Healing of Fresh Frozen Bone Allograft with or without Platelet-Rich Plasma: A Histologic and Histometric Study in Rats

Michel Messora, DDS, PhD;* Laís Braga, DDS;* Gabriela Oliveira, DDS;* Luiz Fernando Oliveira, DDS;* Roselaine Milagres, DDS, MS;* Leandro Kawata, DDS, PhD;* Flávia Furlaneto, DDS, PhD;† Natália Pola, DDS, MS;‡ Natália Campos, DDS, MS;‡ Maria Nagata, DDS, PhD‡

ABSTRACT

Purpose: This study histomorphometrically analyzed the effect of autogenous platelet-rich plasma (PRP) on healing of fresh frozen bone allograft (FFBA) in bony defects in rat calvaria.

Materials and Methods: A 5 mm–diameter defect was created in the calvarium of 30 rats. Animals were divided into three groups: C (defect was filled by blood clot only), FFBA (defect was filled with 0.01 mL of FFBA), and FFBA/PRP (defect was filled with 0.01 mL of FFBA combined with 100 μL of PRP). All animals were euthanized at 30 days postoperatively. Histomorphometry and histology analyses were performed. Data were statistically analyzed (analysis of variance, Tukey, $p < .05$).

Results: FFBA had a statistically smaller new bone area than groups FFBA/PRP and C. No statistically significant differences were observed between groups FFBA and FFBA/PRP with regard to remaining bone graft particle area.

Conclusion: It can be concluded that (1) PRP improved the incorporation of FFBA, increasing the amount of new bone formed; (2) PRP has not influenced the resorption of nonviable particles of the FFBA; and (3) presence of remaining FFBA particles might have accounted for the smaller amount of new bone observed in group FFBA when compared with control group.

KEY WORDS: bone regeneration, homologous, platelet-rich plasma, transplantation

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 three pillars: scaffolds, cells, and growth factors (GFs).[2]

In a search for an adequate substitute for autogenous bone, cadaveric allograft has been a viable option. These grafts provide a structural framework or scaffold for host tissue to grow, hence making allograft osteoconductive.[3] Bone allografts (BAs) 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, BAs are frequently used in combination with platelet-rich plasma (PRP) to improve bone regeneration.[5] PRP contains a number of GFs 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

*Division of Integrated Clinic, Dental School of Lavras, Lavras University Center – UNILAVRAS, Lavras, Brazil; †Division of Periodontics, Federal University of Ceara, Sobral, Brazil; ‡Division of Periodontics, Department of Surgery and Integrated Clinic, Dental School of Araçatuba, UNESP – Univ. Estadual Paulista, Araçatuba, Brazil

Reprint requests: Dr. Michel Reis Messora, DDH, PhD, Lavras University Center – UNILAVRAS, Rua Padre José Poggel 506, CEP: 37200-000 Lavras, MG, Brazil; e-mail: michel_messora@terra.com.br

DOI 10.1111/j.1708-8208.2011.00419.x

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 BA (FDBA) plus PRP versus FDBA plus resorbable membrane.[9] The 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 BAs were combined with PRP in the treatment of critical-size defects (CSDs) 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 non-CSDs 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 are justified remains a topic for further study.[1]

According to Marx,[22] healing of BAs 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 BA (FFBA), contains 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 histologically analyze the effect of autogenous PRP on the healing of FFBA placed in surgically created CSDs in rat calvaria.

MATERIALS AND METHODS

Experimental Model

The experimental protocol was approved by the Lavras University Center – UNILAVRAS, Institutional Animal Care and Use Committee. Experiments were carried out in accordance with the guidelines laid down by the National Institute of Health in the USA regarding the care and use of animals for experimental procedures. Thirty, 5- to 6-month-old male rats (*Rattus norvegicus*, *albinus*, Wistar) weighing 450 to 500 g (UNILAVRAS, Dental School of Lavras, Animal Care Unit, Lavras, Brazil) were used. The rats were kept in a room with a 12-hour light/dark cycle and temperature between 22 and 24°C. They were randomly assigned to one of the three experimental groups: group C (control), group FFBA, and group FFBA/PRP (FFBA with PRP).

BA Preparation

Allografts were prepared by using cortico-cancellous bone from rats 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 animals 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

Before the surgical creation of the CSD, 3.15 mL of autologous blood was drawn from each animal of group FFBA/PRP, via cardiac puncture, into a syringe containing 0.35 mL of 3.2% sodium citrate to prevent coagulation. The blood sample was centrifuged at 160 G for 20 minutes to separate the plasma containing the platelets from the red cells (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The plasma was drawn off the top and centrifuged for an additional 15 minutes at 400 G to separate the platelets. The platelet-poor plasma was drawn off the top leaving the PRP and buffy coat. Then, the buffy coat and PRP (0.35 mL) were resuspended, activated, and used within minutes. A 10% solution of calcium chloride (calcium chloride 10% solution, ScienceLab.com Inc., Houston, TX, USA) was used to activate the PRP samples (0.05 mL of calcium chloride for each 1 mL of PRP). The platelets in the whole blood and PRP samples were counted automatically.

