# Effect of Solely Applied Platelet-Rich Plasma on Osseous Regeneration Compared to Bio-Oss®: A Morphometric and Densitometric Study on Rabbit Calvaria

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## ABSTRACT

*Background:* The use of platelet-rich plasma (PRP) in bone augmentation procedures is well documented; however, the exact benefit of this material is not yet established.

*Purpose:* This study aimed to evaluate the benefits of using PRP, when only used, and compare it to Bio-Oss® (Geistlich Biomaterials, Wolhusen, Switzerland) in vertical bone augmentation capacity.

*Materials and Methods:* The study was performed in calvaria of eight adult female New Zealand rabbits using titanium bone conduction cylinder. Two titanium cylinders were fixed into perforated slits made on the parietal bone of each rabbit. On each rabbit, one chamber was grafted with Bio-Oss, and the contralateral was filled with PRP. Animals were sacrificed 4 weeks after intervention and biopsies were taken. Densitometric, histological, and histomorphometric analyses were performed to evaluate bone mineral density, vertical bone augmentation, and remaining graft volume, respectively. Statistical analyses were performed with Mann–Whitney test, using a significance level of $p < .05$.

*Results:* Densitometric and histomorphometric data analysis revealed that mean bone mineral densities and bone augmentation were significantly lower in the cylinders treated with PRP ($p < .0001$) 4 weeks after implantation.

*Conclusion:* This study showed no beneficial effect of using PRP on osseous regeneration. In addition, it was emphasized that Bio-Oss presents good osteoconductive properties by achieving suitable bone volume values.

KEY WORDS: Bio-Oss, bone regeneration, PRP, rabbits

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Ideal bone graft material should combine osteoconductive, osteoinductive, and osteogenic properties; however, only autologous bone grafts gather all these three properties.[1] Several osteoconductive biomaterials such as allografts, xenografts, and alloplastic grafts have been evaluated for bone regeneration purposes.[2–9] Bio-Oss® (Geistlich Biomaterials, Wolhusen, Switzerland) xenografts are hydroxyapatite granules from bovine origin that have been extensively used in bone augmentation procedures.[3–6] The lack of osteoinductive properties in this biomaterial encourages researchers to find ways for improving its in vivo behavior. Recently, the use of growth factors for stimulating bone regeneration raised great interest.[10–16] Multiple studies had pointed out the advantages of using platelet-rich plasma (PRP)

for stimulating bone regeneration.[17,18] PRP is a platelet concentrate easily obtained by centrifugation of autologous blood and enriched with growth factors. These growth factors were described to be released from alpha granules when platelet activation is induced by Ca ions from the extracellular media. Platelet growth factors play an important role in angiogenesis and tissue regeneration by controlling cell migration, differentiation, and proliferation.[19–21] PRP had been used in combination with other biomaterials such as bovine hydroxyapatite or autologous bone grafts.[22–25]

However, there might be possible benefits from using PRP, especially when it is not combined with other biomaterials. Therefore, the present study aimed to evaluate the benefits of using PRP alone, and compare it to Bio-Oss, as control, in vertical bone augmentation capacity.

## MATERIALS AND METHODS

In this animal study, the protocol was approved by the ethical committee for animal experiments of Complutense University of Madrid (UCM). Experiments were conducted in accordance with the guidelines laid down by the European Communities Council Directive of November 24, 1986 (86/609/EEC), and adequate measurements were taken to minimize pain and discomfort to animals.

Eight healthy 6-month-old female New Zealand rabbits of about 3.5 kg were used in this study. The animals were accommodated in the official stable for animal assays of the UCM at 22 to 24°C with 55 to 70% humidity, light cycles of 12 hours, and air renewal 15 times per hour.

