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Bone healing in critical-size defects treated with platelet-rich plasma activated by two different methods. A histologic and histometric study in rat calvaria

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Background and Objective: The purpose of this study was to analyze histologically the influence of platelet-rich plasma (PRP) coagulated with two different activators on bone healing in surgically created critical-size defects (CSD) in rat calvaria.

Material and Methods: Forty-eight rats were divided into three groups: C, PRP-C and PRP-T. An 8 mm diameter CSD was created in the calvarium of each animal. In group C, the defect was filled by a blood clot only. In groups PRP-C and PRP-T, the defect was filled with PRP activated with either calcium chloride or thromboplastin solution, respectively. Each group was divided into two subgroups ($n = 8$ per subgroup) and killed at either 4 or 12 weeks postoperatively. Histologic and histometric 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's *post hoc* test, $p < 0.05$).

Results: No defect completely regenerated with bone. Group PRP-C had a statistically greater amount of bone formation than groups C and PRP-T at both time points of analysis. No statistically significant differences were observed between groups C and PRP-T.

Conclusion: It can be concluded that the type of activator used to initiate PRP clot formation influences its biological effect on bone healing in CSD in rat calvaria.

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The use of platelet-rich plasma (PRP) as an adjunct to bone grafting procedures in oral and maxillofacial surgery has increased in popularity since its introduction in 1997 by Whitman *et al.* (1). The rationale behind the use of PRP is the assumption that it may improve wound healing by increasing the levels of growth factors (GFs) in

the wound site after degranulation of the platelets. Marx *et al*. (2) have proposed the use of PRP to obtain faster maturation of autogenous bone grafts. According to their clinical study, autogenous bone grafts with PRP demonstrated faster radiographic maturation and greater bone density than grafts without PRP. Since then, this biomaterial has been successfully used in a variety of clinical applications to improve hard and soft tissue formation (3–7). However, other studies have reported no significant benefits of the use of PRP in bone reconstructive surgeries (8–11), reinforcing the need for controlled studies designed to increase understanding of the roles of GFs released from PRP in wound healing (12).

Preparation of PRP requires the concentration of platelets by centrifugation and subsequent polymerization to form a semisolid gel. At present, almost all methods of PRP gelation use calcium and bovine thrombin (13). It has been shown that thrombin is a potent agent for platelet aggregation and induction of release of GFs (12). Based on the known sequence of events that takes place during bone regeneration, it is desirable to have platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) present in the early phases, followed by TGF-β during the intermediate phase, and insulin-like growth factor 1 (IGF-1) and bone morphogenetic proteins (BMPs) during the final phase of bone differentiation and maturation (14). Growth factors activated in the proper temporal sequence and present in the appropriate spatial distribution have a remarkable effect on bone regeneration (13).

Several *in vitro* studies have reported differences in retention and temporal availability of GFs, suggesting that their bioavailability is related to the methods used to obtain PRP gelation (12–16). Martineau *et al*. (12) have shown that the concentration of GFs released from PRP varied over time and was regulated by the amount of calcium and thrombin added. Materials other than thrombin, such as thrombin receptor agonist peptide (TRAP) and bone substitutes, may be more effective in sustaining levels of GFs that are critical for the cascade of events leading to bone formation (13). Other studies have shown that PRP activated with calcium chloride alone has a stimulatory effect on human osteoblasts (17).

Therefore, retention of GFs has been shown to be a function of the substance used to initiate PRP clot formation as well as the specific growth factor examined (12–16). Since bone regeneration undoubtedly depends on the levels of GFs, it could be speculated that the agents used to initiate PRP clot formation may affect the regenerative potency of PRP. Further studies are needed to evaluate this hypothesis with respect to bone formation *in vivo*.

The purpose of this study was to analyze histologically the influence of PRP coagulated with two different activators on bone healing in surgically created critical-size defects (CSD) in rat calvaria.

