Evaluation of the Bone Healing Process Utilizing Platelet-Rich Plasma Activated by Thrombin and Calcium Chloride: A Histologic Study in Rabbit Calvaria

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To evaluate the bone healing of defects filled with particulate bone graft in combination with platelet-rich plasma (PRP), added with a mixture of calcium chloride and thrombin or just calcium chloride. Two 5-mm bone defects were created in the calvaria of 24 rabbits. Each defect was filled with particulate bone graft and PRP. In one defect the PRP was activated by a mixture of calcium chloride and thrombin; in the other, PRP was activated by calcium chloride only. The animals were euthanized 1, 2, 4, and 8 weeks after the surgeries, and the calvaria was submitted to histologic processing for histomorphometric analysis. The qualitative analysis has shown that both defects presented the same histologic characteristics so that a better organized, more mature, and well-vascularized bone tissue was noticed in the eighth week. A good bone repair was achieved using either the mixture of calcium chloride and thrombin or the calcium chloride alone as a restarting agent of the coagulation process.

Key Words: platelet-rich plasma, bone graft, dental implants, growth factors

INTRODUCTION

Developed in the last decade from fibrin adhesives, PRP, or platelet-rich plasma, has been widely studied and applied for tissue repair. It is an autologous source of platelet-derived growth factors which aim to stimulate or accelerate the healing process.1,2

In implantology and oral and maxillofacial surgery and traumatology, its main application is related to the bone repair process, especially in reconstructive procedures.3,4 The addition of PRP to autogenous bone grafts or biomaterials has been showing promising results both concerning the speed5 and the bone neoformation quality.6–8 These characteristics are based upon the liberation of growth factors contained in the platelets’ alpha granules, such as PDGF, PGF-β, PDEGF, IGF, and PF-4, each of them with different activities related to the increase of chemotaxis for undifferentiated mesenchymal cells, macrophages and osteoblasts, stimulus for fibroblasts and osteoblast differentiation and proliferation, collagenous matrix secretion, angiogenesis, and deposition of mineralized tissue.9,10

PRP preparation is accomplished through venous punch and autologous blood collection in Vacutainer tubes containing the anticoagulant sodium citrate. After a centrifugation process, PRP
is separated from red blood cells and from platelet-poor plasma (PPP) by density gradient, and subsequently, a solution of 10% calcium chloride and 10,000 units of topical bovine thrombin (TBT) is added to PRP, which after a few seconds in transformed in gel. When thrombin and calcium are added to PRP, platelets are activated and release growth factors contained in their cytoplasmatic granules that initiate the coagulation process of converting fibrinogen into fibrin. The result of this interaction is a gel consistency that provides a better adhesion of the graft particles, provided it is not displaced in the recipient area.11

The addition of TBT and calcium chloride to PRP aims to initiate the coagulation process through platelet activation12,13; however, the use of TBT has been associated with the development of coagulopathies. According to Landesberg et al,14 TBT preparation produces an immunologic cross-reaction in the bovine antithrombin antibodies against factors V, XI, and human thrombin. The severity of bleeding varies widely from cases without clinical evidence to cases of hemorrhage with risk of death, developed from 7 to 14 days after the exposure to TBT.10,15

The intensity of the side effects would be related to the concentration of TBT used.10 In order to minimize the development of coagulopathies, present methods of TBT extrapurification decrease the concentration of the contaminating factor from 50 to 100 μL to less than 0.2 μL, quantity that would be insufficient for the development of immunologic cross-reaction.16,17 Alternatively, Landsberg et al14 suggest the use of a compound called ITA as a platelet activation media and PRP gelation; however, its composition was not revealed. Due to the importance of platelet-rich plasma in regeneration and the potential risk offered by the use of TBT, the purpose of this study was to evaluate if a solution of thrombin and calcium chloride is necessary for PRP to be effective in bone defect healing, or if calcium chloride separately would reach the same result.