### Preparation of PRP and Platelet Counting

All rabbits were anesthetized with an intramuscular dose of 0.75 mg/kg ketamine (Imalgene 1000®, Rhone Merieux, Toulouse, France) and 0.25 mg/kg xilacine (Rompun®, Bayer, Leverkusen, Germany). Immediately before surgery, 10 cc of whole blood was withdrawn via ear venous aspiration into 4.5 cc test tubes. Directly, it was mixed with a 3.8% sodium citrate solution at a ratio of 1 cc sodium citrate solution to 5 cc whole blood, achieving anticoagulation through calcium binding. The blood was then centrifuged with a Nahita® 280G centrifuge (AUXILAB S.L., Navarra, Spain) into three basic components: red blood cells (RBCs), PRP, and platelet-poor plasma (PPP). Because of differential densities, the RBC layer usually presents at the lowest level, the PRP layer in the middle, and the PPP layer at the top. A pipette (Gilson, Villiers-le-Bel, France) was used to separate each layer from the less dense to the denser. Therefore, PPP was separated first (about 2.25 cc) and PRP second (about 0.9 cc), leaving the residual RBCs (about 2.25 cc).

Platelet counting of the obtained PRP was measured with a flow cytometry device (ADVIA 120, Hematology system, Bayer, Leverkusen, Germany). Before its surgical application, PRP (0.2 mL) was activated with a 30% $CaCl_2$ solution. The preparation of PRP was performed simultaneously during the surgical procedures.

### Surgical Procedure

In the present study, titanium bone conduction cylinder method was applied. The titanium cylinders (Laboratorios Aragoneses s.a., Madrid, Spain) had an inner rough surface with dimensions of 4 mm height, 0.5 mm thickness, and 9 mm diameter. Upon collecting the PRP, animals were placed in sternal recumbency, the head was shaved, and the cutaneous surface was disinfected with povidone iod solution prior to the operation. Surgical steps followed in this protocol were similar to those applied in a previous study.[26] The calvaria bone surface was exposed through a skin incision of approximately 4 cm in length over the linea media. The defects were made on each side of the median sagittal suture without crossing it. A titanium bone conduction chamber was created by fixing a 4-mm-high titanium ring on each slit, and slightly removing the cortical bone surface surrounded by the ring with a rounded burr in order to promote bone regeneration. On each rabbit, one chamber was grafted with Bio-Oss and the contralateral was filled with PRP. Closure and relocation of periosteum, subcutaneous tissues, and skin were achieved according to a previous protocol.[26] Terramicina® (Pfizer, Madrid, Spain) in water was given for 7 days as a postoperative antibiotic. The animals were sacrificed 4 weeks after the intervention with an overdose of sodium pentobarbital IV (Dolethal®, Vétoquinol, Lure, France).

In postmortem phase, tested bone sites were harvested and samples were then preserved fixed in formaldehyde 10% buffer solution at pH 7.0.

### Densitometry

Densitometry for the analysis of bone mineral density (BMD) was achieved on the calvaria blocks using the

XR-26 Norland® densitometer (Norland Corp., Fort Atkinson, WI, USA). A bone area of $0.5 \times 0.5$ cm$^2$ was analyzed inside both the PRP and the Bio-Oss control cylinders with an exploring resolution of $1.0 \times 1.0$ mm$^2$, a measuring resolution of $0.5 \times 0.5$ mm$^2$, and an exploration speed of 40 mm/s. BMD was then calculated.[26]

### Histology and Histomorphometry

Sample dehydration, embedding, sectioning, and staining were performed according to a previous protocol.[26–28] The histological evaluation of bone neoformation was carried out by means of optical microscopy. Light micrographs (at magnification ×6) of the biopsy slices were captured with a digital camera and analyzed with the histomorphometry software MIP-4 (Digital Image System, Barcelona, Spain). Six randomly selected slices were analyzed for each biopsy sample. The area inside the cylinder was included for histomorphometric evaluation, while the original cortical bone and the area outside the cylinder were excluded. The already-existing bone was lamellar, while the regenerated bone was woven and grew inside the cylinder so both types of bone could be easily differentiated in the histological observations.

Total sample volume, newly formed bone, and remaining graft (RG) volume were measured in each cylinder. Collected measurements would enable calculating the average percentage of augmented bone volume (BV) formed in chamber and percentage volume of the RG material. The total of BV and RG is the augmented mineralized tissue (AMT).[26]

### Statistical Analysis

Statistical analyses were achieved using statistical software package (SPSS 7.0, Chicago, IL, USA) for analyzing densitometry and histomorphometric measurements by Mann–Whitney test ($p < .05$).