Surgical Procedure

Animals were anesthetized by intramuscular injection of xylazine (6 mg/kg body weight) and ketamine (70 mg/kg body weight). After aseptic preparation, a semilunar incision was made in the scalp in the anterior region of the calvarium allowing reflection of a full-thickness flap in a posterior direction. A 5 mm in diameter CSD was made with a trephine used in a low-speed hand piece under continuous sterile saline irrigation. The defect included a portion of the sagittal suture.

One L-shaped mark was made 2 mm anterior and one 2 mm posterior to the margins of the surgical defect using a small tapered carbide fissure bur and a surgical stent. The long axes of the L-shaped marks were located on the longitudinal axis bisecting the surgical defect. The marks were filled with amalgam (Figure 1).[25,26] Their purpose was to allow identification of the centerline of the original defect during laboratory processing and also to be used as references to locate the original bone margins of the surgical defect during histometric analysis.

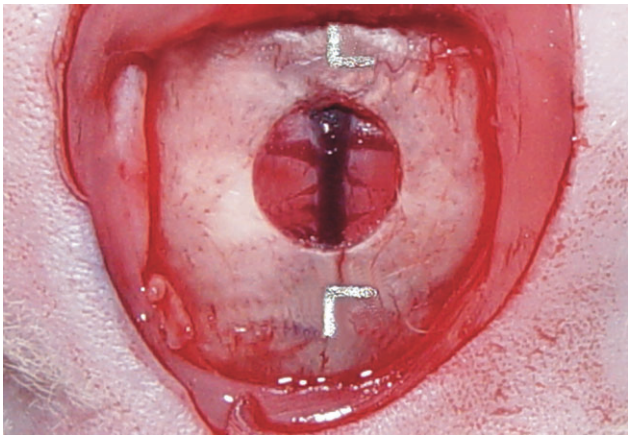

Figure 1 Critical-size defect (5 mm diameter) and the two reference marks created on the calvarium.

In group C, the surgical defect was naturally filled with blood only. In group FFBA, the surgical defect was filled with approximately 0.01 mL of particulate FFBA. In group FFBA/PRP, the surgical defect was filled with approximately 0.01 mL of particulate FFBA combined with 100 µL of PRP. The amount of bone graft was enough to fill the defects up to the level of their original bone margins.

The soft tissues were then repositioned and sutured to achieve primary closure (4–0 Silk, Ethicon, São Paulo, SP, Brazil). Each animal received an intramuscular injection of 24,000 IU penicillin G-benzathine (Pentabiótico* Veterinário Pequeno Porte, Fort Dodge®, Saúde Animal Ltda., Campinas, SP, Brazil) postsurgically.

Tissue Processing

All animals were euthanized at 30 days postoperatively. The area of the original surgical defect and the surrounding tissues were removed en bloc. The blocks were fixed in 10% neutral formalin, rinsed with water, and then decalcified in 18% ethylediaminetetraacetic acid solution. After an initial decalcification, each specimen was divided longitudinally into two blocks exactly along the centerline of the original surgical defect using the long axis of both L marks as references. Transverse cuts were then made using the short axis of each L mark as references. Each specimen then measured 9 mm in length along the longitudinal axis running through the center of the defect, allowing for identification of the original surgical defect margins during both histologic and histometric evaluations (Figure 2). After additional decalcification, they were processed and embedded in paraffin. Serial sections, 6 µm thick, were cut in a

