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Bone healing in critical-size defects treated with platelet-rich plasma: a histologic and histometric study in rat calvaria

M. R. Messora[1], M. J. H. Nagata[1], R. C. Mariano[2], R. C. M. Dornelles[3], S. R. M. Bomfim[4], S. E. Fucini[1], V. G. Garcia[1], A. F. Bosco[1]

[1]Division of Periodontics, Department of Surgery and Integrated Clinic, Dental School of Araçatuba, São Paulo State University – UNESP, Brazil, [2]Division of Surgery and Traumatology Bucco-Maxillo-Facial, Department of Clinic and Surgery, Dental School, Federal University of Alfenas – UNIFAL, Brazil, [3]Division of Physiology, Department of Basic Sciences, Dental School of Araçatuba, São Paulo State University – UNESP, Brazil and [4]Division of Clinic Laboratory, Department of Clinic, Surgery and Animal Reproduction, Veterinary School of Araçatuba, São Paulo State University – UNESP, Brazil

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Background and Objective: The purpose of this study was to analyze histologically the influence of autologous platelet-rich plasma on bone healing in surgically created critical-size defects in rat calvaria.

Material and Methods: Thirty-two rats were divided into two groups: the control group (group C) and the platelet-rich plasma group. An 8-mm-diameter critical-size defect was created in the calvarium of each animal. In group C the defect was filled by a blood clot only. In the platelet-rich plasma group, 0.35 mL of platelet-rich plasma was placed in the defect and covered by 0.35 mL of platelet-poor plasma. Both groups were divided into subgroups ($n = 8$) and killed at either 4 or 12 wk postoperatively. Histometric (using image-analysis software) and histologic analyses were performed. The amount of new bone formed was calculated as a percentage of the total area of the original defect. Percentage data were transformed into arccosine for statistical analysis (analysis of variance, Tukey, $p < 0.05$).

Results: No defect completely regenerated with bone. The platelet-rich plasma group had a statistically greater amount of bone formation than group C at both 4 wk (17.68% vs. 7.20%, respectively) and 12 wk (24.69% vs. 11.65%, respectively) postoperatively.

Conclusion: Within the limits of this study, it can be concluded that platelet-rich plasma placed in the defects and covered by platelet-poor plasma significantly enhanced bone healing in critical-size defects in rat calvaria.

Maria José Hitomi Nagata, Rua Oscar Rodrigues Alves, 625 – Apt. 171, CEP: 16015–030 Araçatuba, SP, Brazil
Tel: +55 18 36217932
Fax: +55 18 36217932
e-mail: mjnagata@uol.com.br

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Researchers continuously strive to improve bone grafting techniques and to provide the means to obtain a faster and denser bony regeneration (1). Marx *et al*. (2) have proposed the use of platelet-rich plasma to obtain faster maturation of autogenous bone grafts. According to their clinical study, autogenous bone grafts with platelet-rich plasma demonstrated faster radiographic maturation and greater bone density than grafts without platelet-rich plasma. The rationale behind the use of platelet-rich plasma is the assumption that it may improve wound healing by increasing the levels of growth factors in the wound site after degranulation of the platelets. Among

the growth factors present in platelet-rich plasma, both platelet-derived growth factor and transforming growth factor-β have demonstrated their potential to increase bone healing in several *in vitro and in vivo* studies (3–6).

Despite the promising results reported by Marx *et al*. (2), clinical studies evaluating the addition of platelet-rich plasma to diverse grafts and alloplastic materials used for sinus lift (7,8), localized ridge augmentation (9), bone restoration of a severely atrophic mandible (10) and treatment of periodontal defects (11,12), have yielded contradictory results regarding bone formation and maturation.

Contradictory results have also been reported in animal studies. The influence of platelet-rich plasma has been evaluated on osseous healing of dental implants (13), in sinus lift combined with either bovine hydroxyapatite (14) or autogenous bone grafts (15), in mandibular defects associated with autogenous bone grafts (16,17) and in cranial defects combined with autogenous bone (18), anorganic bovine bone (19) or freeze-dried bone (20).

However, as the grafts and biomaterials are usually osteoconductive, they could potentially obscure the genuine effect of platelet-rich plasma (21). To date, only a few studies have been conducted to evaluate the influence of platelet-rich plasma alone on bone healing. The treatment of bony defects with platelet-rich plasma alone, in either femurs (22) or calvaria of rabbits (20) or mandibles of dogs (23), showed no improvement in bone formation. Conversely, favorable results were reported by Anitua (24), Sammartino *et al*. (25) and Simon *et al*. (26) in bone healing of extraction sockets treated with platelet-rich plasma only. Therefore, there are still no definitive conclusions about the biologic effect of platelet-rich plasma when it is used alone. It should be noted that in the above-mentioned studies, the lack of histometric analysis of newly formed bone in test sites (24) or in test and control sites (25), and the use of noncritical-size defects (20,24,25) are all potential factors that make it difficult to draw definitive conclusions.