MATERIALS AND METHODS

Before this experiment began, the methodology was submitted for review and approval by the Ethics Committee in Animal Research from 2008–003638. Twenty-four New Zealand rabbits (Oryctolagus cuniculus) weighing approximately 3.5 kg were used in this study. The animals were maintained at the vivarium of the Veterinary Hospital from Cuiabá University for a period of 2 weeks prior to the experiment.

PRP obtainment

After a 12-hour presurgical fasting, all animals received a 0.1 mL/kg prophylactic dose of intramuscular antibiotics (Pentabioticão, Fort Dodge, Campinas - SP, Brazil). After anesthesia with Rompum (Bayer saúde animal, São Paulo, Brazil) and Katalar (Bayer saúde animal), in the dosages of 0.25 and 0.37 mg/kg, respectively, a trichotomy was accomplished on the areas of the calvaria and external jugular, followed by aseptic preparation with iodine alcohol on both areas. A vacuum scalpel #25 was used to puncture the external jugular, obtaining this way two tubes, each one containing 4.5 mL of total blood and 0.5 mL of sodium citrate (anticoagulant). The tubes were put into a homogenizing device for 5 minutes and subsequently centrifuged. After centrifugation, it was possible to observe a lower fraction (red cells) and a yellow upper fraction correspondent to plasma.

The upper fraction was pipetted in each tube as follows:
- 1.0 mL, which presented platelet-poor plasma (PPP)
- 0.8 mL, which presented platelet-medium plasma
- 0.7 mL, which presented platelet-rich plasma (PRP)

From each tube, PRP was separated and stored into a Dappen dish. This way, two Dappen dishes were obtained with 0.7 mL PRP.

From the 24 animals divided into 4 groups of 6 rabbits, 8 were chosen to obtain 1 blood tube more than necessary for the analysis of platelet concentration through optical microscopy with Giemsa staining. The same procedure of platelet counting was accomplished with PRP. This experiment included only animals whose platelet counting varied from 250 to $300 \times 10^3$/mm$^3$ for blood samples and 970 to $1150 \times 10^3$/mm$^3$ for PRP.

Surgical procedure

The animals were maintained in the ventral position on a surgical table, and antisepsis with iodine alcohol was performed in the calvaria region. A
solution of 2% Xylocaine with 1:100 000 adrenaline was infiltrated in this region to achieve local anesthesia and decrease transoperative hemostasis. A semilunar incision was made in the calvaria integument with a scalpel blade #5. Posteriorly, with an instrument proper for detaching the periosteum, a flap was laterally raised, exposing the bone surface of this region. Afterwards, using a 5-mm diameter surgical trephine drill activated by a surgical microengine and under abundant and constant irrigation with 0.9% saline solution, 2 bone defects were done in the right and left frontoparietal regions. The bone block removed was triturated in order to be used as a graft.

**Right Parietal**

After removing and triturating the right parietal bone block, the particulate bone was stored in a Dappen dish with 700 μL of PRP, for the addition of a 100-μL solution of bovine thrombin and 10% calcium chloride.

**Left Parietal**

After removing and triturating the left parietal bone block, it was also stored in a Dappen dish with 700 μL of PRP, for the addition a 28-μL solution of 10% calcium chloride. Then, the flap was closed with nylon 5-0 wire in the periosteal and cutaneous plans. After 7 days, skin sutures were removed.

**Euthanasia and tissue processing**

After 1, 2, 4, and 8 weeks, 6 animals were killed in each group. The animals were anesthetized as previously described, for the accomplishment of an incision, which was done in the calvaria, removing only the animal skin, leaving the perios- teum in the graft area. In the areas lateral to the grafts, other incisions were performed in the periosteam, exposing bone tissue. Osteotomies were accomplished with a #701 low-speed drill, under constant and abundant irrigation with 0.9% saline solution, in order to remove 2 fragments containing the experimental areas. After removing the fragments, the animals were killed in a few seconds with a potassium chloride injection into the heart. In the laboratory, the pieces were processed into 7 μm thick serial cuts and stained with hematoxylin and eosin and Mallory Trichrome techniques for qualitative and quantitative analysis in optical microscopy.