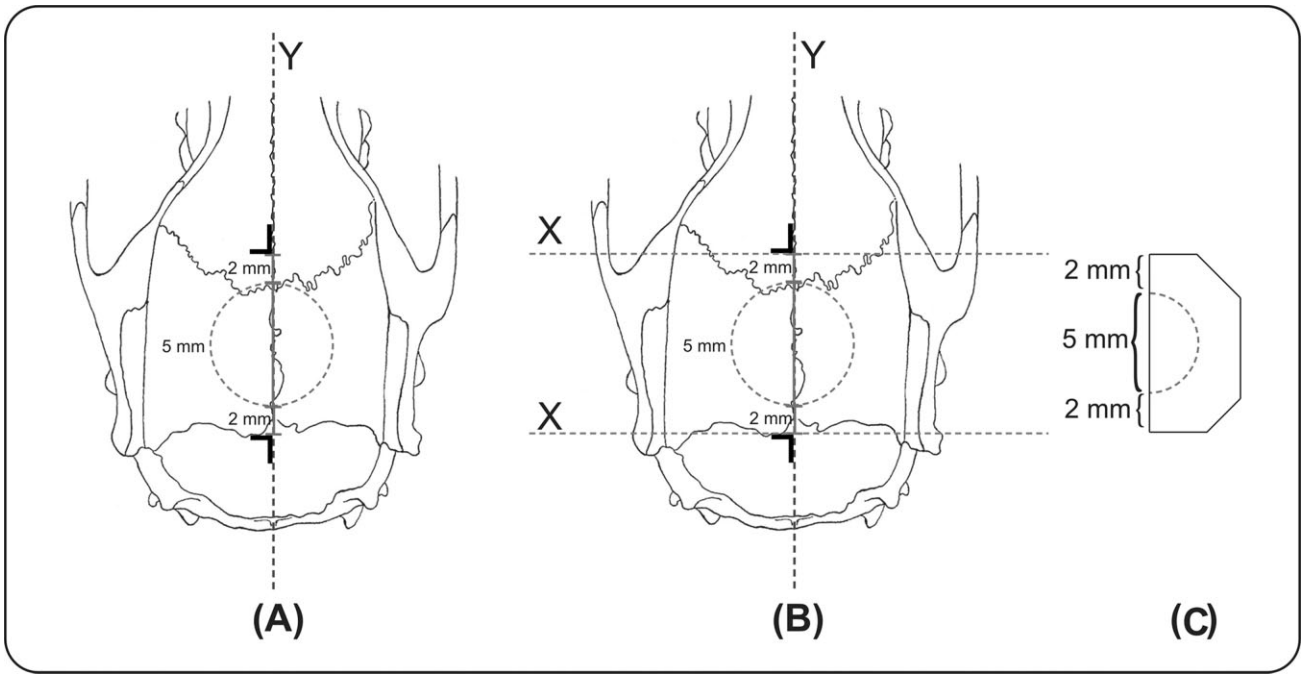

Figure 2 *A*, Longitudinal cut along the centerline (Y) of critical-size defect; *B*, transverse cuts (X); and *C*, dimensions of specimen to be embedded in paraffin.

longitudinal direction starting at the center of the original surgical defect. The sections were stained with either hematoxylin and eosin or Masson's Trichrome for analysis by light microscopy.

Histomorphometric Analysis

Two histologic sections, representing the center of the original surgical defect, were selected for the histologic and histometric analyses in order to increase the reliability of the data used in the statistical analysis. The histologic and histometric analyses were performed by an examiner blinded with respect to the treatment rendered. The images of the histologic sections were captured by a digital camera connected to a light microscope with an original magnification of ×1.6. The digital images were saved on a computer. The "ImageLab 2000" software (Diracon Bio Informática Ltda., Vargem Grande do Sul, SP, Brazil) was used for the histomorphometric analysis. Intra-examiner errors of identification and measurements were calculated by repeating the measurements on a randomly selected 10% sample of all images, 48 hours after the initial measurements were taken. Calibration was accepted if measurements at baseline and at 48 hours later were similar at the >90% level.

The following criteria, based in part on the work of Messora and colleagues,[25,26] were used to standardize the histomorphometric analysis of the digital images:

1. 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 external and internal surfaces of the original calvarium at the right and left margins of the surgical defect, and then connecting them with lines drawn following their respective curvatures. Considering the total length of the histologic specimen, 2 mm was measured from the right and left edges of the specimen toward the center in order to determine the margins of the original surgical defect. The new bone area (NBA) and the remaining bone graft particle area (RPA) were delineated within the confines of the TA.
2. The TA was measured in square millimeter and was considered 100% of the area to be analyzed. The NBA and RPA were also measured in square millimeter and calculated as a percentage of TA.

Statistical Analysis

The values of NBA and RPA for each animal were represented by the mean percentage of the two histologic sections. These percentage data were transformed into arccosine for the statistical analysis. The significance of differences between groups in relation to NBA and RPA was determined by an analysis of variance, followed by a post hoc Tukey's test when the analysis of variance suggested a significant difference between groups. The α

error was set at 0.05. The power of the study, given 15% as a significant difference between the groups, was calculated to be 0.80.

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

RESULTS

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

Platelet Count Study

The platelets exhibited normal morphology. Platelet counts confirmed that the PRP preparation technique used in this study produced samples of highly concentrated platelets. The PRP smears showed higher concentrations of platelets than the whole blood smears. The average whole blood platelet count was $465.90 \pm 47.83 \times 10^3$ platelets/μL, while the average PRP platelet count was $2{,}628.80 \pm 603.07 \times 10^3$ platelets/μL. The concentration of the platelets in PRP was increased by 5.7-fold.

Qualitative Histologic Analysis

Group C (Control). Almost all the surgical defect was occupied by connective tissue with collagen fibers parallel to the wound surface and a moderate number of fibroblasts. Newly formed bone was restricted to areas close to the original borders of the surgical defect (Figure 3A).

