Effect of Platelet-Rich Plasma on the Healing of Mandibular Defects Treated With Fresh Frozen Bone Allograft: A Radiographic Study in Dogs

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The purpose of this study was to radiographically analyze the effect of autologous platelet rich plasma (PRP) on the healing of fresh frozen bone allograft (FFBA) placed in surgically created resection defects in mandibles of dogs. Bilateral resection defects measuring 1.5 cm × 1 cm were surgically created on the inferior border of the mandible in 10 adult male dogs. The defects were randomly divided into three groups: C, FFBA, and FFBA/PRP. In Group C, the defect was filled by blood clot only. In Group FFBA, the defect was filled with particulate fresh frozen bone allograft. In Group FFBA/PRP, it was filled with particulate fresh frozen bone allograft combined with PRP. At 90 days postoperative, standardized radiographs of the mandibles were obtained and results were quantitatively evaluated. Analysis of digitized radiographs indicated that non-PRP grafts were significantly less dense than the PRP grafts. Group FFBA/PRP also presented a statistically greater mineralized tissue area than Groups C and FFBA. Within the limits of this study, it can be concluded that PRP enhanced the healing of FFBA in resection defects in mandibles of dogs.

Key Words: bone regeneration, growth factors, platelets, bone grafting

INTRODUCTION

Autogenous bone, with its osteogenic, osteoinductive, and osteoconductive properties, has long been considered the ideal grafting material in bone reconstructive surgery.[1] However, drawbacks with autogenous bone include morbidity, availability, and unpredictable graft resorption.[1,2] Therefore, bone regeneration by means of tissue engineering has attracted increasing interest. The concept of tissue engineering is based on 3 pillars: scaffolds, cells, and growth factors.[2]

In a search for an adequate substitute for autogenous bone, cadaveric allograft has been a

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viable option. These grafts provide a structural framework or scaffold for host tissue to grow, hence making allograft osteoconductive.[3] Bone allografts (BA) are probably incorporated into existing bone by a process similar to that of autogenous bone grafts, but proceed more slowly as a result of the absence of living cells.[4] Therefore, current approaches are focused on the use of osteoinductive agents to enhance the properties of BA.

In orthopedic and maxillofacial surgery, BA are frequently used in combination with platelet-rich plasma (PRP) to improve bone regeneration.[5] PRP contains a number of growth factors in its natural composition, which can influence the chemotaxis, differentiation, proliferation, and synthetic activity of bone cells.[2,6] However, there are controversies with respect to the regenerative capacity of PRP and the real benefits of its use in bone grafts.[7,8]

A randomized, single-blinded, controlled study was conducted to compare bone formation after subantral maxillary sinus augmentation with freeze-dried bone allograft (FDBA) plus PRP versus FDBA plus resorbable membrane.[9] Results of this study suggested that the combination of FDBA and PRP enhances the rate of formation of bone compared with FDBA and membrane.

Animal studies have also demonstrated an improved bone healing when BA were combined with PRP in the treatment of critical-size defects in femurs of rabbits[10] and in bony defects around implants in dogs.[11] However, in other studies, the combination of BA and PRP did not enhance bone healing when used in maxillary sinus lift in goats,[12] in defects created around implants,[13,14] in defects created in calvarium,[15] in zygomatic arch,[16] or in alveolar ridge[17] of dogs and in noncritical size defects in rabbit calvaria.[18]

In summary, the role of PRP on healing of bone grafts remains controversial.[2] Specifically with allografts, few scientific conclusions have been reached.[18] Recent literature reviews have concluded that there are insufficient data to recommend the clinical use of PRP,[8,19] while others have indicated that well-designed controlled studies are needed to provide evidence of PRP efficacy in bone regenerative procedures.[1,20,21] Whether the extra cost and time spent on the PRP procedure is justified remains a topic for further study.[1]

According to Marx,[22] healing of BA cannot be improved by PRP as they do not contain viable cells. Therefore, BA cannot respond to the GFs in the PRP the same way as the autogenous bone grafts. However, a recent study has shown that a type of BA, the fresh frozen bone allograft (FFBA), contain living cells capable of growing.[23] Samples of FFBA presented capacity to give rise to proliferating cells, using tissue culture methods in vitro. It was observed that the DNA marker patterns of the cultured bone cells and freshly obtained buccal cells from the same donor were identical.[23]