**Analysis**

For qualitative analysis of the graft region, 240 microscopic slides were analyzed for each experimental group, totaling 480 slides observed in the experiment. For the histometric analyses, 3 histologic sections, representing the center of the original surgical defect, were selected in order to increase the reliability of the data used in the statistical analysis. The histometric analyses were performed by an examiner blinded with respect to the treatment rendered. The images of the histologic sections were captured at X4 magnification, using a binocular microscope Olympus BX 40 (Shinjuku-ku, Tokyo, Japan) coupled to an Olympus OLY 200 camera (Center Valley, Pa) connected to a PC computer through a Data Translation 3153 digitization board (Marlboro, Mass). A composite digital image was then created by combining 3 smaller images because it was not possible to capture the entire defect in 1 image at the level of magnification that was used. The composite image was created based on anatomic reference structures (eg, blood vessels and bone trabeculae) within each of the histologic sections, so that the final image will be formed by the whole extension of the defect and its margins. The IMAGELAB 2000 software (Diracon Bio Informática Ltda, Vargem Grande do Sul, SP, Brazil) was used for the histomorphometric analysis.

The following method was used to quantify the neoformed bone area: the total area 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 calvaria at the right and left margins of the surgical defect, and then connecting them with lines drawn following their respective curvatures. This area, in pixels, was considered to represent 100% of the area to be analyzed. Then, the newly formed bone area was also measured and calculated as a percentage of the total area. The mean value of newly formed bone obtained in the 3 histologic sections was assigned to each animal and used for statistical analysis. In the statistical analysis, the data normality was verified using the Shapiro-Wilk W test. Measurements of newly formed bone did not have a normal distribution; therefore, nonparamet-
ric Kruskal-Wallis test and Dunn post-hoc test for 2-by-2 comparisons were used for analysis of these data in both groups with 5% significance level ($P < .05$).

**RESULTS**

The slides from the qualitative analysis demonstrated repair aspects identical in both groups with and without thrombin, in each period studied. Thus, the qualitative results will be described conjointly for both groups in the periods of 1, 2, and 8 weeks.

**Groups with and without thrombin – 1 week**

In the peripheral region of the lesion, a discrete remodeling by resorption and the presence of newly formed bone matrix with thin rudimentary bone spiculae were observed. The spiculae were arranged progressively in thin bone plates, covered by osteoblasts with a morphologic aspect of intense activity of protein synthesis. Near the lesion margin, an impingement of cells originating from the internal side of the periosteum was observed with a morphologic aspect of fibroblasts. In the central region, traces of blood clot in the resolution process were observed, as well as many blood vessels and several disperse cells, with predominance of fibroblasts and the presence of macrophages and osteoclasts. Bone fragments from the graft, covered by osteoblasts and newly formed bone tissue, presented blood vessels into cavities inside it (Figures 1 and 2). Bigger fragments were involved by connective tissue, with some resorption regions. All graft fragments presented the osteocyte spaces empty.

**Groups with and without thrombin – 2 weeks**

Almost the entire lesion was filled by bone tissue. In this period, there was a predominance of primary bone tissue, with thin bone trabeculae, and few and poorly delineated lamellae. It was possible to observe osteoblasts in intense protein synthesis activity covering the bone trabeculae. The small cavities delimited by the bone trabeculae contained a great amount of blood vessels (Figure 3). It was also possible to observe bone neoformation on the surface of the biggest bone fragments and incorporation of the smaller ones by the newly formed matrix. Bone fragments were vascularized, but with empty osteocytic lacunae (Figure 4).