Group FFBA (Particulate FFBA). Almost all the defect region was occupied with remnants of bone graft particles (Figure 3B). Most bone graft particles showed empty osteocyte lacunae and did not demonstrate any new bone formation (Figure 4). Some FFBA particles were surrounded by new bone when they were located next to the borders of the defect. In general, the connective tissue presented a small number of fibroblasts. A small number of lymphocytes, macrophages, and osteoclasts were observed in a few specimens.

Group FFBA/PRP (Particulate FFBA Combined with PRP). An almost complete closure of the surgical defect with newly formed bone at the intracranial layer was seen in two specimens. Remnants of bone graft were observed throughout the defect in all specimens (Figure 3C). Most bone graft particles were surrounded by newly formed bone (Figure 5A). Different from groups FFBA and C, the newly formed bone was surrounded by highly active osteoblasts (Figure 5B). The connective tissue presented a moderate number of fibroblasts and numerous collagen fibers, which were

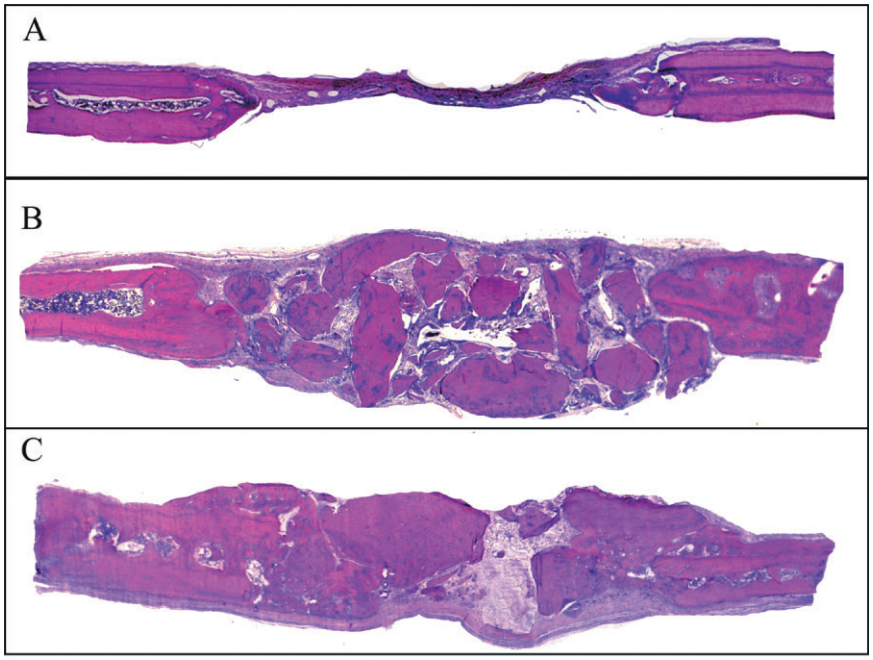

Figure 3 Panoramic views of the surgical defects. *A*, group C; *B*, group FFBA; and *C*, group FFBA/PRP. Hematoxylin and eosin stained; original magnification ×25. FFBA = fresh frozen bone allograft; PRP = platelet-rich plasma.

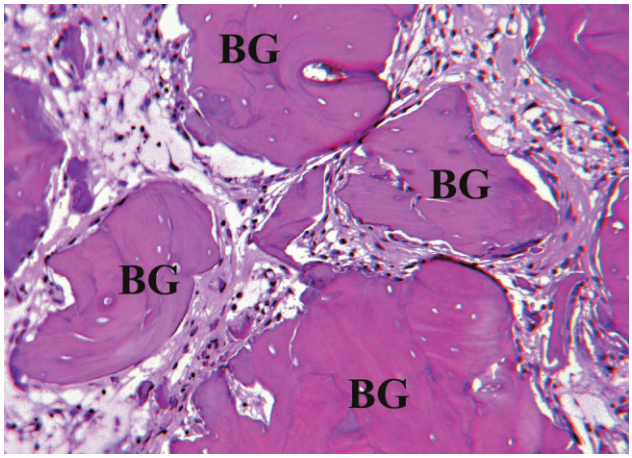

Figure 4 Remnants of bone graft (BG) particles showing empty osteocyte lacunae and surrounded by connective tissue. Group FFBA. Hematoxylin and eosin stained; original magnification ×250. FFBA = fresh frozen bone allograft.

more organized than group FFBA. Areas of provisional connective tissue and osteoid matrix were also seen. A small number of lymphocytes, macrophages, and osteoclasts were observed in a few specimens.

Histometric and Statistical Analyses

The data normality and homogeneity of variances were verified. Means and standard deviations of NBA and RPA for groups C, FFBA, and FFBA/PRP, as well as the comparison between the groups, are documented in Table 1.

