

# Bovine Anorganic Bone Graft Associated with Platelet-Rich Plasma: Histologic Analysis in Rabbit Calvaria

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Autogenous bone tissue has regeneration potential; however, this capacity may not be sufficient in larger bone defects. The aim of this study is to histologically evaluate anorganic bovine bone grafts (GenOx Inorg) with or without platelet-rich plasma (PRP). Two bone lesions were created in calvaria of 12 rabbits. The 24 surgical lesions were separated into 3 groups: coagulous, anorganic, and anorganic with PRP. At the 4-week time point, the animals were euthanized and the grafted area removed, fixed in formalin 10% with phosphate buffered saline, 0.1 M, and embedded in paraffin. The histologic parameters analyzed were new bone filling the defect area, presence of giant cells and particles of the graft, and new bone formation associated with the particles. In the coagulous group, defects were filled with fibrous tissue that attached the periosteum and little bone neof ormation in the periphery. In anorganic groups with or without PRP, little new bone formation in the periphery of the defect was observed; however, in the center of some defects there was new bone. Moderate presence of giant cells and little new bone formation was associated with the innum erous graft particles. Histologic results revealed no statistically significant differences among the defects new bone fill between the studied groups ( $P = .64$ ). There was no significant difference in the number of giant cells ( $P = .60$ ), graft particles ( $P = .46$ ), and new bone formation around graft particles ( $P = .26$ ), whether PRP was added or not. Anorganic bone, isolated or mixed with PRP, was biocompatible and osteoconductive, while maintaining bone volume.

**Key Words:** *anorganic bone graft, platelet-rich plasma, bone repair, bone graft*

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## INTRODUCTION

**A**utogenous bone is currently used for treating bone defects.<sup>1-3</sup> Advantages of autogenous bone are due to its biologic properties, determined by the terms osteoconduction, osteoinduc-

tion, and osteogenesis, and the lack of the possibility of disease transmission or host rejection.<sup>2,4</sup> Nevertheless, donor site morbidity is a factor that limits the autograft procedure,<sup>2</sup> and additional site-specific complications of harvesting must be taken into consideration, particularly if a second surgery is needed.<sup>5</sup>

Researchers continuously strive to improve on current bone grafting techniques and provide faster and denser bone regeneration. In recent years, several therapeutic alternatives have been developed, such as synthetic bone substitutes,<sup>6-8</sup> local growth factors, and composites that behave as repair promoters and carriers of bone induction factors.<sup>4</sup> Anorganic biomaterials are important alternatives to autogenous grafts due to their biocompatibility<sup>9,10</sup> and osteoconduction properties.<sup>11</sup> These anorganic biomaterials can also enhance a high content of calcium and phosphorus essential for bone formation.<sup>12,13</sup>

Bovine bone has been investigated and has shown favorable results.<sup>14,15</sup> In vivo studies have demonstrated an increase in osteoblast activity and bone formation when mineralized,<sup>16-18</sup> and demineralized<sup>2</sup> bone matrices are used in conjunction with growth factors.<sup>19</sup> In vitro studies also demonstrated the benefits of anorganic bone matrices.<sup>20</sup> Anorganic biomaterials require long periods to be resorbed, determining continuous flow of giant cells next to the material surface. The resorption process seems to be related to the amount of newly formed bone.<sup>14</sup>

The use of platelet-rich plasma (PRP) can increase the benefits of bone grafts.<sup>21,22</sup> Platelets release growth factors and cytokines that contribute to bone regeneration and vascular proliferation, essential to bone graft healing.<sup>23,24</sup> Additional advantages include their adhesive nature due to the fibrin, which acts as a tissue glue<sup>25,26</sup> and their capacity to accelerate deposition of

new bone along the graft material.<sup>26</sup> Some authors suggest that the addition of PRP to osteoconductive grafting materials can potentiate osteoinduction<sup>27,28</sup>; however, others did not observe any increase in the bone healing when using PRP.<sup>23,29-31</sup> The current study histologically evaluated bone repair in calvarial defects filled with bovine anorganic bone matrix, with or without PRP.

## MATERIAL AND METHODS

### Material

The tested material was bovine anorganic bone (BAB), thermally deproteinized at 100°C (GenOx Inorg, Baumer SA, Mogi Mirim, São Paulo, Brazil).