The first case reports that presented the use of FFBA in dentistry, with histological and histometric analyses, were recently published by Stacchi et al.[24] These authors demonstrated a good incorporation of FFBA in maxillary sinus in humans. In fact, prospective controlled studies of FFBA in dentistry are still lacking.[1] Studies evaluating the combination of these grafts with PRP are also scarce. Only two studies in animals were conducted to evaluate the healing of FFBA combined with PRP in the treatment of bony defects created around titanium implants placed in humerus or in femoral condyles of dogs.[13,14] The findings of these studies demonstrated no additional benefits in adding PRP to the FFBA. However, the authors attributed these results mainly to the potential of spontaneous healing of the bony defects used and to the biological variation among the animals regarding the measurements of the parameters evaluated.

The purpose of this study was to radiographically analyze the effect of autologous PRP on the healing of fresh frozen bone allograft (FFBA) placed in surgically created resection defects in mandibles of dogs.

Materials and methods

Experimental model

The experimental protocol was approved by the São Paulo State University – UNESP, Dental School of Araçatuba Institutional Animal Care and Use Committee. Experiments were carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. Ten adult male mongrel dogs, weighing 15 to 20 kg, were used in this study at the Animal Facility of the Dental School of Araçatuba – UNESP. The animals were in good systemic and oral health.

Bone allograft preparation

Allografts were prepared by using cortico-cancellous bone from one dog euthanized at the end of other experimental protocol not involving the skeletal tissue or the surgical use of the explanted bone. Femurs were harvested immediately after euthanasia of the animal under rigorous aseptic conditions and shipped on dry ice to the Musculoskeletal Tissue Bank of the Marília University (Unioss, Marília, SP, Brazil). Final cleaning and processing of the femurs were performed following the guidelines set by the American Association of Tissue Banks. The bone was cut and shaped using precision tools. Cells, blood, and adipose tissues were removed using saline solution. The blocks were packed in a triple polyethylene bag and frozen at −80°C. All procedures were done under strictly aseptic conditions and bacterial cultures were taken at all stages of processing.

At the time of use, the bags containing FFBA were opened in a sterile environment. The bone was rehydrated in saline solution and ground with a bone mill (Quentin Bone Mill, Quentin Dental Products, Leimen, BW, Germany) to a uniform particle size.

PRP preparation

40 mL of autologous blood was drawn from each animal, via jugular vein, into a syringe containing 5 mL of Anticoagulant Citrate Dextrose Solution (ACD-A). PRP was prepared according to the PCCS II (BIOMET 3i, Inc, Palm Beach Gardens, Fla) protocol. The blood sample was centrifuged at 3200 rpm for 12 minutes. A 10% solution of calcium chloride (calcium chloride 10% solution, ScienceLab.com Inc, Houston, Tex) was used to activate PRP samples (0.75 mL of calcium chloride for each 1.5 mL of PRP). The PRP samples were then immediately combined with 0.8 mL of particulate FFBA.

