains platelets and leukocytes. Thus, a portion of PPP was pipetted together with the platelets of the other portion and transferred to a new tube. A second round of centrifugation was performed following the same patterns as those of the first and separation between PPP and PRP was obtained. Afterwards, Calcium Chloride was added to the PRP to induce coagulation. To a 10 ml syringe, 6 ml of PRP, 1 ml of calcium chloride and 1 ml of air were added to aid the mixture. Immediately after this, the syringe was agitated for 6 to 10 seconds until the gel had formed. The gel was then applied to Groups BP, CP and GP.

Figure 1 - Surgical protocol.

Histological processing

For histological processing, the samples obtained were fixed in Bouin solution for 48 hours and washed by immersion in 70% alcohol until the fixer was completely removed. Decalcification was performed with a mixture of 20% formic acid, 50% Sodium Citrate, completed with distilled water.

After this, the tissues were washed in running water for twenty-four hours and stored in a 70% alcohol solution for two hours. Then the alveolus of the experiment were sectioned and submitted to the histological routine. To obtain the blades, semi-serial longitudinal cuts were made in the mesio-distal direction. In each group, five blades and three cuts were made sequentially. Only two blades were stained

with picrosirius red and after this they were counter-stained with hematoxylin.

Microscopic analysis

For analysis of bone neoformation, one slide of each group was selected, each of them containing 3 cuts of the alveolus of each animal in 60 and 90 days. Of each cut, 5 images were obtained by means of centralization within the alveolus following its long axis having the adjacent alveolar bone as a guide. Of the four animals a total of 60 images were obtained. These images were captured by means of a polarized light microscope (40× magnification) digital photographic camera coupled to the microscope, with the aid of the software program Elements®. Histomorphometry was performed with the aid of the software *Image Pro Plus®*, version 4.5 (Media Cybernetics, USA).

This program allows one to select the tone of color produced by the different types of collagen by means of a color selection tool. The area occupied by collagen type I was represented by an orangy-red color and type III was characterized by a greenish color (Figure 2). After the color was selected, the area in μm2 of each type of collagen per image was determined. All the images were captured and quantified using the same lighting conditions by a single operator in order to diminish errors of interpretation. Averages of areas (in μm2) obtained of each type of collagen (type I and type III) for all groups in 60 and 90 days were compared.

The results were analyzed by means of delineation in blocks, followed by the Tukey test. The level of significance adopted was 5%.

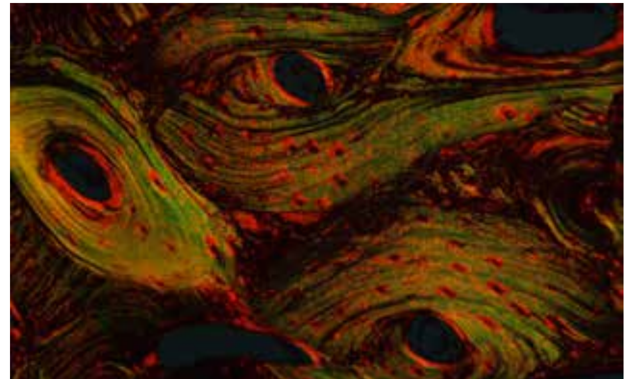

Figure 2 - Photomicrography stained with picrosirius red. The area occupied by collagen type I was represented by an orangy-red color, and type III was characterize by a greenish color. (40× magnification).

RESULTS

Table 1 presents the averages of collagen type I and type III, in μm2, in the period of 60 and 90 days for all the groups. The biomaterial of equine origin associated with PRP (Group BP) presented the highest average of mature collagen in 60 days and when compared with the group B (not associated with PRP) were significantly different ($p < 0.05$). Comparisons of the averages of mature collagen at 60 days indicated significant differences between Group G when compared to Groups C and B ($p < 0.05$).

The general group analysis at 90 days indicated that Group GP presented the greatest average of mature collagen (54.58) and Group G presented the highest average of collagen type I compared with all of the other groups not associated with PRP (Groups B and C). The Group BP presented the highest average of immature collagen (type III) (21.92) ($p < 0.05$).

Table 1 - Averages of areas (in μm²) and standard error obtained of each type of collagen (type I and type III) per image for the groups: C - Blood clot; CP -Blood clot Ü PRP; B - Bio-Gen; BP - Biogen Ü PRP; G - Genox; GP - Genox Ü PRP in 60 and 90 days

	Type of collagen	C	CP	B	BP	G	GP
60 days	I	12.41 ± 1.47	17,85 ± 2.35	10 ± 1.42	21.92 ± 3.28*	16.3 ± 1.71*,**	14.26 ±1.35
	III	10.28 ± 3.22	14,05 ± 1.28	12.66 ± 0.89	16.39 ± 2.71	12.61 ± 1.26	15.65 ± 1.01
90 days	I	19.7 ± 1.95	11,9 ± 0.88	26.46 ± 3.56	39.55 ± 4.78	48.51 ± 8.01*,**	54.58 ± 7.82#, ##
	III	12.41 ± 1.47	17.85 ± 2.35	10 ± 1.42	21.92 ± 3.28*	16.29 ± 1.71	14.26 ± 1.35#

*p < 0.05 when compared with Group B. **p < 0.05 when compared with Group C. #p < 0.05 when compared with Group BP. ##p < 0.05 when compared with Group CP

DISCUSSION

Biomaterials have been extensively studied as bone substitutes. Bone substitutes that present ideal properties are continuously sought. [1,2] Among several materials that are commercially available we chose Bio-Gen® and Genox® in the present study.