### Animal surgical procedure

Twelve New Zealand white rabbits weighing between 2.5 and 3.5 kg were included in this randomized, blind study. The guidelines of the Brazilian College of Animal Experimentation were used in all animal protocols. Each rabbit was anesthetized with ketamine (25 mg/kg), xylazine (10 mg/kg), Acepran (0.2 mg/kg), midazolam (0.2 mg/kg), and local anesthesia with 0.9 mL of mepivacaine with epinephrine. A single prophylactic dose of antibiotic therapy with cephalosporin (30 mg/kg) was administered intravenously. After trichotomy and antisepsis with aqueous solution of povidone-iodine, a 2-cm incision was made along the anteroposterior surface of the bony cranium and a full-thickness flap was raised. Two defects of 8 mm diameter were created in parietal calvarial bone with a trephine burr under continuous irrigation with saline solution. Care was taken to make a full-thickness defect without damaging the underlying dura. The 24 defects were separated in 3 groups and randomly filled with coagulant in the control group, BAB alone, and BAB mixed with PRP. The wound was closed using nylon 4.0 suture, which was removed 7 days after

TABLE 1  
Established criteria for evaluation

	Defect Bone Filling	Giant Cells	Graft particles	Bone Neoformation Around Graft Particles
0	Absent (no bone formation)	Absent	Absent	Absent
1	Little (one-quarter of the defect filled)	Little (present in one-quarter of the graft particles)	Little (present in one-quarter of the defects)	Little (present in one-quarter of the graft particles)
2	Moderate (one-half of the defect filled)	Moderate (present in one-half of the graft particles)	Moderate (present in one-half of the defects)	Moderate (present in one-half of the graft particles)
3	Abundant (more than one-half of the defect filled)	Abundant (present in more than one-half of the graft particles)	Abundant (present in more than one-half of the defects)	Abundant (present in more than one-half of the graft particles)

surgery. All animals received the antibiotic Flotril (enrofloxacin, 2.5%, 2.5 mL/kg) subcutaneously during 5 days after surgery.

### PRP preparation

Four and a half milliliters of autologous blood was drawn from each rabbit from the auricular vein to prepare the PRP several minutes before administration of anesthesia. The 4.5 mL of autologous blood was combined with anticoagulant, 0.5 mL of 3.8% sodium citrate, to prevent coagulation. The blood was centrifuged (206-BL-Fanem; Datamed, São Paulo, Brazil) according to the Sonnleitner modified method<sup>29,32</sup> at 1000 rpm (160G) for 20 minutes to separate the plasma containing the platelets from the red cells. The supernatant and 2 mm below the dividing line between the phases was pipetted and transferred to a tube without anticoagulant. An additional centrifugation for 15 minutes at 1600 rpm (400G) was done to separate the platelets. The precipitate formed in the tube by this second centrifugation was the PRP. For each 0.5 mL of PRP, 25  $\mu$ L of 10% calcium chloride was used as an activator.