## RESULTS

No operative or postoperative complications were noticed, and no animal loss occurred before scarification.

### Platelet Counting

Platelet counts confirmed that the PRP preparation technique used in this study produced a source of highly concentred platelets. The average peripheral blood platelet count was 144,000/mm$^3$ with a range from 70,000 to 260,000. The average PPP count was 45,000/mm$^3$ with a range of 10,000 to 50,000. The average PRP platelet count was 1,050,000/mm$^3$ with a range of 625,000 to 1,465,000.

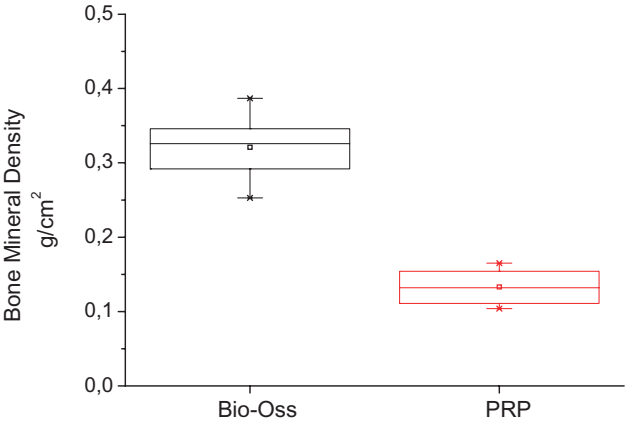


**Figure 1** Box-plot chart of bone mineral density values of inside the cylinders.

### Densitometry

BMD mean values of PRP and Bio-Oss chambers were $0.317 \pm 0.051$ and $0.128 \pm 0.024$ g/cm$^2$, respectively. High statistical significant difference ($p < .0001$) was revealed as Bio-Oss had significantly high mineral density (Figure 1).

### Histology

No inflammatory reactions were observed in the histological slices in bone conduction cylinders for both tested materials. In both groups, bone growth was more pronounced on the titanium walls and occurred from the original bone surface, but not from the periosteum. This was observed more in Bio-Oss cylinders (Figure 2).

In cylinders grafted with PRP, bone formation was scarce, and few isolated newly formed trabeculae were seen on the external cortical surface. The trabeculae

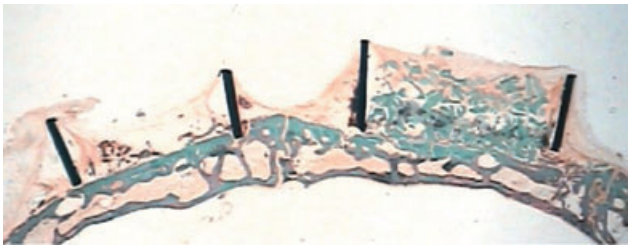

**Figure 2** Higher degree of new bone formation and lesser numbers of interconnecting bone trabeculae noticed in samples of platelet-rich plasma (left cylinder) compared to those grafted with the Bio-Oss (right cylinder) (original magnification × 2).

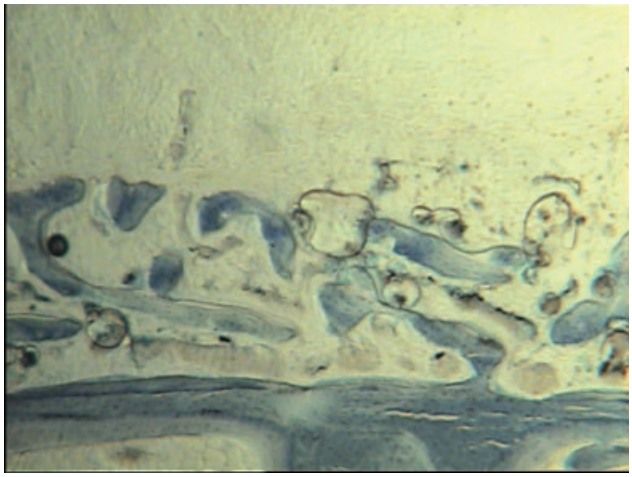

**Figure 3** Few isolated newly formed trabeculae were seen on the external cortical surface (original magnification × 10).