The purpose of this study was to analyze histologically the influence of platelet-rich plasma on bone healing in surgically created critical-size defects in rat calvaria.

Material 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. Thirty-two, 5–6-mo-old male rats (*Rattus norvegicus*, *albinus*, Wistar) weighing 450–500 g (São Paulo State University – UNESP, Dental School of Araçatuba, Animal Care Unit) were used. The rats were kept in a room with a 12-h light/dark cycle and a temperature between 22 and 24°C. They were randomly assigned to one of two experimental groups: group C (control) or the platelet-rich plasma group.

Surgical procedure

The rats were anesthetized by an intramuscular injection of xylazine (6 mg/kg of body weight) and ketamine (70 mg/kg of 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. An 8-mm-diameter critical-size defect was made with a trephine used in a low-speed handpiece 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 (Fig. 1). Their purpose was to allow identification of the center line 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.

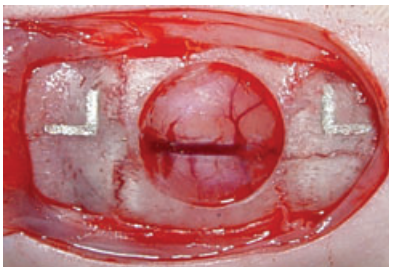

Fig. 1. Critical-size defect (8 mm diameter) and the two reference marks created on the calvarium.

In group C, the surgical defect was filled with a blood clot only. In the platelet-rich plasma group, the surgical defect was filled with 0.35 mL of platelet-rich plasma and covered with 0.35 mL of platelet-poor plasma.

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 of penicillin G-benzathine (Pentabiótico* Veterinário Pequeno Porte; Fort Dodge® Saúde Animal Ltda., Campinas, SP, Brazil) postsurgically.

Platelet-rich plasma preparation

A 3.15-mL volume of autologous blood was drawn from each animal, via cannulation of the jugular vein, into a syringe containing 0.35 mL of 3.2% sodium citrate to prevent coagulation. The same volume of saline (3.15 mL) was then injected through the jugular vein to maintain the systemic blood volume of the animal. The blood sample was centrifuged at 160 *g* for 20 min at 22°C to separate the plasma containing the platelets from the red cells (Beckman J-6M Induction Drive Centrifuge; Beckman Instruments Inc., Palo Alto, CA, USA). The plasma was drawn off the top and centrifuged at 22°C for an additional 15 min at 400 *g* to separate the platelets. The platelet-poor plasma was then drawn off the top, leaving the platelet-rich plasma and buffy coat. Then, the buffy coat and platelet-rich plasma (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 both the platelet-rich plasma and the platelet-poor plasma samples (0.05 mL of calcium chloride for each 1 mL of platelet-rich plasma or platelet-poor plasma).

Platelet counts

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

Tissue processing

Each group of animals was divided into two subgroups for killing either 4 or 12 wk 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% EDTA solution. After an initial decalcification, each specimen was divided longitudinally into two blocks, exactly along the center line 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 12 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 (Fig. 2). After additional decalcification, they were processed and embedded in paraffin. Serial sections, 6 μm thick, were cut in a 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.

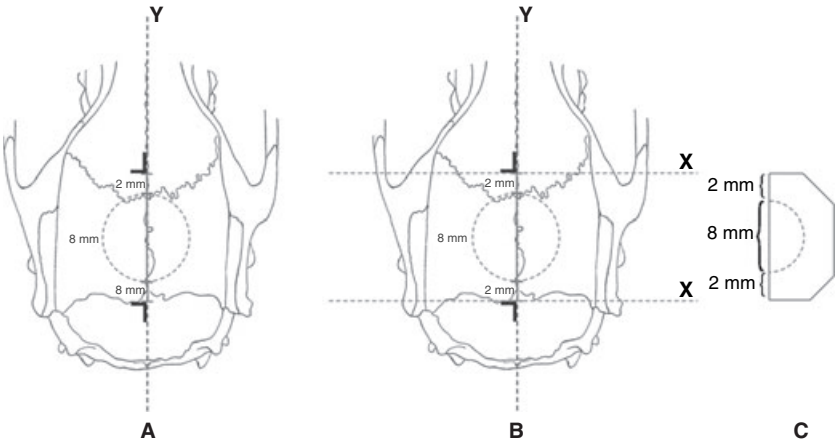

Fig. 2. (A) Longitudinal cut along the center line (Y) of the critical-size defect. (B) Transverse cuts (X). (C) Dimensions of the specimen to be embedded in paraffin.