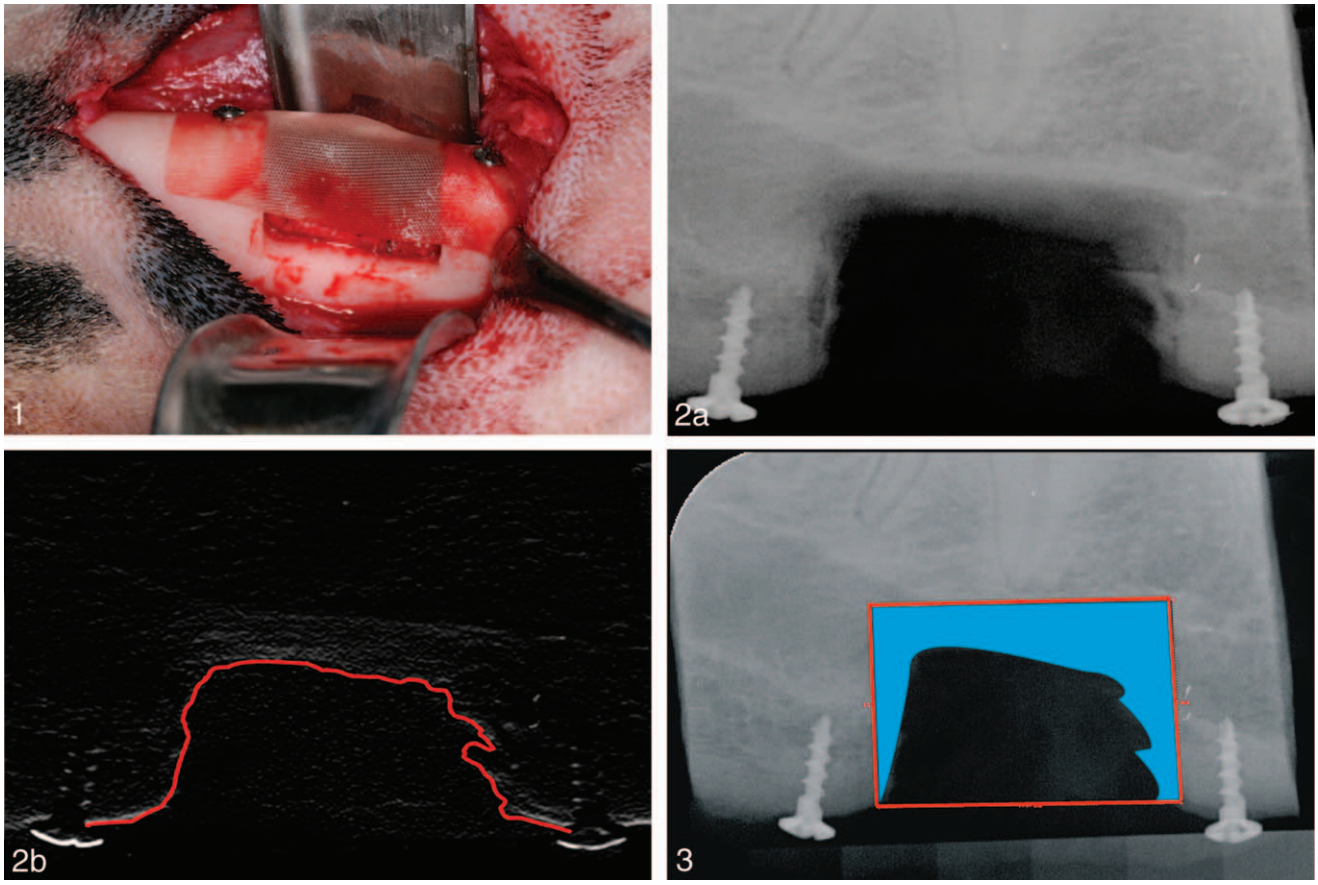
Platelet counts

Brecher liquid was used to lyse the erythrocytes and dilute the whole blood and PRP samples. The platelets in the diluted whole blood and PRP samples were then counted manually in a Neubauer chamber. In addition, PRP and whole blood smears were stained with "Panótico Rápido LB" (LaborClin, Pinhais, PR, Brazil) in order to see the morphology of the platelets. The platelet counts and the analysis of the platelet morphology were performed by a veterinary hematologist.

Surgical procedure

All animals were given an oral dosage of 1 g of amoxicillin and clavulanate potassium (Clavulin 500 mg; GlaxoSmithKline, Rio de Janeiro, RJ, Brazil) 1 hour before the experimental procedures. The surgeries were performed under general anesthesia. The animals received acepromazine 0.2% (0.05 mg/kg of body weight intramuscularly [IM]; Acepran, Univer S/A, São Paulo, SP, Brazil) and morphine 10 mg/mL (0.5 mg/kg of body weight IM; Dimorf, Cristália Produtos Quimicos Farmacêuticos Ltda, São Paulo, SP, Brazil) as pre-anesthetic medications. The general anesthesia was induced by an intravenous (IV) injection of propofol 10 mg/mL (5 mg/kg of body weight; propofol Cristália Produtos Quimicos Farmacêuticos Ltda) and maintained by halothane and oxygen through a constant volume ventilator, administered through an endotracheal tube. Dogs were connected to a heart monitor and a standard monitoring of general anesthesia was performed during the entire course of the surgery.

