Original

Evaluation of bioactive glass and platelet-rich plasma for bone healing in rabbit calvarial defects

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Abstract: Bone regeneration is an important objective in clinical dental practice and has been used for different applications. The aim of this study was to evaluate the effectiveness of platelet-rich plasma (PRP) and bioactive glass (BG) for bone healing of surgical calvarial defects in rabbits. Two 8-mm defects were prepared in the parietal bones of ten animals, and the animals were randomly assigned to two groups. In each group, two subgroups were created with five defects each: BC - blood clot, BG, PRP and PRP + BG. Thus, four treatments were performed with five specimens each. The animals were sacrificed after 12 weeks and the specimens were analyzed radiographically, histologically and histomorphometrically. Data were subjected to ANOVA and Tukey's tests (α = 0.05). Outcomes demonstrated that the PRP group had higher bone density (%) values than the groups not treated with PRP (P < 0.05). Histometrically, both groups treated with PRP (PRP: 25.6 ± 9.9; PRP+BG: 25.8 ± 12.4) demonstrated higher percentages of new bone formation than the groups not treated with PRP (BG: 6.1 ± 4.3; BC: 7.8 ± 5.6) (P < 0.05). The results suggested that PRP improved bone repair and that bioactive glass alone, or in association with PRP, did not improve bone healing.

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Introduction

Bone regeneration is a desirable objective in dental clinical practice. Regeneration of bone can be used for correction of traumatic bone loss or atrophic changes in the alveolar processes, or even for bone reconstruction after bone loss due to progression of periodontal disease. Several studies, in both animals and humans, have reported that bone regeneration can be achieved by bone grafting, with or without the use of growth factors (1-5).

Growth factors have an important role in the regulation and stimulation of wound healing, affecting cellular processes such as mitogenesis, chemotaxis, differentiation and metabolism (5-7). Platelets contain many of these growth factors (platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF- β), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF-I) and epithelial growth factor (EGF)) and platelet-rich plasma (PRP) can be obtained in high concentrations by centrifugation. The application of PRP was initially proposed by Marx et al. (6) and Whitman et al. (8) to enhance wound healing. Currently, PRP is used during implant placement, for the treatment of periimplant bone defects, and for sinus-lift procedures, as well as for maxillofacial and periodontal reconstructive surgery (9,10).

When used alone, PRP does not seem to exert any additional effects when compared with blood clots (11-14). For this reason, PRP has been used in association

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with bone grafts, as it can act as a scaffold material, since a high concentration of platelets can increase the local concentration of secreted growth factors inside bone defects and enhance bone regeneration. Marx et al. (6) demonstrated that a combination of bone graft substitutes and growth factors such as cytokines contained in PRP might be suitable for enhancing bone healing and density.

Basic studies have shown that application of PRP enhances early bone formation (12,15-17) or might have additional beneficial effects on bone regeneration (18-23), when used alone or mixed with bone grafts (4,14,24-26). However, the use of highly concentrated PRP has also been considered prejudicial to the repair of non-critical defects in the rabbit calvaria (27). Nagata et al. (19) have also indicated that there might be an optimal concentration of PRP beyond which harmful effects may occur.

Autogenous bone grafts are the gold standard for regeneration of bony defects in the craniofacial region. However, a variety of osteoconductive bone substitutes have been reported. With regard to clinical applications, other studies have used PRP in association with some bone substitutes, such as chitosan (28), particulate bone grafts (29-31), anorganic bovine bone allografts (1,3,17) and bioactive glass (32). Bioactive glass (BG) is a type of bioactive ceramic that has also been used in some studies to enhance bone formation (4,32-35). Demir et al. (4) tested a combination of BG and platelet-rich plasma for use in bone defects, and suggested that both the PRP/ BG combination and BG alone were effective. Keles et al. (34) also demonstrated a significant effect when bone defects were filled with PRP and BG. These studies demonstrated that PRP and BG were effective for reduction of pocket depth, filling of defects, and enhancement of clinical attachment gain.

Our hypothesis in designing the present study was that healing of a bone defect filled with BG would be more rapid when PRP was added. Accordingly, using radiographic, histologic and histomorphometric techniques, we evaluated the effects of BG and PRP for healing of calvarial defects in rabbits.