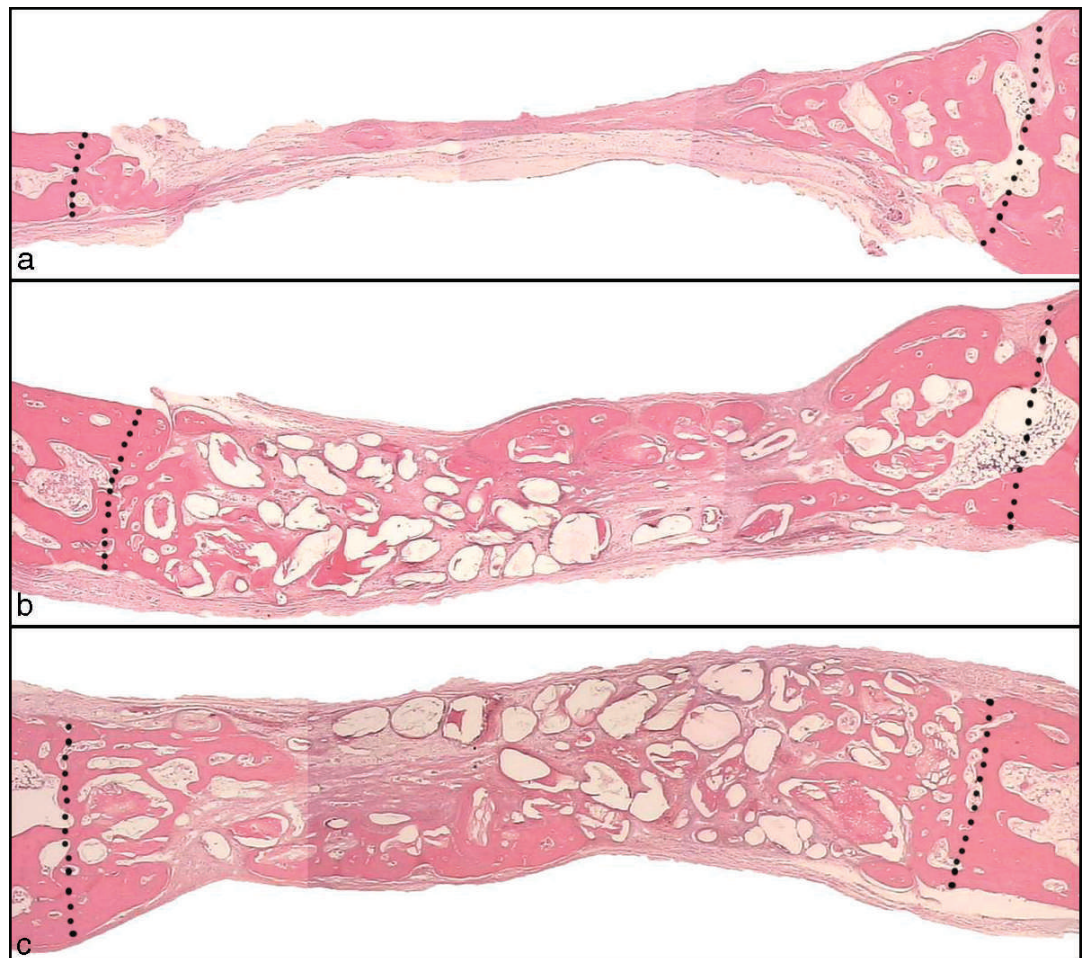
### Sample evaluation

The animals received a normal diet consisting of granular food and water ad libitum. At the 4-week survival period they were anes-

thetized with Pentothal sodium 2.5% and euthanized with an overdose of potassium chloride, 19.1%. The calvarial defects and surrounding tissue were removed and immediately fixed in 10% phosphate buffered formaldehyde solution during hour 48. Subsequently, the tissue blocks were decalcified in EDTA, 4.13% for 4 weeks, dehydrated with graded alcohols, and embedded in paraffin. The histologic specimens were prepared in the usual fashion, and the semi-serial sections of 5- $\mu$ m thickness were stained with hematoxylin-eosin and Mallory trichrome. Histologic analysis of the bone defect area, performed under light microscope at  $\times 10$  and  $\times 40$  magnification in 3 sections for each animal revealed the defects new bone fill, presence of giant cells/graft particles, and new bone formation associated with the graft particles. The scores in Table 1 were employed for evaluation. The results obtained were submitted to normality test, Kruskal-Wallis, and Mann-Whitney *U* tests. Differences were considered statistically significant at  $P \leq .05$ .

### RESULTS

During the experiment all animals remained in good health and did not experience complications. The histologic analysis of the defect area exhibited normal healing pro-



**FIGURE 1.** Micrography of the defect (delimited by dotted). (a) Coagulous group. (b) Anorganic group. (c) Anorganic with PRP group. Hematoxylin-eosin,  $\times 4$ .

cess. No inflammatory signs or adverse tissue was observed regardless of the evaluated groups.

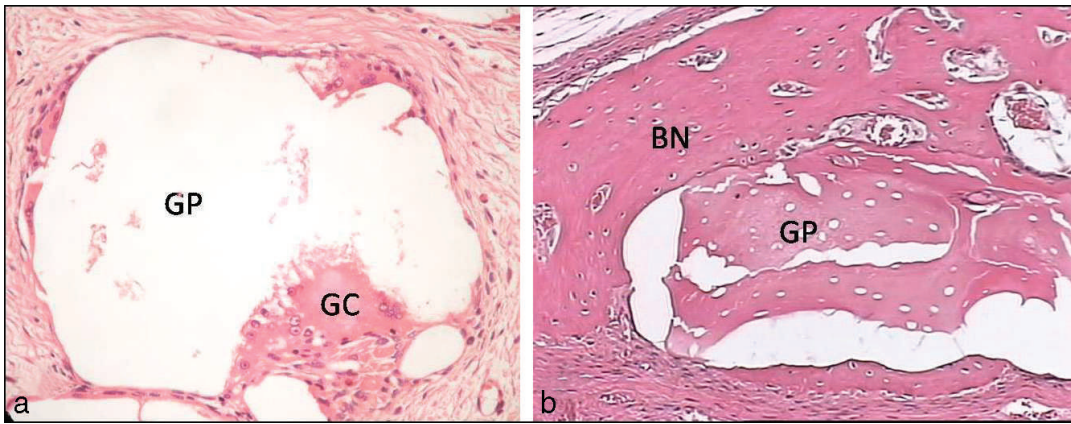
In the coagulous group, the defects presented an extensive fibrous connective tissue and little bone ingrowth from the periphery of the defects (Figure 1a). The presence of giant cells was not observed. In the BAB and BAB/PRP groups, we observed little new bone formation, mainly from the periphery of the defects (Figure 1b,c). A moderate amount of multinucleated giant cells among an abundant quantity of graft particles (Figure 2a) and little new bone formation associated with these particles (Figure 2b) could be noted.

Histologic results revealed no statistically significant differences in defect bone filling

between all studied groups ( $P = .64$ ). There was no significant difference in the number of giant cells ( $P = .60$ ), graft particles ( $P = .46$ ), and new bone formation around graft particles ( $P = .26$ ) between the grafted materials, whether PRP was added or not (Table 2).