All surgeries were performed by the same surgeon. Regional anesthesia of the inferior alveolar nerve was obtained with 2% lidocaine and 0.5% bupivacaine containing epinephrine (1:200 000). Surgical sites were also locally infiltrated with 2% mepivacaine containing epinephrine (1:100 000) to reduce hemorrhaging. After aseptic preparation, a 5-cm midline incision was made in the neck through the skin and subcutaneous tissues, followed by a deeper dissection to the fascia of the midline geniohyoid muscles. At this point, dissection proceeded superficial to the muscle fascia laterally so as to encounter the inferior border of the mandible on both sides, right and left. The periosteum was divided sharply between the angle of the mandible and the mental foramen region bilaterally. A subperiosteal dissection was performed with strict attention to avoid the oral cavity. Standardized marginal resection defects measuring 1.5 cm × 1 cm were surgically created on the inferior border of the mandible bilaterally. The bone was removed with burs and osteotomes. Care was taken to protect the inferior alveolar neurovascular bundle. A surgical mesh (Vicryl Mesh Woven, Ethicon Inc, Somerville, NJ) was then placed on the resection defect and fixed to the inferior border



Figures 1–3. Figure 1. Surgical defect (1.5 cm × 1 cm) created on the inferior border of the mandible and protected with an absorbable surgical mesh. **Figure 2.** Computer-aided method for edge detection. (a) Digital radiographic image without Sobel filter; (b) Digital radiographic image with Sobel filter. The margins of the surgical defects (red line) are defined. **Figure 3.** The total area is delineated by the red line and corresponds to the area of the mandible where the surgical defect was originally created. The mineralized tissue area is delineated by the blue line.

of the mandible with 6 mm × 1.5 mm screws (SIN, São Paulo, SP, Brazil). One screw was placed 2 mm anterior and one 2 mm posterior to the margins of the surgical defect (Figure 1). These screws were also used as references to locate the original bone margins of the surgical defect during radiographic analysis.

The mandibular defects were randomly divided into three groups: C (n = 7), FFBA (n = 7) and FFBA/PRP (n = 6). In Group C, the defect was filled with a blood clot only. In Group FFBA, the defect was filled with 0.8 mL of particulate fresh frozen bone allograft. In Group FFBA/PRP, the defect was filled with 0.8 mL of particulate fresh frozen bone allograft combined with 1.5 mL of PRP.

The tissues then were closed in multiple layers using resorbable sutures (5-0 Vicryl, Ethicon) and the skin closure was achieved using silk sutures (4-0 Silk, Ethicon).

Postsurgical procedures

Following the surgeries, each animal received: (1) meloxican 10mg/ml (0.2 mg/kg of body weight IM; Movatec, Eurofarma Laboratórias Ltda, São Paulo, SP, Brazil) once a day for 5 days; (2) tramadol chloride 50 mg/ml (2 mg/kg of body weight IM; Tramal, Pfizer Ltda, São Paulo, SP, Brazil) once a day for 3 days; (3) oral dosage of amoxicillin and clavulanate potassium (Clavulin 500 mg; Glaxo-SmithKline) twice a day for 7 days.

All animals were placed on a soft diet for 15 days following surgery. Sutures were removed 10 days postsurgery. The animals were monitored for signs of infection and discomfort throughout the experimental period.

Radiographic processing and evaluation

Standardized radiographic images of the dogs mandibles were obtained using a dental radiographic unit (70 kVp, 10 mA; General Electric Company, Milwaukee, Wis) and photostimulable phosphor plates (Digora system, Soredex/Orion Corp, Helsinki, Finland). The dog mandible was positioned in such a way that the long axis of the surgical defect was parallel to the plate. The X-ray beam was then positioned perpendicular to the plate and, consequently, to the surgical defect. The distance between the X-ray source and plates was standardized at 40 cm for all specimens. Digitization of the photostimulable phosphor plates was performed immediately after X-ray exposure using a digital dental radiography system (Digora system, Soredex/Orion Corp).

The digital images were evaluated with the software "ImageLab 2000" (Diracon Bio Informática Ltda, Vargem Grande do Sul, SP, Brazil). The investigator who performed radiographic analysis was blinded with respect to the treatment rendered.