Material and methods

Experimental model

The experimental protocol was approved by the UNESP – Dental School of Araçatuba Institutional Animal Care and Use Committee. Forty-eight, 5- to 6-month-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–12 h light–dark cycle and temperature between 22 and 24°C. They were randomly assigned to one of three experimental groups: group C (control), group PRP-C (platelet-rich plasma activated with 10% solution of calcium chloride) and group PRP-T (platelet-rich plasma activated with thromboplastin solution).

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. An 8 mm diameter CSD was made with a trephine used in a low-speed handpiece under continuous irrigation with sterile saline. 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.

In group C, the surgical defect was filled with a blood clot only. In groups PRP-C and PRP-T, the surgical defects were filled with 0.35 mL of PRP activated with either a solution of 10% calcium chloride or 25% thromboplastin, respectively. The PRP was then covered with 0.35 mL of platelet-poor plasma (PPP).

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

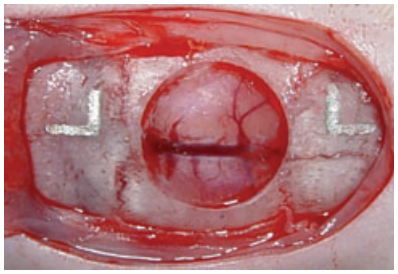

Fig. 1. Critical-size defect (8 mm diameter) and the two reference marks created on the calvarium. Suprerior view.

Ltda., Campinas, SP, Brazil) post-surgically.

Preparation of PRP

Just before the surgical procedures, under anesthesia, a sample of 3.15 mL 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. The blood sample was centrifuged at 160*g* for 20 min 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 for an additional 15 min at 400*g* to separate the platelets. The PPP 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. Next, 0.017 mL of 10% solution of calcium chloride (calcium chloride 10% solution, ScienceLab.com Inc., Houston, TX, USA) and 0.7 mL of 25% solution of thromboplastin solution (Thromboplastin C^{TM}, Baxter Diagnostics, Miami, FL, USA) were used to activate the PRP samples in groups PRP-C and PRP-T, respectively.

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.

Clot retraction analysis

Aliquots of PRP were dispensed into 18-well plates. Nine wells were activated with 10% calcium chloride solution (group PRP-C), while the remaining wells were activated with thromboplastin solution (group PRP-T).

At 30 min, if still attached, the clots were gently released from the sides of the well with a pipette tip. Clot retraction was determined by measuring the clot diameter at 24 h, and then comparing that value with the well diameter. Clot retraction analysis was performed by an examiner blinded with respect to the treatment rendered. The 'ImageLab 2000' software (Diracon Bio Informática Ltda., Vargem Grande do Sul, SP, Brazil) was used for the analysis.

Tissue processing

Each group of animals was divided into two subgroups and euthanized with an overdose of pentobarbital (Thiopentax®, Cristália Produtos Químicos e Farmacêuticos Ltda., São Paulo, SP, Brazil) at either 4 or 12 weeks post-operative. 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 center line of the original surgical defect using the long axis of both L-shaped marks as references. Transverse cuts were then made using the short axis of each L-shaped 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