No statistically significant correlation was observed between the platelet count from the PRP samples and the NBA (rp = 0.245, $p = .3116$).

DISCUSSION

Bone grafting has increased dramatically in recent years, and in clinical practice bone is the second most transplanted tissue after blood transfusion.[27] Different animal models have been developed to study bone graft incorporation,[28–30] and different conclusions have been drawn from these studies. Bone graft incorporation is a complicated process with multiple variables influencing rate, pattern, and completeness. A deep frozen necrotic allograft lacks osteogenetic and osteoinductive properties and only serves as a scaffold for bone ingrowths. The incorporation of an allograft, then, will only take place from the host bone site, and the initial healing processes will be slower than for a fresh autograft.[27] The findings of some studies suggest that the process of bone graft incorporation may be manipulated by, for example, the addition or removal of cells (and, indirectly, of cytokines).[31] This observation raises the possibility of enhancing the incorporation of FFBA.

In the present study, group FFBA presented NBA significantly lower than group C. This finding may be in part attributed to the degree of histocompatibility of the graft. Immunological responses have been observed after implantation of BAs,[32–34] and this slows down the incorporation of the graft.[35] Virolainen and colleagues[36] suggest that a major antigen mismatch between donor and recipient affects the temporal gene expression of extracellular bone matrix and delays new bone formation at the graft-host interface of cortical BAs.

When FFBA was associated to PRP, a significantly greater amount of new bone was observed compared with the isolated use of FFBA. Therefore, PRP improved the incorporation of FFBA. The use of PRP is based on

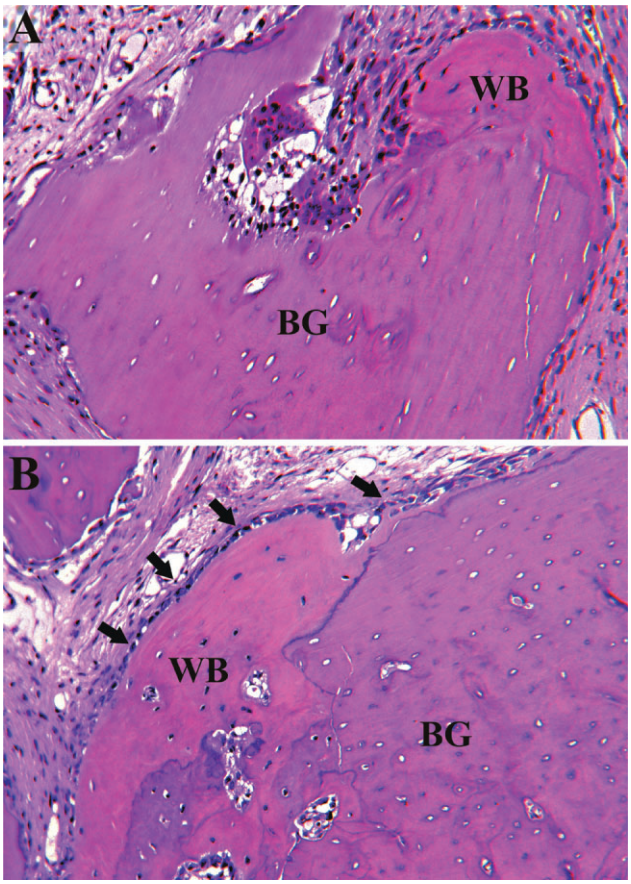

Figure 5 *A*, Remnants of bone graft (BG) particles surrounded by newly formed woven bone (WB). *B*, Newly formed WB surrounded by highly active osteoblasts (*arrows*). Group FFBA/PRP. Hematoxylin and eosin stained; original magnification ×250. FFBA/PRP = fresh frozen bone allograft with platelet-rich plasma.

TABLE 1 Mean Percentage (%) of New Bone Area (NBA) and Remaining Bone Graft Particle Area (RPA) within the Surgically Created Defects with Comparison between the Groups; Mean (SD)

Parameter	Group C	Group FFBA	Group FFBA/PRP
NBA	11.78 (2.22)	5.51 (4.33)*	11.76 (4.97)
RPA	–	51.48 (13.17)	48.94 (10.48)
N	10	10	10

Between-group comparisons: *Compared with groups C and FFBA/PRP ($p < .05$).
C = control; FFBA = fresh frozen bone allograft; PRP = platelet-rich plasma; SD = standard deviation; N = sample size.

the premise that platelets constitute a reservoir of critical GFs, such as platelet derived GF, transforming GF-b, insulin-like GF-I, and vascular endothelial GF, which, once released, may positively regulate the wound healing process.[22,37,38] Some studies have demonstrated that PRP may increase the proliferation of endothelial cells,[39] pre-osteoblasts,[40] and osteoblasts[41,42] in the surgical site. It could be speculated that these PRP effects might have contributed to the greater NBA in group FFBA/PRP when compared with group FFBA. PRP also has adhesive properties due to the presence of fibrin and fibronectin, which may have improved the handling of the particulate FFBA and facilitated its placement and stability. The stability of the graft is one of the important factors for their successful incorporation.[1]

No significant differences were observed between groups C and FFBA/PRP in relation to NBA. One fact that could help explain this finding is the period of observation selected, which may not reflect the final reparative response. While in group C the reparative process was basically concluded, tissue healing was still in intense activity in group FFBA/PRP. In the latest, highly active cuboid osteoblasts depositing bone matrix were observed lining the borders of the newly formed bone. There was also a greater amount of osteoblasts and osteoid matrix in process of organization than group C.

