

## Original Article

# Occlusive barriers in combination with particulate Bio-Oss® graft: a pilot study on rabbit calvaria

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**Abstract:** The aim of this study was to histologically evaluate the potential for vertical bone augmentation of the Bio-Oss® graft compared to a blood clot in conjunction with an occlusive barrier in the rabbit calvaria defect model. Metallic dome shaped barriers with 4.5 mm width and 3.5 mm height were positioned in six adult rabbit skulls. At the right side, the barrier was filled with Bio-Oss®, and the left side was filled with a blood clot. After a healing period of three months, the animals were sacrificed, and the samples were prepared for histological and histomorphometric analyses. The total mineralized area (TMA) as well as the newly formed bone (NBA) was calculated as the percentage of the bone augmentation inside the metallic barriers, and parametric statistical analysis was used to describe the findings. The samples with blood clots exhibited significantly less TMA formation than the Bio-Oss® group. However, the difference in the amount of NBA was not statistically significant. Furthermore, the Bio-Oss® specimens exhibited remaining graft particles within the sample. In conclusion, the barriers filled with Bio-Oss® exhibited significantly higher TMA than those with only blood clots, and the remaining Bio-Oss® particles were integrated into newly formed bone tissue to fill the spaces and promote a greater volume than the samples from the blood clot groups.

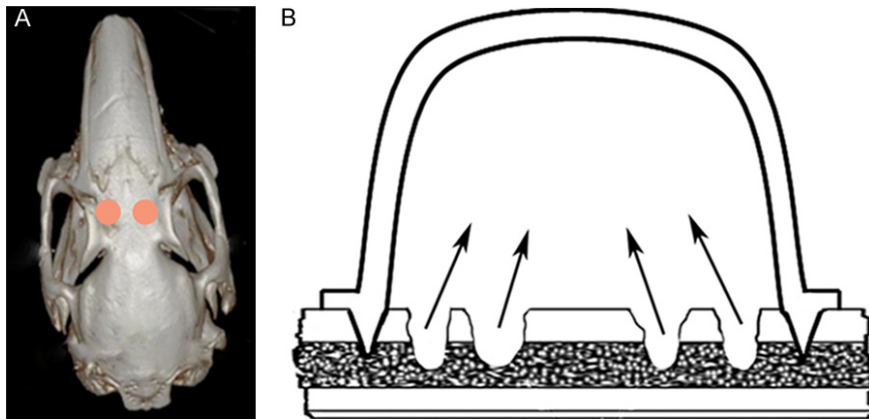
**Keywords:** Bone regeneration, bone graft, Bio-Oss, barriers

## Introduction

Bone augmentation procedures require complex planning, are costly and may involve multi-step long-lasting therapy. Various surgical techniques have been proposed with controversial results. As an alternative to increase bone volume with autografts from a separate donor site, the use of a subperiosteal barrier was developed to allow blood clots to promote bone tissue development [1]. This technique provides a space that allows the immigration of osteogenic and angiogenic cells and allows the underlying blood clot to mineralize [2]. Furthermore, this technique can be enhanced combining the barrier with some biomaterial underneath the defect [3, 4]. Previous researches [5] studied the behavior of the barrier technique on humans and rabbits, observing that a

large bone volume augmentation could be accomplished with this technique. The main advantage of a rigid occlusive barrier is that the surgery is less traumatic compared with removing bone grafts and does not require a donor site [5].

Bone substitute materials are used to fill bone defects adjacent to dental implants and may support bone augmentation [6, 7] without use of bone grafts. In this context, xenografts, such as the anorganic bovine bone Bio-Oss®, have been widely used for filling of bone defects in the alveolar ridge. Bio-Oss® is a deproteinized anorganic bovine xenograft with a low resorption rate and intercrystallite bonding similar to human cancellous bone [8, 9]. However, some reports [10] concluded that the amount of bone regeneration using this material seems to be



**Figure 1.** A. Rabbit skull scheme when the barriers were placed; B. Barrier with a space inside to be filled with blood clot or Bio-Oss® particulate graft to achieve bone augmentation. The arrows are showing the active bleeding of decortication procedure.

variable. In addition, further studies are necessary to evaluate the potential benefits of an anorganic bovine bone as Bio-Oss®, particularly in combination with other devices [11]. In particular the value of Bio-oss® for vertical augmentation of alveolar sites still remains unclear.