Histomorphometric analysis

Four 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 ×32. The digital images were saved on a computer. A composite digital image was then created by combining three smaller images because it was not possible to capture the entire defect in one image at the level of magnification that was used. The composite image was created based on anatomic reference structures (e.g. blood vessels and bone trabeculae) within each of the histologic sections. The IMAGELAB 2000 software (Diracon Bio Informática Ltda., Vargem Grande do Sul, SP, Brazil) was used for the histomorphometric analysis.

The following criteria, based in part on the work of Melo *et al.* (27), were used to standardize the histomorphometric analysis of the digital images.

(a) The total area 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 towards the center in order to determine the margins of the original surgical defect. The newly formed bone area was delineated within the confines of the total area.
(b) The total area was measured in mm^2 and was considered to represent 100% of the area to be analyzed. The newly formed bone area was also measured in mm^2 and calculated as a percentage of the total area.

Statistical analysis

The values of newly formed bone area for each animal were represented by the mean percentage of the four histologic sections. These percentage data were transformed into arccosine for the statistical analysis. The significance of differences between groups in relation to the newly formed bone area 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 ($p < 0.05$).

Pearson's correlation coefficient (r_p) was used to demonstrate the relationship between the newly formed bone area and the platelet counts from the platelet-rich plasma samples, as well as between the platelet counts from the platelet-rich plasma and whole-blood 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 platelet-rich plasma preparation technique used in this study produced samples of highly concentrated platelets. The platelet-rich plasma smears showed higher concentrations of platelets than the whole-blood smears. The average whole-blood platelet count was $676.06 \pm 157.37 \times 10^3/\mu L$, whereas the average platelet-rich plasma platelet count was $2644.06 \pm 1084.85 \times 10^3/\mu L$ (Fig. 3). The concentration of the platelets in platelet-rich plasma was increased by almost fourfold.

Qualitative histologic analysis

Group C — At 4 wk, almost all of 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 surrounded by a small number of osteoblasts was restricted to areas close to the borders of the surgical defect (Fig. 4A). At 12 wk, most specimens presented similar bone formation when compared with the 4-wk specimens (Fig. 4B), whereas a few showed increased bone formation. The connective tissue presented a moderate number of fibroblasts and many collagen fibers.

Platelet-rich plasma group — At 4 wk, most specimens presented a greater amount of newly formed bone close to the borders of the surgical defect when compared with the specimens of group C (Fig. 4C). The newly formed bone was surrounded by a small number of osteoblasts. The connective tissue presented a moderate number of fibroblasts and numerous collagen fibers, which were thicker and more organized than the ones observed in the specimens of group C. At 12 wk, all specimens presented newly formed bone, surrounded by a small number of osteoblasts, extending towards the center of the defect. In three specimens, the newly formed bone extended almost the entire length of the surgical defect. However, it was thinner than that of the original calvarium (Fig. 4D). The connective tissue showed similar characteristics to those observed in the 4-wk specimens.

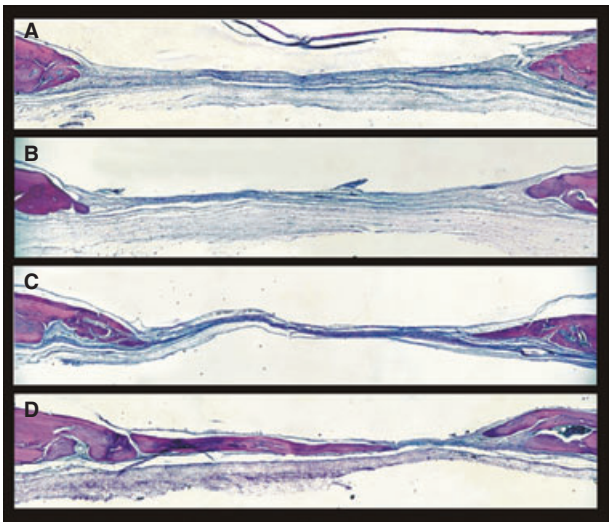

Fig. 4. Panoramic views of the surgical defects. (A) Control group (group C), 4 wk; (B) group C, 12 wk; (C) platelet-rich plasma group, 4 wk; (D) platelet-rich plasma group, 12 wk. Hematoxylin and eosin staining; original magnification ×25.