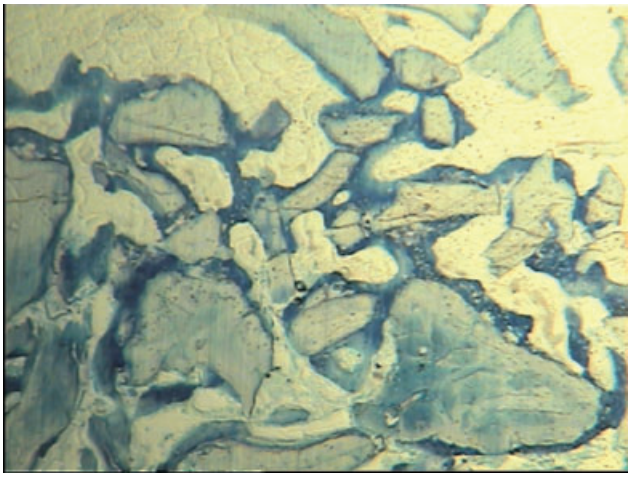

**Figure 4** Micrograph of bone conduction chamber grafted with Bio-Oss showing the second half of the bone conduction chamber away from the original bone surface. Bio-Oss granules (*) are surrounded mainly by fibrous tissue (+) and scarce newly formed bone (arrowhead) (original magnification × 10).

were often connected to the original bone surface. No osteoblast activity was observed (Figure 3).

Regarding chambers grafted with Bio-Oss, a large number of the implanted granules was observed distributed over the whole specimen area. Acidophilic Bio-Oss was dyed gray-blue with TB. Bio-Oss particles present lacunae free from osteocytes because of their animal origin; nevertheless, their artificial shape differentiates them from any bone trabeculae that might be growing around. Bio-Oss granules were mostly surrounded by a thin layer of fibrous tissue, especially at the medium and upper tiers, while in the lower tier (corresponding to the area in contact with calvaria) bone trabeculae were observed surrounding the smooth surface of the Bio-Oss particles (Figure 4). Bone regeneration was observed from the external surface of the cortical bone to approximately one-third of the height of the cylinder confirming the osteoconductive properties of Bio-Oss (Figure 5). However, typical signs of biomaterial resorption such as osteoclasts forming Howship's lacunae on its surface, etching, pits, or resorptive trail formation could not be identified on Bio-Oss granules.

### Histomorphometry

The data obtained from histomorphometry analyses are shown in Table 1. Obtained BV, RG, and AMT values were significantly lower for PRP chambers ($p < .0001$).

## DISCUSSION

A clear differentiation was revealed by the result of the present study between the effect on osseous regeneration of solely applied PRP and Bio-Oss. The investigated densitometric and histomorphometric parameters showed statistically significant high influence of the control (Bio-Oss) when compared with PRP (see Figure 1 and Table 1).

In recent studies, PRP had been found to influence bone matrix protein expression during early stages of bone regeneration, and a significant increase in bone formation occurs 2 weeks after its implantation.[29,30] A possible role in local regulation of fracture healing and bone regeneration was suggested and that might be probable because of the synergic effect of growth

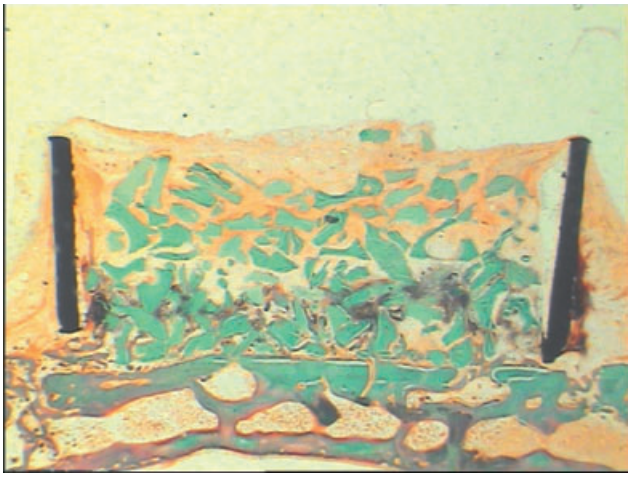

**Figure 5** Bone regeneration was observed from the external surface of cortical bone to approximately one-third of the height of the cylinder (original magnification × 3).