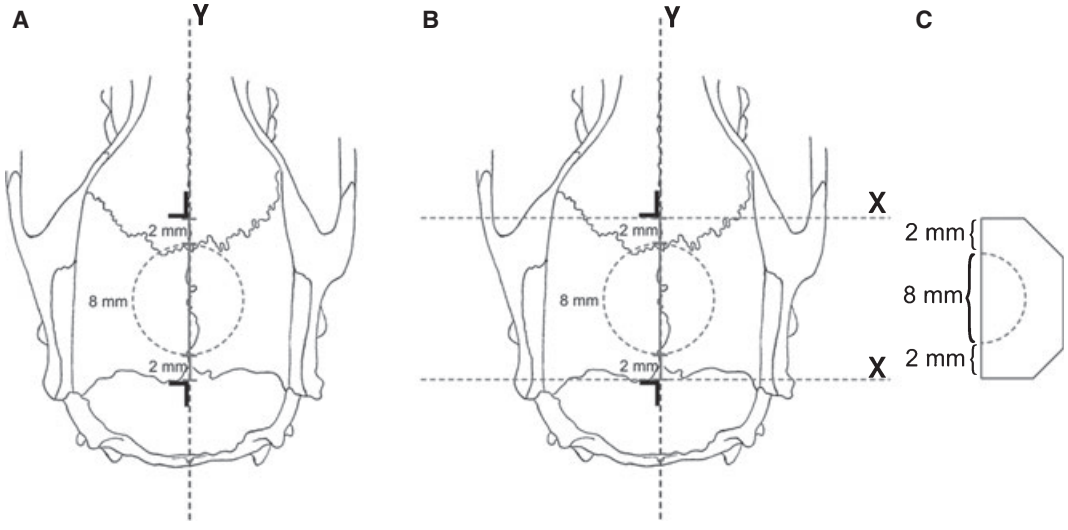

Fig. 2. (A) Longitudinal cut along the center line (Y) of critical-size defect; (B) transverse cuts (X); and (C) dimensions of specimen to be embedded in paraffin.

center of the original surgical defect. The sections were stained with either hematoxylin and eosin (H&E) or Masson's Trichrome for analysis by light microscopy.

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.) was used for the histomorphometric analysis.

The following criteria, based in part on the work of Melo *et al.* (18), 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 were 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 (NFBA) was delineated within the confines of the TA.
2 The TA was measured in millimetres squared and was considered 100% of the area to be analyzed. The NFBA was also measured in millimetres squared and calculated as a percentage of TA.

Statistical analysis

The values of NFBA 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 NFBA was determined by an analysis of variance, followed by Tukey's *post hoc* 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 NFBA and the platelet counts from the PRP samples, as well as between the platelet counts from the PRP and whole blood samples.

The clot retraction data were analyzed using Student's unpaired *t*-test. A significance level of 0.05 was chosen.

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 681.25 ± 120.77 × $10^3/\mu L$ while the average PRP platelet

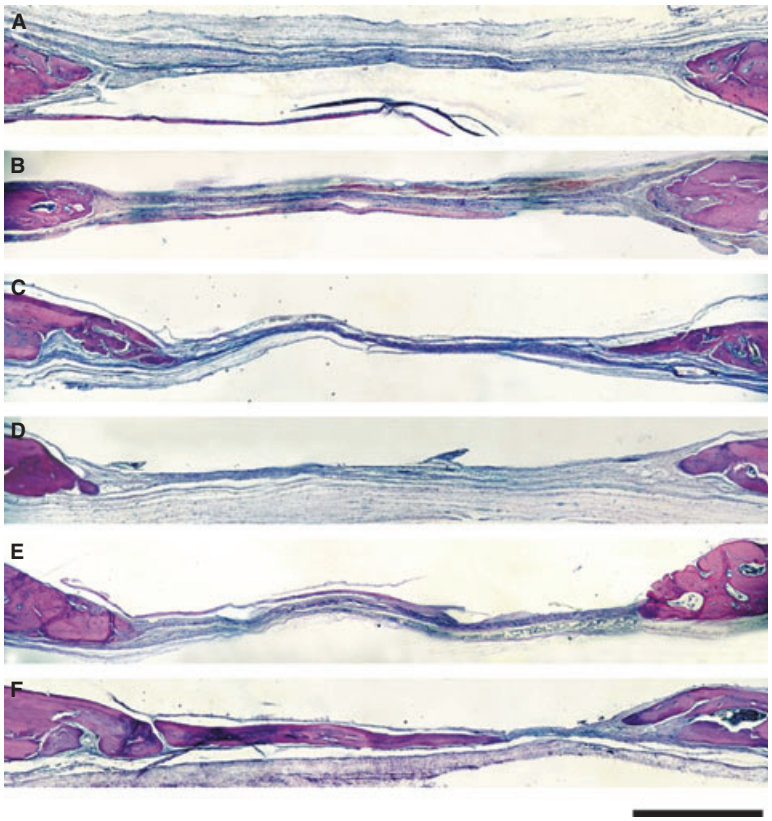

Fig. 3. Panoramic views of the surgical defects showing newly formed bone. (A) Group C at 4 weeks; (B) group PRP-T at 4 weeks; (C) group PRP-C at 4 weeks; (D) group C at 12 weeks; (E) group PRP-T at 12 weeks; and (F) group PRP-C at 12 weeks. H&E. Original magnification ×25. Scale bar = 2mm.