**Groups with and without thrombin – 4 weeks**

Secondary or mature bone tissue was observed, substituting the primary or immature bone tissue. However, there was a predominance of primary bone tissue with thicker trabeculae than in the second week. These trabeculae delimited smaller medullar cavities. Bone neoformation filled the entire lesion with new bone tissue, and graft fragments were still present (Figure 5). Bone graft fragments presented empty lacunae and blood vessels and were incorporated by the newly formed bone tissue (Figure 6). Periosteum was less corrugated than in the other periods, but it was still possible to verify cellular impingement in its internal side.

**Groups with and without thrombin – 8 weeks**

The defect was presented completely filled by bone tissue. Moreover, it was possible to observe a great amount of primary bone tissue, with several regions presenting bone remodeling through the substitution of the primary tissue by the secondary or lamellar (Figure 7). Bone tissue was less cellularized and the trabeculae were thicker, delimiting smaller medullar cavities (Figure 8). Graft bone fragments were not differentiated from the mature bone anymore, and this one was a product of the substitution by primary bone tissue. In some regions, empty lacunae were verified, which suggests that it was a fragment of incorporated graft. The periosteum was less corrugated than in the 1-month group, and cellular impingement was not noticed.

**Quantitative analysis**

Quantitative results are expressed in percentage of newly formed bone in the Table. Statistical analysis did not show any significant difference ($P > .05$) between the groups with and without thrombin in any of the studied periods.

**DISCUSSION**

In the presence of an ample utilization of platelet rich-plasma and knowing the need for an initiator agent for the PRP coagulation process, studies were
proposed to investigate the PRP efficiency in bone repair when calcium chloride and TBT or calcium chloride only were used as activators. In this study, in order to ensure the platelets’ integrity and PRP viability, the variables from the PRP obtainment process, such as amount and concentration of anticoagulant solution, centrifugation speed, amount of platelets contained in the peripheral blood and in the PRP, and time period between the plasma obtainment and its clinical use were carefully controlled in such a way that the study variable was only the presence or absence of TBT. According to Messora et al.,\textsuperscript{18} quantitative or qualitative platelet alterations could directly alter the effect of PRP.

According to Marx et al.,\textsuperscript{11} a solution of 10% calcium chloride and 10 000 units of topical bovine thrombin are necessary for the coagulation process to be initiated; after a few seconds, a gel consistency is formed through platelet activation and conversion of fibrinogen into fibrin. Anitua\textsuperscript{19} described the use of only calcium chloride as an initiator agent of the PRP coagulation process, obtaining the gel consistency in approximately 15 to 20 minutes. Gel consistency is desirable when PRP is utilized in conjunction with particulate grafts; once the graft particles are agglutinated by the gel, manipulation and graft stability at the recipient site during flap suture are easy. In this investigation, fibrin gel formation was observed in both groups; however, in the group where TBT was used, the coagulation process occurred in a few seconds, while in the group without thrombin this period varied between 6 and 17 minutes, similar to the results obtained by Anitua.\textsuperscript{19} Previous studies had demonstrated that the PRP activation with calcium...
chloride alone produces a blood clot that is much poorer with regard to its structural integrity when compared to that obtained through the addition of TBT. Moreover, the use of calcium chloride alone produces a smaller blood clot retraction of the PRP, which, according to Landesberg et al., would be favorable to the bioavailability of growth factors once they would be retained by a longer time period in the surgical bed, increasing the action time.