The following criteria were used to standardize the radiographic analysis of the digital images:

1) In each digital image, the radiographic distortion was corrected using the real dimensions of the screws as references;
2) A computer-aided method for edge detection (Sobel filter) was used to improve the standardization of the radiographic densities of the mineralized tissues (Figure 2);
3) Intra-examiner errors of identification and measurements were calculated by repeating the measurements on a randomly selected 10% sample of all images, 48 h after the initial measurements were taken. Calibration was accepted if measurements at baseline and at 48 h later were similar at the $>$ 90% level.
4) The total area (TA) to be analyzed corresponded to the entire area of the original surgical defect. This area was determined by first identifying the inferior border of the original mandible at the right and left margins of the surgical defect, and then connecting them with a line (Line A). Another line (Line B) was then drawn parallel to Line A, located 1 cm (original height of the surgically created defects) coronally to this line. Considering both screws as references, 2 mm were measured from the right and left screws towards the center in order to determine the side margins of the original surgical defect (Lines C and D). Therefore, a rectangle was delineated with the original dimensions of the surgically created defect (1.5 cm in length $\times$ 1 cm in height). The mineralized tissue area (MTA) was delineated within the confines of the TA (Figure 3);
5) The TA was measured in mm^2 and was considered 100% of the area to be analyzed. The MTA was also measured in mm^2 and calculated as a percentage of TA.
6) For the analysis of the optical density (OD) of MTA, an internal standardization of the gray scale values was performed using fields over the screws as the upper limiting gray scale value and fields over the radiographic film outside the sample as the lower limiting value.

Statistical analysis

These percentage data were transformed into arccosine for the statistical analysis. The significance of differences between groups in relation to MTA and OD was determined by an analysis of variance, followed by a post-hoc Tukey's test for independent samples when the analysis of variance suggested a significant difference between groups ($P < .05$).

Pearson's correlation coefficient (r_p) was used to demonstrate the relationship between the platelet counts from PRP and whole blood samples.

Results

All animals tolerated the surgical procedures well and were healthy during the entire experimental period.

Platelet count study

The PRP smears showed higher concentrations of platelets than the whole blood smears. Platelets exhibited normal morphology in the whole blood and PRP smears. Platelet counts confirmed that the PRP preparation technique used in this study produced samples of highly concentrated platelets. The average whole blood platelet count was $175.083 \pm 15.59 \times 10^3/\mu L$ while the average PRP platelet count was $758.167 \pm 98.62 \times 10^3/\mu L$ (Figure 4). Thus, the concentration of the platelets in PRP was increased by almost 4.5-fold (Figure 4).

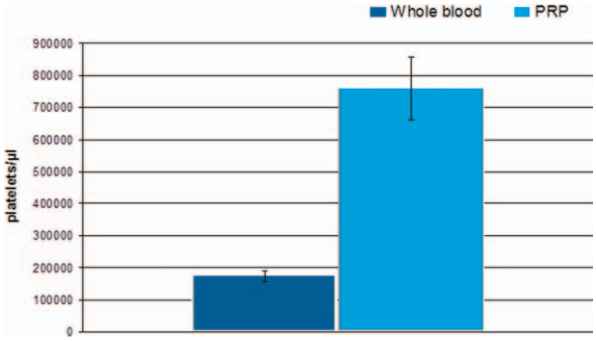


Figure 4. Mean number of platelets per microliter (µl) and standard deviations in the samples of platelet rich plasma and whole blood.

A statistically significant correlation was observed between the platelet count from whole blood and PRP samples ($r_p = 0.74$, $P = .0055$).

Radiographic and statistical analyses

The data normality and homogeneity of variances were verified. Means and standard-deviations of MTA and OD for groups C, FFBA, FFBA/PRP, as well as the comparison between the groups are documented in Table 1. Typical examples of radiographic images of each group are presented in Figure 5.