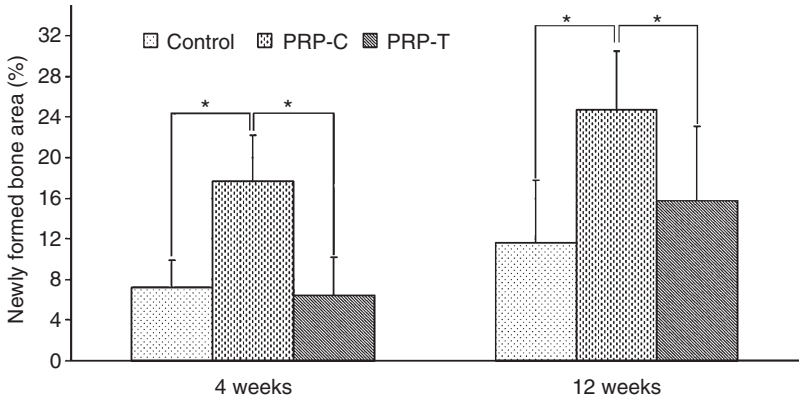

Fig. 4. Means of newly formed bone area (% of total defect area) and standard deviations for groups C, PRP-C and PRP-T, 4 and 12 weeks post-operative. *Statistical difference between the groups ($p < 0.05$).

count was $2977.66 \pm 1174.83 \times 10^3/\mu L$. The concentration of the platelets in PRP was increased by more than fourfold.

Clot retraction analysis

Group PRP-T showed significantly more clot retraction ($46.85 \pm 8.02\%$) than group PRP-C ($29.88 \pm 5.38\%$) at 24 h ($p < 0.05$).

Qualitative histologic analysis

The histologic events observed both at 4 and 12 weeks post-operative are presented in Fig. 3. Greater bone formation was seen in group PRP-C than in groups C and PRP-T at both time points of analysis. At 4 and 12 weeks almost all of the surgical defect in groups C and PRP-T was occupied by connective tissue.

Histometric and statistical analyses

The data normality was verified. Means and standard deviations of NFBA for both groups, as well as the comparison between the groups, at 4 and 12 weeks post-operative are documented in Fig. 4. No statistically significant correlation was observed between the platelet count from the whole blood and PRP samples ($r_p = 0.2362$, $p = 0.2007$) nor between the platelet count from the PRP samples and the NFBA ($r_p = -0.1457$, $p = 0.4423$).

Discussion

Recent *in vitro* studies have shown that some methods used to coagulate PRP can interfere with the release of some GFs by the platelets (12–16), and thus may also affect the biological response of PRP *in vivo*. The present study histologically analyzed the influence of PRP coagulated with either calcium chloride or thromboplastin on bone healing in surgically created CSD in rat calvaria.

Qualitative and/or quantitative alterations of the platelets may affect the regenerative potential of PRP. Thus, the protocol used in this study was designed to optimise the quantity and quality of the platelets in PRP samples in a number of ways. With regard to the quality of platelets, several fundamental aspects were considered in the present study to guarantee their integrity in the PRP samples, such as the choice of the anticoagulant, the speed of the centrifugation and the amount of time between activation of the PRP and its clinical use (19–21). According to Marx (22), platelets damaged or rendered non-viable by PRP processing will not secrete bioactive growth factors and may result in disappointing outcomes. With regard to the quantity of platelets, the double-centrifugation protocol used in this study resulted in an average increase of approximately 390% in the concentration of platelets in the PRP when compared with that observed in the whole blood. Previous animal studies (23,24) have demonstrated that the use of PRP samples with similar platelet concentration increased bone formation.