Bio-Gen® is a biomaterial of equine origin composed of small granules of cortical or cancellous bone tissue that is free of proteins and lipids. It presents bone-conductive actions and is principally indicated forraising the maxillary sinus, vertically increasing maxillary bones, filling cystic cavities, repairing peri-implant defects and post-extractions. [15]

Genox® is a reabsorbable biomaterial that is composed of demineralized and lyophilized bovine bone. It has osteoconductive action and is indicated for filling bone defects by professionals in the dental and medical fields. It is a biocompatible bone substitute, acellular, non-cytotoxic, non-immunogenic, non-pyrogenic and has a high degree of purity. It acts as a base for depositing new bone, is reabsorbed and leaves the new bone formation in place. [16]

In the present study, criteria for the quantitative analysis of mature (type I) and immature (type III) collagen were used to determine the stage of bone regeneration.

Collagen type I, II, III and V act directly on dental alveolus repair. [13,17,18] Collagen type II is a predominant protein in the matrix of hyaline cartilage, and plays and essential role in endochondral ossification. [13] However, the dental alveolus follows the characteristics of intramembranous ossification, in which there is no cartilage formation before bone calcification. In spite of the expression of collagen type II in the alveolus, the authors concluded that this protein does not participate in the structure of collagen fibers on which the immature bone forms. Therefore, to verify the different stages in bone neoformation, one mus verify the presence of collagen type I (mature) and type III (immature).

The picrosirius red technique is used specifically for staining the different types of collagen. The sirius red dye, a strong acid, contains six sulfonic groups that react with the amine groups of the lysine molecules present in collagen. The molecules of the dye dispose themselves in parallel to the elongated collagen molecules, normally oriented in a single direction. This normal orientation of the collagen molecules is responsible for its weak birefringence, however, after staining with sirius red the addition of a large quantity of elongated molecules of the dye leads to a considerable increase in birefringence of the collagen fibers making it possible to visualize and study them by polarized light microscopy. [19]

Group BP obtained the highest number of collagen type I fibers in 60 days with statistically significant difference only compared with Group B. The Group G presented the highest rate of mature collagen in 60 days when compared with others groups not associated (B and C). Genox® was the most effective biomaterial because presented the highest average of mature collagen at 90 days associated (54.58) or not (48.51) to PRP with statistically significant results when compared to the Biogen®. Furthermore, the biomaterial of equine origin associated with PRP (Group BP) was the one that presented the highest rate of immature collagen at 90 days (21.92) ($p < 0.05$).

GenOx® is of bovine origin and is obtained by means of deproteinization at high temperatures (between 950 and 1000°C). The growing rise in temperature increases the crystalline material of the bone matrix. [20] This high temperature is capable of changing the biological response and this process favors bone repair. [21]

In order to verify more studies about these biomaterials we reviewed the literature concerning the issue. The source was conducted in Medline database, the texts should have

been written in English, and the searching terms were Biogen AND Genox. Nothing was found until 2015.

A study [22] made a radiographic and histologic comparison of bone neoformation after the implantation of Bio-Gen® and Bio-Oss® (bovine origin), the former being of equine origin and the latter of bovine origin, in bone defects. After six months the result was that there was no histological difference between them but only for Bio-Oss® a greater bone density was shown radiographically.

The association of PRP was shown to be effective for greater mature collagen production for the alveolus with Biogen at 60 days and at 90 days with collagen immature ($p < 0.05$). In the literature, this association is controversial, and many studies such as those of Obarrio et al. [23], Schimitz et al. [24] and Sanchez et al. [25] stated that the association of biomaterials with PRP showed no effective action on bone regeneration.

A split-mouth study [26] compared the post-extraction alveolar sockets filled with Bio-Oss and Biogran either associated or not with PRP and concluded that in 30 days the association with PRP accelerated the bone repair process when compared with the insertions of biomaterials only.

PRP associated with bone grafts acts as a biologic adhesive to keep the particles together. [27] Osteoprogenitor cells are stimulated when associated with bone grafts and autogenous bone. [6] Plasma rich in platelets is more than only a concentration of platelets. It also contains three blood proteins that are known to act as cell adhesion molecules and allow osteoconduction as a matrix for bone and connective tissue and epithelial migration. These cell adhesion molecules include fibrin, fibronectin and vitronectin. [6,7] PRP is safe, low cost, convenient for the patient and has an effective action on regeneration, therefore, it is advantageous for clinical use [6,27].

CONCLUSION

- Groups associated to PRP were effective in the production of mature and immature collagen.
- Genox® associated with PRP demonstrated a more advanced stage of bone regeneration, presenting as an alternative to fill post-extraction alveolus.

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Date submitted: 2016 Jul 13
Accept submission: 2016 Sep 20