**TABLE 1 Results in Percentage of Histomorphometric Analysis for Both Tested Materials**

| Tested Material | BV % (±SD) | RG % (±SD) | AMT % (±SD) |
|---|---|---|---|
| PRP | 6.24 (±1.87) | 0.00 | 6.24 (±1.87) |
| Bio-Oss | 13.18 (±4.09) | 35.31 (±6.48) | 46.81 (±10.55) |

AMT = augmented mineralized tissue; BV = bone volume; PRP = platelet-rich plasma; RG = remaining graft.

factors present in the alpha granules of PRP.[31–33] Similarly, several authors claimed PRP capacity to improve bone regeneration quality and quantity.[17] It has been suggested that PRP improves bone regeneration in its early phases, between 3 and 6 weeks after implantation.[34] In addition, it has recently been reported that PRP alone could help in increasing bone augmentation in sinus lifting procedures.[35] However, in this experiment, the aforementioned could not be confirmed when PRP was solely applied. The present study came in agreement with other recent studies that reported no benefits from using PRP on bone regeneration in bicortical defects on rabbit calvaria.[30,36] It was reported that PRP has no effect in early bone healing, and its combination with collagen shows no benefits on bone regeneration over using collagen alone.[30] Moreover, in another study, PRP was combined with collagen sponges and used to fill critical size defects on rabbit calvaria, but no significant improvement on the bone regeneration was observed.[36] Yamada and colleagues[37] stated that PRP is only useful when it is used combined with mesenchymal cells; otherwise, it has no better effect than controls.

Nevertheless, the histomorphometric results of the present study reveal little bone formation in PRP groups. These results resemble those reported elsewhere in the literature, where only a 6.24% augmented BV was obtained after 4 weeks of locally applying peripheral blood in a similar-guided bone regeneration model.[38] The BV values obtained in samples treated with Bio-Oss were comparable to that found in the literature where Bio-Oss was grafted into silicone cylinders on rat's calvaria (18.1%),[39] or in titanium cylinders on rabbits' calvaria (11.7[26] and 19.9%[27]) (see Table 1).

Most of the animal studies that evaluate bone regeneration obtained by using PRP were done in critical-sized defect models.[40,41] In the evaluation of osteoconductive materials, the defect created should be large enough to challenge the adjacent bone with a space that can hardly fill spontaneously. However, the effects of growth factors on bone conductive materials are usually seen at an early stage during bone ingrowth. It is then difficult to find the right time to measure these effects, if new ingrown bone rapidly filled the defect.[42] The "bone conduction chamber" (or cylinder) appeared to be a useful tool for quantifying bone regeneration under the most variable conditions in both rats, goats, and rabbits.[27,41,42] This bone chamber is unlikely to be completely filled with bone. Therefore, the effects of growth factors, processing of bone and biomaterials within these chambers, can be evaluated as differing final amounts of bone are formed. Moreover, using bone conduction chambers, vertical bone augmentation obtained by tested biomaterials can be evaluated.

Several authors consider that a 4-week period of implantation is enough time to observe angiogenesis and bone formation in several animal models, including rabbits, where hydroxyapatite polymers or brushite were grafted into bone.[26,38,43,44] In the present experiment, histological changes that occurred in grafted areas during the 4 weeks of implantation were pronounced and permit to evaluate the differences in bone regeneration capacity of both assayed biomaterials.

Regarding signs of biomaterial resorption such as osteoclasts forming Howship's lacunae on its surface, etching, pits, or resorptive trail formation could not be identified on Bio-Oss granules. From the present observations, it can be inferred that after 4 weeks in rabbits' calvaria, Bio-Oss resorption did not take place.

## CONCLUSIONS

Within the limits of the present study, it can be concluded that no beneficial effect of using PRP on osseous regeneration as it was solely applied. In addition, it was emphasized that Bio-Oss presents good osteoconductive properties by achieving suitable BV values.

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