The role of PRP on healing of BA remains controversial. Several studies[9–11] have shown positive effects while others[12–14,18] have not. These controversies may be discussed with regard to the variations in the protocols for PRP preparation. An important parameter for the performance of PRP is the method of its preparation, as this can significantly influence the concentrations of platelets and GFs, and consequently their osteogenic capacity.[2] The selection of an appropriate PRP preparation protocol is fundamental to evaluate the actual biological effects of PRP. Thus, our protocol was designed to optimize the quality of the platelets in PRP samples in a number of ways. Several aspects were considered to guarantee their integrity in the PRP samples, such as the choice of the anticoagulant, the speed of the centrifugation, the activator used to initiate PRP clot formation, and the amount of time between activation of the PRP and its clinical use.[22,26,43,44] According to Marx,[37] platelets damaged or rendered nonviable by PRP processing will not secrete bioactive GFs and may result in disappointing outcomes.

An interesting finding of the present study is that PRPs have not contributed to a faster resorption of FFBA particles. No significant differences regarding RPA were observed between groups FFBA and FFBA/PRP, which indicates that PRP may have not stimulated the production of osteoclasts. In fact, conflicting results have been reported as regard to the effect of PRP on osteoclastogenesis. According to Maitz and colleagues,[45] supernatants of activated platelets induced an increase in osteoclast differentiation of murine bone marrow cells in vitro. However, Ogino and colleagues[46] have recently demonstrated that PRP inhibited the formation of tartrate-resistant acid phosphatase positive multinucleated cells from rat bone marrow. According to Cenni and colleagues,[47] this effect of the PRP seems to be related to its concentration in the environment. In a previous study performed by our group,[48] the volume of PRP used has directly influenced RPA values in osseous defects created in rat calvaria and treated with autogenous bone grafts associated or not with 50, 100, and 150 µL of PRP. In this study,[48] no significant differences regarding RPA were found between osseous defects treated with autogenous bone grafts only or with autogenous bone grafts combined with 50 and 100 µL of PRP. However, when 150 µL of PRP was combined with

autogenous bone grafts, RPA was significantly greater when compared with the other groups, indicating a possible inhibitory effect of PRP on osteoclastogenesis.[48]

Grageda[49] suggested a standardized protocol for future studies evaluating the biological effects of PRP. As part of this protocol, Grageda[49] suggested assessing the correlation between the histormorphometric analysis and the number of platelets in the PRP. In the present study, no significant correlation could be found between the platelet count in the PRP samples and the NBA. Because the regenerative potency of PRP undoubtedly depends on its GF levels, it could be inferred that this result supports the findings of Weibrich and colleagues[50] who demonstrated that neither whole blood nor PRP platelet counts are reliably predictive of the resultant GF levels in PRP. These findings are also corroborated by recent studies that have observed differences in GF levels in samples with the same concentration of platelets.[39,51]

Within its limitation, the present study has indicated that (1) PRP improved the incorporation of FFBA, increasing the amount of new bone formed; (2) PRP has not influenced the resorption of nonviable particles of the FFBA; and (3) presence of remaining FFBA particles might have accounted for the smaller amount of new bone observed in group FFBA when compared with control group at 30 days postoperatively.

ACKNOWLEDGMENTS

Lais Braga received a scholarship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