On the other hand, the rabbit calvaria defect model is usually used to test the osteogenic potential of different biomaterials [12, 13]. However, there are no reports about the use of barriers in combination with deproteinized anorganic bovine xenograft as Bio-Oss®. Thus, the aim of this study was to histologically evaluate the potential for vertical bone augmentation of the Bio-Oss® graft compared to a blood clot in conjunction with an occlusive barrier in the rabbit calvaria defect model.

## Materials and methods

### Study design

Six rabbits (*Oryctolagus cuniculus*) were selected for this descriptive transversal study with a mean weight of  $3.0 \pm 0.8$  kg to minimize the growth effect. Two 4.5-mm defects in the rabbit calvaria of six randomly chosen rabbits were created to place an occlusive barrier with deproteinized bovine bone mineral particles (Bio-Oss®, Granules 0.25-1 mm) or a blood clot inside.

This study was approved by the ethical committee, Universidad de La Frontera, Temuco, Chile (N° 06/008). The animals were maintained with

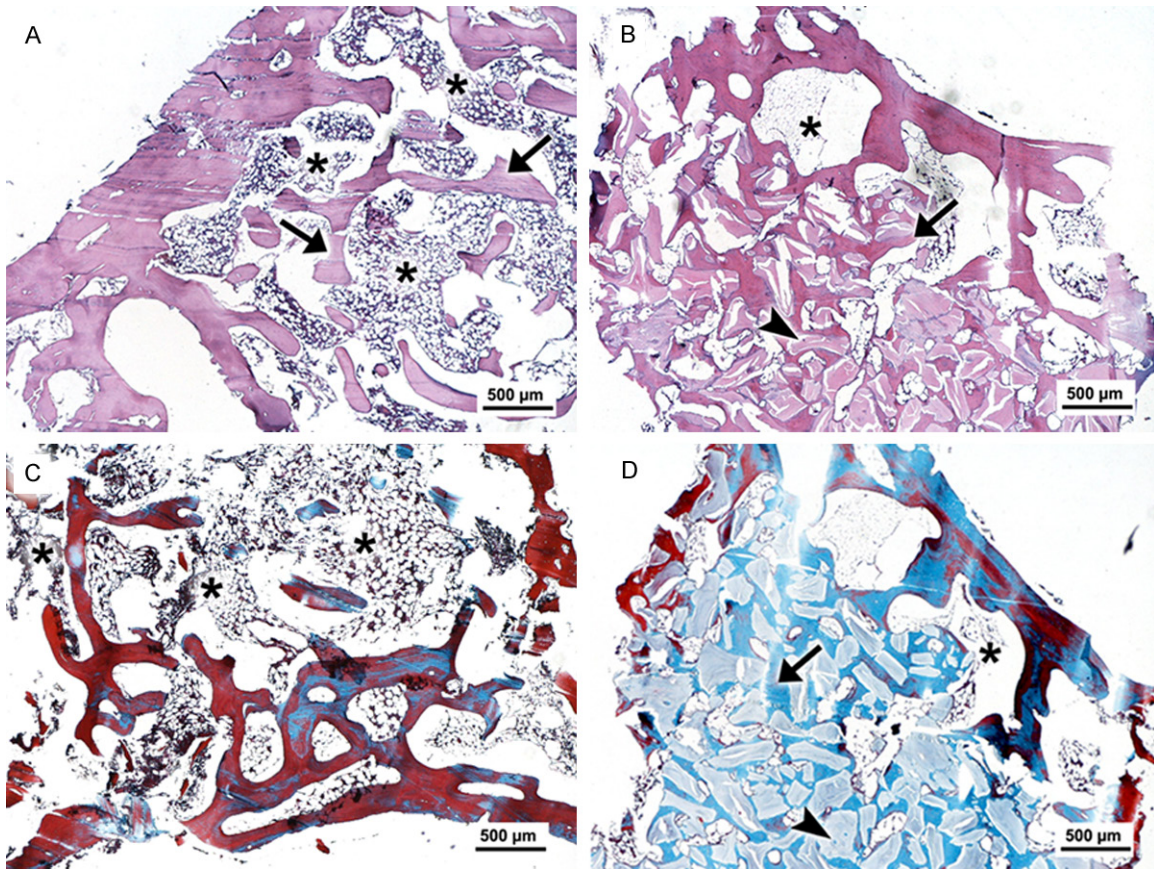
ad libitum feeding in separated closed cages with controlled temperatures.