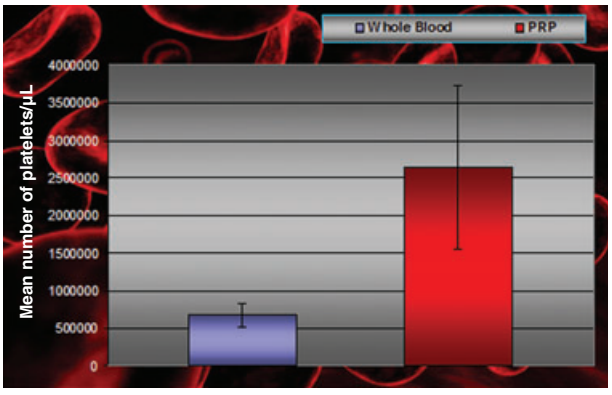

Fig. 3. Mean number of platelets per microliter and standard deviations in the samples of platelet-rich plasma and whole blood. PRP, platelet-rich plasma.

Histometric and statistical analyses

The normality and homogeneity of variance of the data were verified. Means and standard deviations of the area of newly formed bone for both groups, as well as the comparison between the groups, at postoperative weeks 4 and 12, are documented in Fig. 5. No statistically significant correlation was observed between the platelet count from the whole-blood and platelet-rich plasma samples ($r_p = 0.36$, $p = 0.167$) or between the platelet

count from the platelet-rich plasma samples and the newly formed bone area ($r_p = 0.21$, $p = 0.441$).

Discussion

Qualitative and/or quantitative alterations of the platelets may affect the regenerative potential of platelet-rich plasma. Therefore, several fundamental aspects should be considered in the platelet-rich plasma preparation. The urgent need for studies using standardized protocols to evaluate the real biological effects of platelet-rich plasma has been emphasized by Grageda (28) and Marx (29). Thus, the protocol of the present study was designed to optimize the quantity and quality of the platelets in platelet-rich plasma samples in a number of ways. With regard to the quantity of platelets, the double-centrifugation technique used in this study, as recommended by Marx (30), resulted in an average increase of $\approx$390% in the concentration of platelets in the platelet-rich plasma when compared with that observed in the whole blood. According to Marx (29), platelet-rich plasma with this concentration of platelets could be considered a 'therapeutic platelet-rich plasma'. Animal studies have demonstrated that the use of 'therapeutic platelet-rich plasma' increased bone formation (31,32). With regard to the quality of platelets, several fundamental aspects were considered, in the present study, to guarantee their integrity in the platelet-rich plasma samples, such as the choice of anticoagulant and activator, the speed of the centrifugation and the amount of time between activation of the platelet-rich plasma and its clinical use (30,33,34). According to Marx (29), platelets damaged or rendered nonviable by platelet-rich plasma processing will not secrete bioactive growth factors and may result in disappointing outcomes.

In the present study, the platelet-rich plasma samples were covered by platelet-poor plasma. The platelet-poor plasma was used to confine the platelet-rich plasma inside the limits of the surgical defect because platelet-poor plasma has a firmer consistency than platelet-rich plasma. The presence of fibronectin, fibrin and factor XIII in platelet-poor plasma (35) may have increased stability and healing of the flap over the surgical defect. In addition, the platelet-poor plasma may have also contributed to a greater bioavailability of the growth factors present in the platelet-rich plasma. This inference is based on the results of *in vitro* studies, which demonstrated that the retention and temporal availability of growth factors in platelet-rich plasma samples were affected by the use of other biomaterials with platelet-rich plasma (36,37).

The definition of 'therapeutic platelet-rich plasma', proposed by Marx (29), was based only on the concentration of platelets. However, other aspects should also be taken into consideration. Grageda (28) suggested a standardized protocol for future studies evaluating the biological effects of platelet-rich plasma. As part of this protocol, Grageda (28) suggested assessing the correlation between the histormorphometric analysis and the number of platelets in the platelet-rich plasma. In the present study, no significant correlation could be found between the platelet count in the platelet-rich plasma samples and in the newly formed bone area. As the regenerative potency of platelet-rich plasma undoubtedly depends on its growth factor levels, it could be inferred that this result supports the findings of Weibrich *et al.* (38), who demonstrated that neither whole-blood nor platelet-rich plasma platelet counts are reliably predictive of the resultant growth factor levels in platelet-rich plasma. These findings are also corroborated by recent studies that observed differences in growth factors levels in platelet-rich plasma samples with the same concentration of platelets (39,40). According to Weibrich *et al.* (38), knowledge of the growth factor levels in platelet-rich plasma samples is necessary to ensure reliable and reproducible use of platelet-rich plasma for clinical treatment. Unfortunately, there is no simple procedure available for obtaining pre-operative estimates of individual growth factor levels in platelet-rich plasma samples. Therefore, further studies on this topic are needed.