REFERENCES

1. Hallman M, Thor A. Bone substitutes and growth factors as an alternative/complement to autogenous bone for grafting in implant dentistry. Periodontol 2000 2008; 47:172–192.
2. Kasten P, Vogel J, Geiger F, Niemeyer P, Luginbühl R, Szalay K. The effect of platelet-rich plasma on healing in critical-size long-bone defects. Biomaterials 2008; 29:3983–3992.
3. Bostrom MP, Seigerman DA. The clinical use of allografts, demineralized bone matrices, synthetic bone graft substitutes and osteoinductive growth factors: a survey study. HSS J 2005; 1:9–18.
4. Urist MR. Bone transplants and implants. Philadelphia, PA: JB Lipicott, 1980:331–368.
5. Cenni E, Perut F, Ciapetti G, et al. In vitro evaluation of freeze-dried bone allografts combined with platelet rich plasma and human bone marrow stromal cells for tissue engineering. J Mater Sci Mater Med 2009; 20:45–50.
6. Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, Georgeff KR. Platelet-rich plasma: growth factor enhancement for bone grafts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998; 85:638–646.
7. Filho Cerruti H, Kerkis I, Kerkis A, et al. Allogenous bone grafts improved by bone marrow stem cells and platelet growth factors: clinical case reports. Artif Organs 2007; 31:268–273.
8. Plachokova AS, Nikolidakis D, Mulder J, Jansen JA, Creugers NH. Effect of platelet-rich plasma on bone regeneration in dentistry: a systematic review. Clin Oral Implants Res 2008; 19:539–545.
9. Kassolis JD, Reynolds MA. Evaluation of the adjunctive benefits of platelet-rich plasma in subantral sinus augmentation. J Craniofac Surg 2005; 16:280–287.
10. Dallari D, Fini M, Stagni C, et al. In vivo study on the healing of bone defects treated with bone marrow stromal cells, platelet-rich plasma, and freeze-dried bone allografts, alone and in combination. J Orthop Res 2006; 24:877–888.
11. Kim SG, Kim WK, Park JC, Kim HJ. A comparative study of osseointegration of Avana implants in a demineralized freeze-dried bone alone or with platelet-rich plasma. J Oral Maxillofac Surg 2002; 60:1018–1025.
12. Grageda E, Lozada JL, Boyne PJ, Caplanis N, McMillan PJ. Bone formation in the maxillary sinus by using platelet-rich plasma: an experimental study in sheep. J Oral Implantol 2005; 31:2–17.
13. Jensen TB, Rahbek O, Overgaard S, Søballe K. Platelet rich plasma and fresh frozen bone allograft as enhancement of implant fixation. An experimental study in dogs. J Orthop Res 2004; 22:653–658.
14. Jensen TB, Rahbek O, Overgaard S, Søballe K. No effect of platelet-rich plasma with frozen or processed bone allograft around noncemented implants. Int Orthop 2005; 29:67–72.
15. Palmisano W, Degen M, Block MS. Craniotomy bone defect healing with PRP combined with mineralized bone. J Oral Maxillofac Surg 2002; 60:39.
16. Harris D, Farrell B, Block MS. Zygomatic arch defects grafted with mineralized bone with PRP or PPP in dogs. J Oral Maxillofac Surg 2003; 61:42.
17. Dudziak ME, Block MS. Ridge augmentation with PRP/PPP and mineralized bone in dogs. J Oral Maxillofac Surg 2003; 61:41–42.
18. Aghaloo TL, Moy PK, Freymiller EG. Evaluation of platelet-rich plasma in combination with freeze-dried bone in the rabbit cranium. A pilot study. Clin Oral Implants Res 2005; 16:250–257.
19. Wallace SS, Froum SJ. Effect of maxillary sinus augmentation on the survival of endosseous dental implants. A systematic review. Ann Periodontol 2003; 8:328–343.