Materials and Methods

Animals

Ten male adult New Zealand rabbits (3.5 kg) were employed in the study. The animals were kept in plastic cages with access to food and water *ad libitum*. Prior to surgical creation of the calvarial defects, all animals were allowed to acclimate to the laboratory environment for a period of 7 days. The protocol was approved by the institutional Ethics Committee (027A/2006).

Surgical procedure

Twenty-four hours before the surgical procedures, the animals were fasted. General anesthesia was induced by intramuscular administration of 65 mg/kg ketamine and 4 mg/kg xylazine (36). Anesthesia was monitored and maintened by a veterinarian during all surgical processes. After removal of hair over the dorsal cranium, the surgical site was scrubbed with 70% alcohol followed by 10% iodine, and a mid-sagittal linear incision was made through the skin of the scalp from the nasal bone to the mid-sagittal cristae. A full-thickness flap including the periosteum was reflected, exposing the calvarial bone (parietal and frontal bones). After dissection and bone exposure, two standardized circular defects, each 8 mm in diameter, were created on two sides of the parietal bones using a trephine drill, with a 170 x g velocity, 45N torque, and abundant irrigation with sterile saline solution, exposing the dura mater.

The animals were then randomly assigned to two groups to avoid any inter-group crossover effect: Group 1 - blood clot only (BC) and BG only, Perioglas (Nova-Bone Products, Alachua, FL, USA); Group 2 - PRP only and PRP + BG in combination (PRP + BG). Four treatments were thus performed with five specimens each.

PRP preparation

Before the surgical procedure, the animals were subjected to blood collection and hemogram analysis to determine the platelet count. Blood was obtained from all animals several minutes after induction of anesthesia, including those in the non-PRP group, and the same procedures were conducted. Blood was collected for hemogram analysis by puncture of the auricular superficial vein and cardiac puncture for PRP preparation.

For PRP preparation, blood was drawn from each rabbit into two 5-mL silicone tubes containing 0.5 mL of 3.2% sodium citrate solution as an anticoagulant. The tubes were centrifuged at 170c for 10 min at room temperature, and the blood was thus separated into three basic fractions: red blood cells (at the bottom of the tube), PRP (a discrete gray layer in the middle of the tube) and platelet-poor plasma (PPP; at the top of the tube). The portion corresponding to the platelet plasma was transferred by pipette from the tube to another empty tube. PPP was centrifuged again at $170 \times g$ for 10 min, and approximately 50% of the supernatant, corresponding to the PPP, was removed; the platelet concentrate remaining was placed in a sterile vat at 37°C. Approximately 20 µL of 10% calcium chloride was then added to the preparation to activate the platelets and to form a platelet gel, after 10 to 15 min. When BG was

used, the graft was added immediately after addition of 10% calcium chloride. After formation of the gel, it was applied to predetermined bone defects, either alone or in combination with the BG.

After the bone defects had been filled, the soft tissues and periosteum were repositioned for total coverage and sewn with 4.0 resorbable suture (Vicryl suture 4.0, Ethicon Inc. /Johnson & Johnson, São José dos Campos, Brazil). After surgery, 1 mg/kg Flunixin Meglumine (Banamine, Schering-Plough Veterinary, Rio de Janeiro, Brazil) was administered by subcutaneous injection on three consecutive days, as well as antibiotics (Flotril 10% injection, Schering-Plough Veterinary) on the first and fourth days after surgery.

Histology and histomorphometry

After 3 months of healing, the animals were killed under general anesthesia, as described previously, followed by intravascular injection of 10 mL of 0.19% potassium chloride. The calvaria was removed and immediately fixed in buffered 10% formaldehyde solution (Lillie, pH 7.2), together with 100 mL of pure formaldehyde, 900 mL of deionized water, 6.5 g of Na₂HPO₄ and 4.0 g of NaH₂PO₄ H₂O, for a period of 48 h.

Radiographic analysis

Radiographs (Kodak Ultra-speed) of each specimen were taken with a Gnatus Timex 70 dispositive (70 kVp, 7 mA), with a time exposure of 0.32 s and a standard object-focus distance. The radiographs were developed in an automatic developer (Air Techniques, A/T 2000 XR) and digitalized with an HP Scanjet 3670 scanner (600 dpi, 8 bit gray scale). The images were saved and the gray density was further analyzed using Image J Software (version 1.37, http://rsb.info.nih.gov/ij).