### Surgical technique

The anesthetic procedure was performed with ketamine 30 mg/kg and xylazine 5 mg/kg supplemented with intra-operative analgesia with buprenorphine 0.3 ml/kg at 30 minutes after the first application. Additionally,

diazepam 5 mg/mL, with a dose of 1 mg/Kg i.m., was used to maintain neuroleptanalgesia levels. Neuroleptanalgesia was complemented with Hypnorm 0.1 ml/kg i.m. at intervals of 30 minutes during surgery. In addition, as a local anesthetic, 0.4 ml of lidocaine 2% was applied with a dose of 1:100.000 of epinephrine (Octocaine-100, Novocol Pharmaceutical, Ontario, Canada).

During surgery, the rabbit's skin was shaved and treated with polyvidone-iodine to perform the procedure under sterile environment. A median lineal incision was made from the frontal to the occipital region, separating the skin and periosteum 3 to 4 cm laterally. Subsequently, two osteotomies were performed with a 4.5-mm trephine bur and continuous irrigation. Then, multiple perforations were created in the external cortical trephined area of the calvaria with low-speed round burs, with safety stops to prevent intracranial perforation. Therefore, at the left side (blood clot group), the barrier was filled with blood from the spontaneous bleeding that occurred by the calvaria perforations. Conversely, the barrier placed at the right side was filled with anorganic bovine bone (Bio-Oss®, Granules 0.25-1 mm, Geistlich Pharma AG, Wolhusen, Switzerland). Both barriers were placed on each side of the sagittal suture in the same animal without contact (**Figure 1**). Each barrier was 4.5 mm in diameter (3.7-mm internal diameter) and 3.5 mm in height, with smooth internal and smooth surface walls. Following surgery, the rabbits were medicated for the three first days with one daily dose of oxy-



**Figure 2.** Histological analysis through light microscopy: A. Blood clot group (HE) shows newly formed bone (arrow) and connective tissue (\*); B. Bio-Oss® group (HE) reveals newly formed bone (arrow), graft particles (arrowhead) integrated into the bone and connective tissue (\*) in the trabecular region; C. Blood clot group (Masson trichrome) reveals a large area of connective tissue (\*) between the areas of new bone formation (arrow); D. Bio-Oss® group (Masson trichrome) shows the integration of graft particles (arrowhead) with the newly formed bone (arrow) and connective tissue (\*) in the trabecular region.

tetracycline 200 mg/kg i.m and meloxicam 0.2 mg/kg s.c.

#### *Specimen processing*

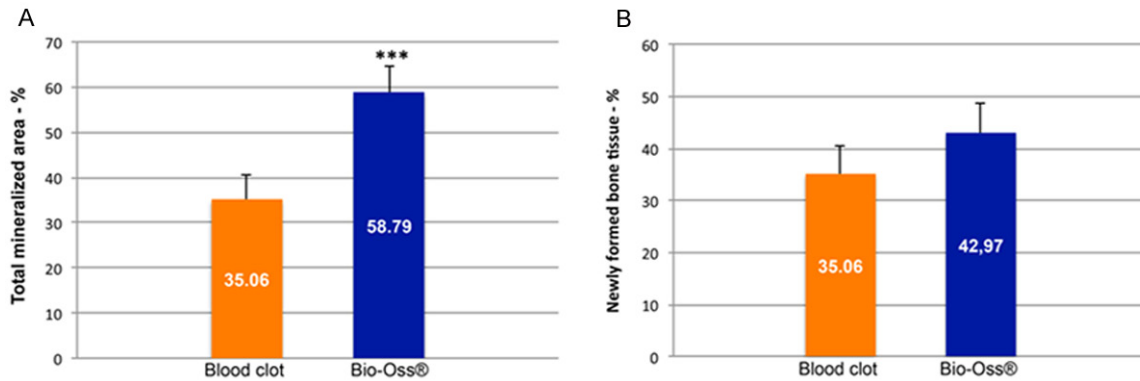
After three months, the animals were sacrificed with an overdose of 1 ml of Hypnorm (i.m.) and 10 ml of sodium pentobarbital (i.v.). Biopsies were obtained from each rabbit calvaria that were composed of the two previously inserted barriers. The biopsies were fixed by immersion in 4% buffered formaldehyde for 48 h and refrigerated at 5°C (41°F). Subsequently, the capsules were cut with a circular saw (0.2 mm thick) in the antero-posterior direction. The first cut was performed to eliminate the lateral metal wall, the second was made at the center of the sample, and the third was made to eliminate the contralateral metal wall (each cut was parallel to the one above). In this way, two sections of the samples were obtained. Subse-