Table 1

Mean percentage (%) of mineralized tissue area (MTA) and optical density (OD) within the surgically created defects with comparison among groups; mean (SD)*

Parameter	Group C	Group FFBA	Group FFBA/PRP
MTA	42.20 (5.10)	44.82 (10.85)	60.93 (12.19)†
OD	44.76 (4.31)	53.88 (4.20)§	80.63 (8.73)‡
N	7	7	6

*C indicates control; FFBA, fresh frozen bone allograft; PRP, platelet rich plasma; SD, standard-deviation; N, number of specimens analyzed.

†Between-group comparisons for MTA: compared with Groups C and FFBA ($P < 0.05$).

‡Between-group comparisons for OD: compared with Groups C ($P < 0.001$) and FFBA ($P < 0.01$);

§Between-group comparisons for OD: compared with Group C ($P < 0.05$).

Discussion

From a clinical point of view, BA combined with osteogenic factors is of great relevance.[10] This approach is particularly attractive in the treatment of patients who have fractures or bone defects that are difficult to heal or a decline in their potentiality of spontaneous healing because of age, osteoporosis or other metabolic pathologies.[10] In the present study, Group FFBA/PRP presented greater MTA and OD than either Group FFBA or Group C.

Osseointegration and remodeling are biological processes by which the allograft bone is resorbed step by step by osteoclasts and is replaced by the ingrowth of a new vascularized osteoid.[25] It is very likely that these processes, in Group FFBA/PRP, were accelerated due to the effects of growth factors (GFs) available in PRP. GFs released by platelets which stimulate osteoblast differentiation and function also stimulate osteoclast precursor cells to divide and differentiate.[26] The stimulation of osteoclasts probably contributed to faster removal of the graft particles and their substitution by newly formed bone. In addition, PRP may have also enhanced the mineralization of newly formed bone. In vitro and in vivo studies have demonstrated that PRP may modulate the expression of some bone matrix proteins (eg, osteopontin and osteocalcin) which are directly related to the maturation of osteoblasts and, consequently, the mineralization of newly formed bone.[27–29] In an immunohistochemical analysis conducted by Nagata et al,[30] critical-size defects surgically created in rat calvaria and treated with bone graft combined with PRP showed higher expressions of osteocalcin and osteopontin than defects treated with bone grafts only.

PRP also presents adhesive properties due to the presence of fibrin and fibronectin, which may have improved the handling of the particulate FFBA in Group FFBA/PRP and facilitated its placement and stability. The stability of the graft is one of the important factors for their successful incorporation.[1] In the present study, the radiographic analysis has shown that the specimens of Group FFBA/PRP presented a bone tissue with improved density when compared with the ones of Group FFBA. This is an extremely important aspect to be considered in the bone reconstruction of sites where implants will be placed, since the quality of the regenerated bone tissue is one of the factors that determine the implants survival rates.[31] It is also important to consider that, in the present study, the mean of OD of the defects treated with FFBA/PRP was similar to the OD of the bony defects treated with autogenous bone grafts combined with PRP in a study in dogs by Gerard et al.[26] Considering this finding, it

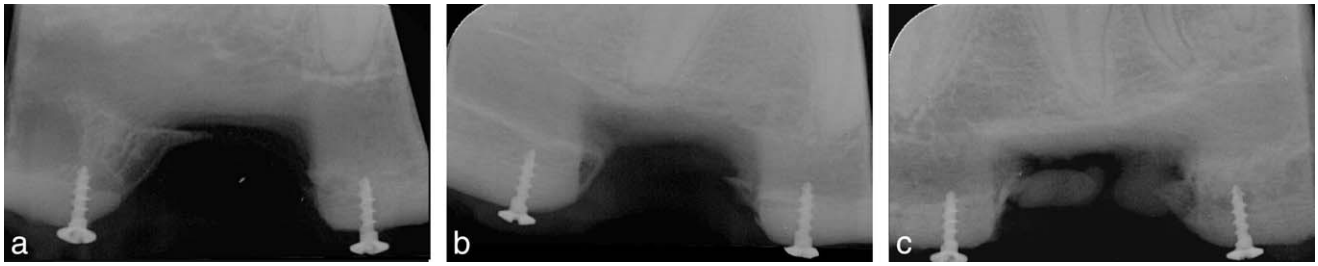


Figure 5. Digital radiographic images of the surgical defects at 90 days postoperative. (a) Group C; (b) Group FFBA; and (c) Group FFBA/PRP.

seems that cadaveric allograft combined with PRP may be a viable option to successfully substitute the use of autogenous bone in reconstructive surgeries.