In the present study, although similar platelet concentrations were used in groups PRP-C and PRP-T, two distinct healing outcomes were observed. These results may possibly be attributed to two factors. The first factor is that the activator used to initiate PRP clot formation could have had a direct influence on the wound healing response. It has been demonstrated that thromboplastin is not only a coagulation agent, but is also a strong chemotactic factor for cultured vascular smooth muscle cells (25). It has also been demonstrated that application of calcium chloride to a wound surface resulted in a significant increase in granulation tissue production (26). The second factor that may help to explain the results observed in the present study is the variations in the levels of GFs. This hypothesis is based on the results of other studies that have shown differences in the levels of GFs in PRP samples with similar platelet concentrations (12,15,27). In the present study, no significant correlation could be found between the platelet count in the PRP samples and the NFBA. Since the regenerative potency of PRP undoubtedly depends on its levels of GFs, it could be inferred that this result supports the findings of Weibrich *et al.* (27), who demonstrated that neither whole blood nor PRP platelet counts are reliably predictive of the resultant growth factor levels in PRP.

It should be taken into account that the efficiency of GFs in enhancing bone regeneration is likely to be dependent on their dosage, spatial distribution and temporal release (14). The bioavailability of GFs may be affected by the use of other biomaterials with PRP (14) and by the type of activator used to initiate PRP clot formation (12,13). Considering that in the present study the PRP was used alone in surgical defects, the variations in bioavailability of the GFs may have occurred owing to the type of activator used in the PRP samples. In contrast with what may have

occurred in group PRP-T, it is likely that the activator used in the PRP samples of group PRP-C promoted GFs levels, spatial distribution and temporal sequencing that favored bony healing of the surgical defects. Since the levels of GFs in the PRP samples of the present study were not measured owing to the experimental model used, further studies are necessary to confirm this hypothesis. In this study, a technique was developed to allow the production of autologous PRP even though rats have a very small blood volume (28). However, the PRP volume obtained from each animal was only enough to fill the surgical defects.

A positive biological effect of PRP activated only by calcium chloride solution has been previously demonstrated in clinical (3) as well as *in vitro* studies (17). It is important to emphasise that PRP activation by calcium chloride alone maintains its autogenous nature, avoiding the use of bovine thrombin and the thus the risk of developing potential life-threatening coagulopathies (29–31). Most studies that have evaluated the influence of PRP on bone healing used bovine thrombin and calcium chloride to initiate PRP clot formation (2,9,32,33). It has been shown that PRP samples activated with calcium chloride alone resulted in a clot with very poor structural integrity (13). In the present study, the calcium chloride itself was sufficient to initiate PRP clot formation. In addition, it also produced less clot retraction than the thromboplastin solution. This smaller clot retraction observed in group PRP-C may be intimately related to the greater amount of newly formed bone in this group. According to Landesberg *et al*. (13), decreasing the clot retraction in PRP coagulation will potentially retain optimal amounts of GFs over a longer period of time by delaying their bioavailability.

In order to favor bone healing, the PRP activator should ideally provide release of GFs from the platelets in a sequence that is strictly related to the chronology of events in bone formation. A key event in bone healing is angiogenesis. A greater stimulation of angiogenesis in the early phases of the healing process would increase bone formation at a later stage (15). The choice of thromboplastin solution as the activator in group PRP-T was based on this hypothesis. The intense chemotactic effect of thromboplastin on the vascular cells (25) could favor bone healing. However, the PRP activated by thromboplastin solution did not favor bone healing in the present study. Since the release and degradation of some GFs may also depend on the amount of the activator used to initiate PRP clot formation (12,34), further studies are needed to evaluate the effect of different dosages of thromboplastin solution.

In summary, this study demonstrated that PRP samples with similar platelet concentrations may produce different biological effects. These differences may be explained, in part, by the results of some *in vitro* studies which demonstrated that the activator used to initiate PRP clot formation influences GF levels, distribution and release kinetics.

Within the limits of this study, it can be concluded that the type of activator used to initiate PRP clot formation influences its biological effect on bone healing in CSD in rat calvaria.

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