Radiographic density, using a gray scale, was obtained as the mean of three measurements performed for each bone defect, with a standardized cursor delimiting the entire defect. These measurements were performed by a blinded and previously calibrated evaluator ($R^2 = 0.9488$).

Preparation of histological sections

After fixing, the specimens were demineralized for 5 days in a mixture of two solutions: equal parts of 88% formic acid in distilled water and 20% sodium citrate in distilled water. For paraffin embedding, each defect was separated into two halves and included in the same block. Semi-serial paraffin sections (6 μ m) were obtained from the middle to the margin of the defect, in an anterior-posterior direction, at six levels within a distance of 100

 μ m, totaling twelve levels for each block. From each level, two glass microscope slides containing two or three sections were prepared. Histological sections were stained with hematoxilin and eosin.

Histologic and histomorphometric evaluation

Histological sections were uniformly selected from the beginning to the end of the defect and analyzed by light microscopy at $\times 10$ magnification. Bone defects were evaluated histologically for linear closure of the bone defect, new bone trabeculae and blood vessel formation, bone tissue cells, bone marrow and possible inflammatory response.

Two histologic sections from each of the twelve levels obtained from the defect were subjected to histomorphometric analysis. New bone formation within the region bounded by the reversal lines in the area of the defect was measured using a light microscope at $\times 100$ magnification and an image analysis system (Image J). The percentage area of new bone corresponded to the mean value for the 12 levels analyzed in each defect for each treatment.

Statistical analysis

Means and standard deviations of radiographic bone density (%) and histomorphometric percentages of new bone formation were calculated for each group. Comparisons between groups were subjected to one-way analysis of variance (ANOVA) and Tukey's test (Bio Estat 4.0) at a significance level of 5% ($\alpha = 0.05$) and a 95% confidence interval.

Results

Radiographic analysis

Data from radiographic analyses are shown in Table 1. The outcomes demonstrated that the groups treated with PRP had higher bone density values than the groups not treated with PRP, and that the differences between the groups were statistically significant (Fig. 1).

Histological analysis

PRP + BG association

Histological evaluation of the defects revealed a central region rich in dense connective tissue, particles of BG and a large number of macrophages, mainly associated with the glass particles. At the edge of the defects, intense de novo bone formation activity was observed, and areas of new bone had already formed in the middle of the defect (Fig. 2).

PRP

Histological sections from the central region of the defect

Treatment	$Mean \pm SD$	Homogenous groups	F	Р
PRP+BG	$66.2 \pm 4.3 \text{ A}$	А	14.9322	0.0003
PRP	$60.7\pm3.5~A$	А		
BG	$51.3\pm4.9~\mathrm{B}$	В		
BC	$47.1\pm5.4~\mathrm{B}$	В		

 Table 1
 Mean percentages and standard deviations (SD) of bone density in defects

Means followed by the same letter did not differ significantly in ANOVA and Tukey's test (P < 0.05) CI = 95%.



Fig. 1 Radiographic images of parietal bones and bone defects in the respective groups. Bone densities were calculated for all groups. (I) Bioactive glass; (II) Blood clot; (III) PRP + BG; (IV) PRP. Groups treated with PRP (III and IV) showed significantly higher bone density than the groups not treated with PRP (I and II).

 Table 2
 Mean percentages and standard deviations (SD) for new bone formation according to treatments

Treatment	$Mean \pm SD$	Homogenous groups	F	Р
PRP+BG	25.6 ± 9.9	А	7.6673	0.0032
PRP	25.8 ± 12.4	А		
BG	6.1 ± 4.3	В		
BC	7.8 ± 5.6	В		

Means followed by the same letter did not differ significantly in ANOVA and Tukey's test (P < 0.05) CI = 95%.

showed dense connective tissue and several foci of new bone formation within the area of the defect. However, the new bone was thin and did not fill the entire defect.

Bioactive glass

Initial histological sections were completely filled with dense connective tissue; there were many glass particles and macrophages, mainly around the biomaterial. Intense activity and areas of new bone formation were only observed at the edge of the defect, while in the middle of the defect there was evidence of connective tissue associated with bioactive glass and macrophages, but without bone formation (Fig. 3).