20. Sánchez AR, Sheridan PJ, Kupp LI. Is platelet-rich plasma the perfect enhancement factor? A current review. Int J Oral Maxillofac Implants 2003; 18:93–103.
21. Anitua E, Sánchez M, Nurden AT, Nurden P, Orive G, Andía I. New insights into and novel applications for platelet-rich fibrin therapies. Trends Biotechnol 2006; 24:227–234.
22. Marx RE. Platelet-rich plasma (PRP): what is PRP and what is not PRP? Implant Dent 2001; 10:225–228.
23. Heyligers IC, Klein-Nulend J. Detection of living cells in non-processed but deep-frozen bone allografts. Cell Tissue Bank 2005; 6:25–31.
24. Stacchi C, Orsini G, Di Iorio D, Breschi L, Di Lenarda R. Clinical, histological, and histomorphometric analyses of regenerated bone in maxillary sinus augmentation using fresh frozen human bone allografts. J Periodontol 2008; 79:1789–1796.
25. Messora MR, Nagata MJH, Mariano RC, et al. Bone healing in critical-size defects treated with platelet-rich plasma: a histologic and histometric study in rat calvaria. J Periodontal Res 2008; 43:217–223.
26. Messora MR, Nagata MJ, Dornelles RC, et al. Bone healing in critical-size defects treated with platelet-rich plasma activated by two different methods. A histologic and histometric study in rat calvaria. J Periodontal Res 2008; 43:723–729.
27. Reikerås O, Sigurdsen UW, Shegarfi H. Impact of freezing on immunology and incorporation of bone allograft. J Orthop Res 2010; 28:1215–1219.
28. Bos GD, Goldberg VM, Zika JM. Immune responses of rats to frozen bone allografts. J Bone Joint Surg Am 1983; 65:239–246.
29. Muscolo DL, Kawai S, Ray RD. In vitro studies of transplantation antigens present on bone cells in the rat. J Bone Joint Surg Br 1977; 59:342–348.
30. Stevenson S, Li XQ, Martin B. The fate of cancellous and cortical bone after transplantation of fresh and frozen tissue-antigen-matched and mismatched osteochondral allografts in dogs. J Bone Joint Surg Am 1991; 73:1143–1156.
31. Stevenson S, Li XQ, Davy DT, Klein L, Goldberg VM. Critical biological determinants of incorporation of non-vascularized cortical bone grafts. Quantification of a complex process and structure. J Bone Joint Surg Am 1997; 79:1–16.
32. Muscolo DL, Ayerza MA, Calabrese ME, et al. Human leukocyte antigen matching, radiographic score, and histological findings in massive frozen bone allografts. Clin Orthop Relat Res 1996; 326:115–126.
33. Stevenson S. The immune response to osteochondral allografts in dogs. J Bone Joint Surg Am 1987; 69:573–582.
34. Strong DM, Friedlaender GE, Tomford WW, et al. Immunologic responses in human recipients of osseous and osteochondral allografts. Clin Orthop Relat Res 1996; 326: 107–114.
35. Reikerås O, Shegarfi H, Naper C, Reinholt FP, Rolstad B. Impact of MHC mismatch and freezing on bone graft incorporation: an experimental study in rats. J Orthop Res 2008; 26:925–931.
36. Virolainen P, Vuorio E, Aro HT. Different healing rates of bone autografts, syngeneic grafts, and allografts in an experimental rat model. Arch Orthop Trauma Surg 1997; 116:486–491.
37. Marx RE. Platelet-rich plasma: evidence to support its use. J Oral Maxillofac Surg 2004; 62:489–496.
38. Roussy Y, Bertrand Duchesne MP, Gagnon G. Activation of human platelet-rich plasmas: effect on growth factors release, cell division and in vivo bone formation. Clin Oral Implants Res 2007; 18:639–648.
39. Fréchette JP, Martineau I, Gagnon G. Platelet-rich plasmas: growth factor content and roles in wound healing. J Dent Res 2005; 84:434–439.
40. Gruber R, Kandler B, Fischer MB, Watzek G. Osteogenic differentiation induced by bone morphogenetic proteins can be suppressed by platelet-released supernatant in vitro. Clin Oral Implants Res 2006; 17:188–193.
41. Choi BH, Zhu SJ, Kim BY, Huh JY, Lee SH, Jung JH. Effect of platelet-rich plasma (PRP) concentration on the viability and proliferation alveolar bone cells: an in vitro study. Int J Oral Maxillofac Surg 2005; 34:420–424.
42. Ogino Y, Ayukawa Y, Kukita T, Koyano K. The contribution of platelet derived growth factor, transforming growth factor-beta1, and insulin-like growth factor-I in platelet-rich plasma to the proliferation of osteoblast-like cells. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006; 101:724–729.
43. Efeoglu C, Akcay YD, Erturk S. A modified method for preparing platelet-rich plasma: an experimental study. J Oral Maxillofac Surg 2004; 62:1403–1407.
44. Dugrillon A, Eichler H, Kern S, et al. Autologous concentrated platelet-rich plasma (cPRP) for local application in bone regeneration. Int J Oral Maxillofac Surg 2002; 31:615–619.
45. Maitz P, Kandler B, Fischer MB, et al. Activated platelets retain their potential to induce osteoclast-like cell formation in murine bone marrow cultures. Platelets 2006; 17:477–483.
46. Ogino Y, Ayukawa Y, Kukita T, et al. Platelet-rich plasma suppresses osteoclastogenesis by promoting the secretion of osteoprotegerin. J Periodontal Res 2009; 44:217–224.
47. Cenni E, Avnet S, Fotia C, Salerno M, Baldini N. Platelet-rich plasma impairs osteoclast generation from human precursors of peripheral blood. J Orthop Res 2010; 28:792–797.
48. Nagata MJ, Messora M, Pola N, et al. Influence of the ratio of particulate autogenous bone graft/platelet-rich plasma on bone healing in critical-size defects: a histologic and histometric study in rat calvaria. J Orthop Res 2010; 28:468–473.

49. Grageda E. Platelet-rich plasma and bone graft materials: a review and a standardized research protocol. Implant Dent 2004; 13:301–309.
50. Weibrich G, Kleis WK, Hafner G, Hitzler WE. Growth factor levels in platelet-rich plasma and correlations with donor age, sex, and platelet count. J Craniomaxillofac Surg 2002; 30:97–102.
51. Martineau I, Lacoste E, Gagnon G. Effects of calcium and thrombin on growth factor release from platelet concentrates: kinetics and regulation of endothelial cell proliferation. Biomaterials 2004; 25:4489–4502.