## DISCUSSION

Rabbits, used in the current study, are useful animal models for preparation of platelet concentrates and bone repair.<sup>29</sup> Generally, platelets of human and other mammals have a similar ultrastructure and constituents. Physiologic and metabolic similarities are also seen between the bone tissues of both species.<sup>33,34</sup> Additional advantages of



**FIGURE 2.** (a) Micrography evidencing graft particles (GP), surrounded by multinucleated giant cells (GC). Hematoxylin-eosin,  $\times 4$ . (b) Micrography evidencing bone neoformation (BN) associated with graft particles (GP). Hematoxylin-eosin,  $\times 10$ .

using a rabbit include easy manipulation and sufficient blood volume for preparation of platelet concentrates.<sup>27</sup>

In this study, care was taken to avoid damaging the underlying dura and the periosteum. Periosteum provides blood supply for bone and osteoprogenitor cells for bone regeneration, and its preservation is found to favorably influence graft revascularization and integrity.<sup>35</sup>

Bovine anorganic bone is comparable to the mineralized matrix of human bone<sup>9,10</sup>; therefore, this material has been used as an alternative to autogenous bone grafts. Previous studies using anorganic bovine bone in bone defects demonstrate significant long-term bone formation, attributing osteoconductive properties to this biomaterial.<sup>14,15,17,18</sup> In our study, the bone defects exhibited new bone formation in both experimental groups, varying between the central and peripheral areas, suggesting the

influence of local tissue and the calvarial bone in providing osteogenic cells,<sup>36</sup> and also the biomaterial osteoconductive capacity, which clearly maintained the original calvarial bone volume. In both experimental groups, there was bone ingrowth in contact with the surface of the biomaterial's particles. These particles seem to act like a scaffold, supporting the formation of new bone.<sup>37</sup> Osteoconductive potential of bovine anorganic bone was confirmed, and the material porosity contributed to an increase in the surface area, favoring the recruitment of a large number of cells surrounding the graft particles.<sup>37</sup>

Bovine anorganic bone resulted in a foreign body reaction with moderate presence of giant cells. However, the presence of these cells occurs in an attempt by the organism to resorb the material. These cells act as phagocytic macrophages, cleaning the graft surface and preparing it for deposition

TABLE 2

Mean values and standard deviation of histologic scoring of treated defects

	Defect Bone Filling	Giant Cells	Graft Particles	Bone Neoformation Around Graft Particles
Coagulous	1.37 ± 0.49	0 ± 0	0 ± 0	0 ± 0
Bovine anorganic bone	1.25 ± 0.44	2.00 ± 0.51	2.62 ± 0.49	1.5 ± 0.72
Bovine anorganic bone with PRP*	1.29 ± 0.46	2.08 ± 0.58	2.75 ± 0.44	1.25 ± 0.44

\*PRP indicates platelet-rich plasma.

of newly formed bone. The presence of giant cells around the biomaterial particles and bone ingrowth along with graft material after a 4-week period suggest that anorganic bone may be slowly resorbed.<sup>38</sup> The resorption rate appears to be directly related to the amount of new bone formation.<sup>14</sup>

Marx<sup>39,40</sup> demonstrated promising results when treating mandibular defects using a combination of autogenous bone graft with growth factors contained in PRP. It is reasonable to think that increasing the concentration of platelets in a bone defect may improve bone formation. The association of the biomaterial to the PRP allows the use of biomaterial's osteoconductive potential<sup>14,15</sup> in conjunction with the osteoinductive properties of the PRP.<sup>27</sup> In vivo studies claimed the successful use of PRP associated with artificial bone substitutes for sinus floor augmentation<sup>41</sup> and treatment of intraosseous periodontal defects.<sup>28</sup> Nevertheless, corroborating with our findings, other studies have failed to provide evidence of the positive effect of PRP combined with various artificial bone materials on bone regeneration.<sup>23,42,43</sup>

Platelets are known to be effective during the early stage of bone graft healing<sup>44,45</sup> because the life span of a platelet in a wound and the period of direct influence of its growth factors are less than 5 days.<sup>46</sup> Therefore, a pronounced effect of PRP supposedly occurs, especially during the early stages of bone regeneration.<sup>44,45</sup> This suggests that its effectiveness cannot be seen in long-term evaluations<sup>47,48</sup> of 4-week survival like in our study, but probably would be more significant if the analysis was made during the first weeks of bone healing.

### CONCLUSION

Bovine anorganic bone, isolated or mixed with PRP, was biocompatible and osteoconductive, maintaining bone volume at the 4-week postoperative time point.

### ABBREVIATIONS

BAB: bovine anorganic bone  
PRP: platelet-rich plasma

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