Blood clot

Initial histological sections from bone defects showed



Fig. 2 Histological aspects of the PRP + BG group. (A) Central region rich in dense connective tissue and particles of BG PRP+BG. Magnification $\times 40$; (B) Middle of the defect with new bone formation and dense connective tissue. Magnification $\times 100$; (C) Particle of bioactive glass with macrophages (arrow). Magnification $\times 400$; (D) Edge of the defect and presence of osteoblasts (arrow). Magnification $\times 400$.



Fig. 3 Histological evaluation of the BG group. (A) Histological aspect of the middle of the defect treated with BG showing a large quantity of glass particles as well as evidence of connective tissue; (B) Edge of the defect showing new bone formation. Magnification $\times 100$.



Fig. 4 Histological aspects of the blood clot group at the edge of the defect. (A and B) Discrete new bone formation is evident only at the edge, and dense connective tissue fills the middle of the defect. Magnification $\times 10$.

complete filling by dense connective tissue without new bone formation in the middle of the defects. Discrete new bone formation was observed only at the edges. However, new bone formation was observed in deep areas of the defects, while the central portion was still filled with dense connective tissue (Fig. 4).

Histomorphometric analysis

The data obtained by histomorphometric analyses

showed significant differences in the percentage of new bone formation among the groups (P = 0.0032). Both of the PRP groups demonstrated higher values of new bone formation than the groups not treated with PRP. On the other hand, no differences were observed between the groups with and without PRP treatment (P > 0.05) (Table 2).

Discussion

Several regenerative procedures have been proposed for evaluation of bone formation in bone defects. The use of bone substitutes, membranes and growth factors, together or separately, is the most common approach used to achieve bone regeneration. The objective of the present study was to clarify whether addition of platelet-rich plasma (PRP) and/or bioactive glass (BG) would have a positive effect on bone formation in rabbit calvarial defects. The results of radiographic, histological and histomorphometric examinations showed that PRP enhanced bone repair, either alone or in combination with BG.

PRP is an autologous source of several growth factors (PDGF, TGF- β , IGF) that have an important role in wound healing, promoting initial coagulation (37) and exerting strong angiogenic, mitogenic and capillary growth effects (38). As such, PRP has become a focus of interest because of its potential to accelerate cell proliferation, initiate mineralization *in vitro* (39), and enhance bone regeneration in humans (40). Kanno et al. (41) reported that PRP promoted the expression of an osteoblast-specific transcription factor (*cbfa-1*) that regulates the transcription of osteopontin, bone sialoprotein, osteoprotegerin and osteocalcin, which are essential for osteoblast differentiation (42,43).

Studies of the application of PRP to bone regeneration in oral surgery have yielded conflicting results, suggesting that factors such as the surgical site, type of bone used, diffusion of PRP and/or growth factors, as well as the concentration of blood platelets, may modulate responses (6,9,10,44,45). According to Mooren et al. (44), the critical effective amount of platelets for each type of animal, as well standardization of the platelet concentration in PRP, should be defined by experimental studies. Mooren et al. (45) also suggested that the difference between the metabolisms of small and large animals may also play a role in the rate of early bone healing.

The results of the present study are in agreement with the results of previous ones demonstrating that PRP significantly enhances bone formation in rabbit calvarial defects (17,19,28,31). After 3 months of implantation, the present study confirmed that no full defect closures were evident in controls treated with a blood clot alone, as described previously (46). In addition, Aghaloo et al. (15) did not find any significant radiographic or histomorphometric improvement in non-critically sized defects in a rabbit cranial model when PRP was added to enhance bone formation. However, at 1, 2, and 4 months, a histomorphometric tendency for increased bone formation was observed when bone and bone/PRP were utilized.

In addition to the effect of PRP on bone formation in calvarial defects, other mechanisms involved in this process could explain the results obtained in the present study. Several studies of cranial suture biology have supported a role for the dura mater in the regulation of calvarial osteogenesis (30,47). Petrie et al. (48) showed that the dura mater has the ability to transform into cells characteristic of the two surrounding tissue types (bone and nerve) due to its neural crest origin, characterizing it as a multipotent cell type.