Another important factor in evaluating the biological effect of platelet-rich plasma is to use only autologous platelet-rich plasma. According to Marx (29), there have been some studies evaluating platelet-rich plasma in animal models with blood volumes that were too small to produce platelet-rich plasma, and thus donor blood had to be used. Marx (29) does not consider this to be true platelet-rich plasma because it is homologous, rather then autologous, platelet-rich plasma. The biological responses may have been influenced by immune reactions leading to false-negative results. In the present study, a technique was developed to allow the production of autologous platelet-rich plasma, bearing in mind that rats have a very small blood volume.

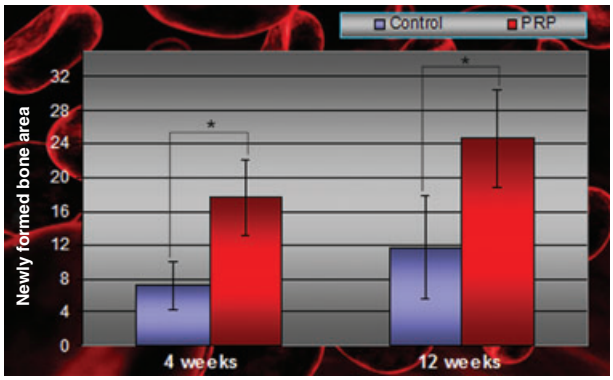

Fig. 5. Means of the newly formed bone area (expressed as a percentage of the total defect area) and standard deviations for the control group (group C) and the platelet-rich plasma group, 4 and 12 wk postoperatively. PRP, platelet-rich plasma. *Statistical difference between the groups ($p < 0.05$).

Besides all of the previously mentioned factors related to the platelet-rich plasma sample itself, the use of a critical-size defect is necessary to evaluate the real influence of any biomaterial on bone healing. In the present study, a critical-size defect was used, as confirmed by the limited bone formation observed in group C (Fig. 5).

In summary, this study evaluated the use of autologous platelet-rich plasma on bone healing in critical-size defects, with great care having been taken to guarantee the quantity and quality of the platelets. Therefore, the significant increase in bone formation observed in the platelet-rich plasma group when compared with group C at both time-points of analyses (Fig. 5) was a result of the inherent biological properties of the platelet-rich plasma. This increase in bone formation was possibly caused by the interaction of bone-forming cells in the dura mater of the surgical defects with an increased concentration of growth factors in the platelet-rich plasma (29,30). Because the dura mater is the main source of bone-forming cells in the calvarium (41), preservation of its integrity during the creation of the surgical defects was fundamentally important to the results obtained in this study. Besides growth factors, the platelet-rich plasma contains fibrin, fibronectin and vitronectin. These proteins act as cell-adhesion molecules for osteconduction and as a matrix for bone formation (29). Also, the platelet membranes can stimulate the mitogenic activity of bone cells, thereby contributing to the regeneration of mineralized tissue (42).

In this study, it is reasonable to speculate that platelet-rich plasma may have acted as a biological membrane in the treated defects. This hypothesis was proposed by Lekovic *et al*. (12) in a clinical study that evaluated the treatment of intrabony defects either with platelet-rich plasma, bovine porous bone mineral and guided tissue regeneration, or with platelet-rich plasma and bovine porous bone mineral only. As guided tissue regeneration has not shown any additional benefit to the result obtained with platelet-rich plasma and bovine porous mineral only, the authors suggested that platelet-rich plasma may have exerted a guided tissue regeneration effect in the treated defects, impeding the apical migration of epithelial cells and connective tissue cells from the flap.

The results of the present study corroborate the findings of previous studies that have also demonstrated improved bone formation when platelet-rich plasma was used alone around implants (13,43) or in third molar extraction sockets (26). Conversely, no additional benefit in bone healing was observed by Yamada *et al*. (23) when platelet-rich plasma was used alone in mandibular defects when compared with controls in a study conducted in dogs. Variations in the platelet-rich plasma concentration (44), as well as in the level of growth factors available in the platelet-rich plasma used, may be some of the factors that could explain these contradictory results.

Within the limits of this study, it can be concluded that autologous platelet-rich plasma placed in the defects and covered by platelet-poor plasma significantly enhanced bone healing in critical-size defects in rat calvaria.

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