In a recent study, Nagata et al. evaluated the healing of critical defects in rats' calvaria with PRP utilizing 10% calcium chloride and 25% thromboplastin for its activation. The results showed a higher bone formation in the calcium chloride group when compared to thromboplastin and to a control group. According to the authors, this difference would be directly related to higher levels of growth factors and oxytocin release by the platelets' activation with calcium chloride. The same characteristic could not be observed in this study, where tissue responses to PRP activated by calcium chloride and calcium chloride and TBT were similar. This difference could be related to a higher efficiency of TBT when compared to thromboplastin, when utilized as a PRP activator. According to Martineau et al., thrombin is a potent platelet aggregator and inductor of growth factor release. The microscopic results in both groups along the 4 periods showed that the histologic structures were similar, but it was not possible to distinguish them qualitatively or the superiority of the repair process in any of the groups. The same was found at the quantitative evaluation, where both groups did not present any significant difference in the statistical analysis.
With 7 days, it was possible in both groups to observe that the lesion presented several blood vessels. This fact can be explained by the presence of TGF-β, which is able to increase the production of endothelial growth factor 20 and of PDGF, which stimulates the endothelial mitosis inside the capillary.21 In the period of 2 weeks, it was already possible to observe almost the entire lesion filled by bone tissue, which was found more organized than the one from the first week. It was possible to verify several blood vessels and cells from the cellularized periosteum, which continued to impinge the entire lesion. In this period, osteoblasts were seen covering the bone trabeculae and in intense activity of protein synthesis. In the periods of 4 and 8 weeks, the entire lesion was filled by bone tissue, and both groups presented the same histologic characteristics and, statistically, did not present any significant difference.

Lind22 described the effects of PRP on the recipient site as a biochemical stimulation. The purpose of this biochemical stimulation is chemotaxis, mitogenesis of the osteoblast precursors, and the ability to stimulate the collagenous matrix deposition6,21; angiogenesis21,23 and the ability to stimulate the bone matrix formation by the differentiated osteoblasts in order to accelerate the bone deposition.11,19 In this study, immediately at the period of 1 week, it was possible to verify a great amount of uninucleated cells from the periosteum deepest layer, which invaded the entire lesion, probably differentiating in osteoblasts. These characteristics are in accordance with previous studies that demonstrated that the PRP activity is given, especially at the initial periods of the healing process.5,24,25 Although many studies confirm the PRP efficiency in the bone repair acceleration, some research does not show any benefit in its use,26–28 or even show the delay in bone healing.29 Recent molecular studies have been investigating this difference by analyzing the real effect of PRP on cellular activity. Kanno et al30 through reverse transcription polymerase chain reaction analysis demonstrated a dose-dependent increase in the synthesis of alkaline phosphatase RNAm, type I procollagen, osteopontin, osteoprotegerin, and Cbfa1 when cultures of osteoblastic cells were stimulated through a concentrate rich in platelets. Nagata et al8 observed, through immunohistochemical analysis, an increase in the expression of osteopontin and osteocalcin with the use of PRP, as compared with a control group without PRP. Moreover, in a histomorphometric analysis, Torres et al,5 utilizing only PRP without any type of graft material, demonstrated an increase in bone healing of defects created in rabbit calvaria, compared to the defects filled only with blood clot. Although these results offer important evidence that seems to support the PRP efficiency to accelerate the bone repair process, there is no standardization in the studies with regard to the obtainment methods, centrifugation, and platelet activation. Probably the differences in the PRP obtained in different methodologies produce divergent results.

The platelet activation method is an important factor to be considered in the clinical efficiency of PRP because it can directly influence the amount of growth factors released in tissue. Weibrich et al31 have demonstrated that neither the platelets counting from the peripheral blood nor the platelets counting from the PRP are able to predict the resultant levels of growth factors in the PRP after its activation. These levels of growth factors would be related directly to different biologic responses in the healing process. Future studies in

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molecular level, measuring the availability of growth factors with different types of PRP activators must be carried for the choice of an ideal activator.

**Conclusions**

Within the experimental conditions of this study, it is possible to conclude that both initiator agents of the coagulation process are able to achieve the gel consistency when added to PRP. Both groups presented a bone repair of good quality. Concerning the amount of newly formed bone matrix, there is no significant difference if we add to PRP a solution of calcium chloride and thrombin or calcium chloride alone, as an initiator agent of the coagulation process.

**Abbreviations**

PPP: platelet-poor plasma  
PRP: platelet-rich plasma  
TBT: topical bovine thrombin

**References**