Gagan et al. (49) reported that cranial sutures are composed of a centrally-located group of proliferating osteoprogenitor cells, surrounded peripherally by the same cells undergoing osteogenic differentiation and programmed cell death. In addition, according to these authors, interactions of the dura mater with the brain and skull are very dynamic; mechanical and biochemical reciprocity has been observed in which the cells of the dura mater can influence cell migration and differentiation in several regions of the embryonic and infant brain and skull. These authors also suggested that the cells involved in the healing of a defect are derived from those located closest, and that differences in various populations of adult stem cells may reflect reparative capacity that is dependent on the organ involved and the tissue source of the stem cells. Due to their close proximity to both the bone and brain, it is possible that dura mater cells could be effective for treatment of cranial bone and neural deficits in vivo.

As PRP is an autologous source of concentrated growth factors, including TGF β , which affects bone formation as well as regulating and stimulating other growth factors, including FGF-2 (50,51), it can be suggested that this growth factor may have mediated the effects observed in the present study. Gosain et al. (52) showed that these specific growth factors (TGF β and FGF-2) have important roles in cranial suture development in the embryogenic and postnatal phases. Mehrara et al. (47) suggested that basic fibroblast growth factor (FGF-2) and transforming growth factor-beta1 (TGF- β 1) have osteogenic actions that are involved in regulating bone formation in calvarial bone defects. The present study demonstrated increased immunohistochemical staining

for FGF-2 and TGF- β 1 within calvarial osteoblasts with increasing age, especially in cells that were in contact with the developing dura mater. Based on the application of these modern principles to tissue regeneration and wound healing, a recent study of 10 patients with advanced frontal sinus disease treated with PRP and PPP and a bioactive scaffold demonstrated a favorable outcome within a follow-up period of 6-10 years, with no complications or sequelae (5).

The addition of BG has been used to achieve bone regeneration, based on the osteoconductive properties of this material (53). BG has become an alternative to autologous and allogeneic bone grafts, and has been employed in various clinical and animal studies (4,14,18-23,26). As shown by some previous authors (32,53), in the present study, no foreign body reaction was observed in the histological specimens, which even showed intense activity and areas of new bone formation, especially at the edge of the defects. These results imply that BG is biocompatible with the surrounding tissues.

A recently published systematic review evaluating the efficacy of PRP, combined with various therapeutic bioactive agents/procedures for the treatment of periodontal intraosseous defects found diverse outcomes (54). More than 6,000 potentially relevant titles and abstracts were examined in the research; however only 20 full-text articles were thoroughly evaluated. The authors concluded that in view of the limited and heterogeneous data available, additional research on the efficacy of these combinations is needed.

Although the present study radiographically demonstrated significantly higher bone density in the groups treated with PRP (as also reported by Marx et al. (6)) when it was used alone or combined with bone grafts, radiographic evaluation of bone repair should be done with caution. Pryor et al. (55) showed that radiographs had low accuracy for assessment of bone healing, for example in critical-size defects in rats. The authors considered that radiography does not reflect the actual stage of healing, and may underestimate the final process as well as overestimating healing at the intermediate stage. They also recommended that, before clinical application, this treatment method should be confirmed using histological analysis in animal models. However, it is important to point out that, in clinical situations, histological examinations are rarely possible and that radiographs can be of help in assessing bone formation. The histological outcomes in the present study indicated that bone formation occurred in all groups; however, the PRP groups demonstrated central focal areas of vital new bone formation, with or without BG. In both groups treated with BG, macrophages were observed surrounding these particles.

With the evolution of animal and clinical studies, new knowledge and concepts have led to the addition, substitution, and alteration of therapies and clinical protocols. According to Simonpieri et al. (10) there was a transitional period in periodontology and oral maxillofacial surgery concerning the use of PRP and platelet-rich fibrin (PRF). The literature on this topic is still contradictory and published data showing actual benefits of PRP and BG for bone regeneration are limited. However, the use of platelet growth factors in association with cell or molecular therapies should be explored further, since these options may improve the process of periodontal or bone regeneration.

On the basis of the present results, it can be concluded that PRP improves bone repair in calvarial defects and that BG, alone or in combination with PRP, is ineffective for bone healing.

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