The role of PRP on the healing of BA remains controversial. Several studies[9–11] have shown a positive effect while others[12–14,18] have not. These controversies may be discussed with regard to two different aspects.

The first pertains to the variations in the protocols for PRP preparation. An important parameter for the performance of PRP is the method of its preparation, since this can significantly influence the concentrations of platelets and growth factors, and consequently their osteogenic capacity.[2] Selection of an appropriate PRP preparation protocol is fundamental to evaluate the actual biological effects of PRP. In the present study, the PRP was prepared according to the PCCS II protocol. The first version of this system (PCCS) has been evaluated in clinical,[32,33] animal[27,34] and in vitro[35,36] studies. The in vitro studies have shown that the PCCS can create a high concentration of platelets, work with a small blood volume and do not damage the platelets. This semiautomatic system has been recommended by Marx[36] because it can create a "therapeutic" concentration of platelets (average percentage increase of approximately 400% in the platelet count). The PCCS II used in the present study consistently resulted in such an increase in the number of platelets in the PRP samples when compared to the whole blood. In addition, the analysis of platelet morphology in the PRP smears demonstrated that the PCCS II also seemed to preserve the integrity of the platelet cell membranes during PRP preparation. According to Marx,[37] platelets damaged or rendered nonviable by PRP processing will not secrete bioactive growth factors. Thus, their use may result in disappointing clinical outcomes.

The second aspect relates to the processing methods for BA used in many studies. One of the main determinants for the amount of new bone formation at the site of an allograft is (among several others) the processing technique applied.[38,39] Advances in allograft processing methods have been important for their successful use. The aim of the current development is to prepare allografts that are sterile while still preserving the biologic and biomechanical properties of the natural bone tissue.[38] The "best" method for bone allograft processing has been the subject of controversy.[25] A study conducted by Hofman et al[25] assessed the influence of 8 different sterilization and disinfection methods for BA on adhesion, proliferation, and differentiation of human bone marrow stromal cells. One of these methods, the fresh frozen bone, did not promote any inhibitory effects on cell proliferation. This processing method is very similar to the one used in the present study. Fresh frozen bone was also evaluated in a study conducted in dogs, resulting in greater bone formation when compared to freeze-dried bone allograft (FDBA).[14]

The mandibular defect created in the present study was protected by a surgical mesh and care was taken to preserve the periosteum throughout the surgical procedure, including the moment of wound closure. This may have favored the spontaneous healing of defects in Group C, what could help to explain the absence of significant differences in MTA between this Group and Group FFBA. An in vivo study lends support to this hypothesis.[40] Histologic and histometric analyses were conducted to evaluate spontaneous bone regeneration, osteoconduction and bone autografting in critical-size mandibular defects protected from soft-tissue

interposition in dogs.[40] In this study, segmental defects created in the midbody of the edentulated mandible were either left empty, implanted with coralline hydroxyapatite blocks or autografted with illiac cancellous bone. All defects were protected with a macroporous titanium mesh. The empty defects exhibited the greatest amount of bone formation at 4 months postoperative. According to the authors, this study demonstrated a remarkable ability of defect protection with a macroporous protective sheet to facilitate bone regeneration in critical-size mandibular defects. The authors have also emphasized that their study identified the need to differentiate critical-size defects into those with and without defect protection and periosteum.

Although statistically significant differences regarding MTA between Groups C and FFBA were not observed in this study, it should be considered that radiographic analysis showed that the specimens of Group FFBA presented a bone tissue with improved density when compared with the ones of Group C. Therefore FFBA were biocompatible and provided a structural framework or scaffold for host tissue to grow.

While some studies[41,42,43] used subjective criteria to evaluate the radiographic images (score system), the present study used objective parameters to evaluate them. The screws fixed to the inferior border of the mandible allowed a precise identification of the original surgical defect margins during radiographic evaluation. Image-analysis software (Diracon Bio Informática Ltda) was then used to measure the mineralized tissues inside the original surgical defect area.

Within the limits of this study, it can be concluded that PRP enhanced the healing of FFBA in resection defects in mandibles of dogs.

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