quently, the metal part was eliminated carefully, and the samples were decalcified for conventional histology. Only the middle portions of the samples were used for histological analysis.

For histological analysis, hematoxylin-eosin and Masson trichrome techniques were used, and the total mineralized area (TMA) and newly formed bone areas (NBA) were calculated as percentages from the various cuts of the bone inside the metallic barriers. Is important to point out that TMA was considered in the histomorphometric analysis as the bone tissue inside the metallic barrier including the graft particles. In contrast, the NBA was considered as only trabecular bone inside the barrier.

In the histomorphometric analysis, the samples were analyzed in detail at all segments of the newly formed bone tissue using a camera





**Figure 3.** A. Scheme of total mineralized area, observing statistically differences between both groups (\*\*\* $P = 0.007$ ); B. Scheme of newly formed bone area in both groups. It does not exist significantly differences between them ( $P = 0.0812$ ).

(NIKON DS-Fi1 (Sight) and a Stemi 2000-C stereomicroscope) with 1280x960 pixels. The ImageJ software was used to measure and analyze the amount of TMA, differentiating the NBA and the particles of Bio-Oss® graft. For this purpose, the regions of microphotographs that did not correspond to bone tissue were deleted, then photomicrographs were transformed into binary images (black and white) and thus the TMA was measured by pixel counting method. Furthermore, by the same method the areas occupied by newly formed bone and particles of Bio-Oss® were measured.

#### Statistical analysis

The Shapiro-Wilk test was applied to determine the normality of the data using Assistat v. 7.7 software. Subsequently, the paired t-test with Welch correction was used to determine the statistically differences ( $P \leq 0.001$ ) using the GraphPad InStat v. 3.0 software.

## Results

#### Animal behavior

No signs of discomfort or suffering were observed in the animals. Feeding was normal and the weight was considered to be within normal parameters for all of the animals. The surgical site exhibited normal healing, and the suture was reabsorbed 20 to 25 days post-surgical intervention without signs of infection. Additionally, when the barriers were removed, the augmented volume had the same shape and contours of barrier.

#### Histological and histomorphometric analyses

Histological analysis using light microscopy identified newly formed bone and a large area of connective tissue in the blood clot group. The Bio-Oss® group exhibited newly formed bone, and graft particles integrated into the bone and a small amount connective tissue was found between the bone trabeculae (**Figure 2**).

The samples with a blood clot ( $n = 6$ ) exhibited 35.06% ( $\pm 5.41$ ) of TMA, representing a bone surface ranging from 3076110  $\mu\text{m}^2$  to 5072645  $\mu\text{m}^2$ . In contrast, the samples with Bio-Oss® inside the capsules exhibited a mean of TMA of approximately 58.79% ( $\pm 5.74$ ), with the bone surface ranging between 6082259 and 9688298  $\mu\text{m}^2$ . Furthermore, the difference between both groups (**Figure 3**) was statistically significantly ( $P = 0.0007$ ).

The NBA represented 35.06% ( $\pm 5.42$ ) and 42.97% ( $\pm 5.74$ ) of the total mineralized area in the blood clot samples and Bio-Oss® specimens, respectively. Although the latter group exhibited a tendency to augment the NBA, the difference was not statistically significant ( $P = 0.0812$ ).

Furthermore, the mean area of the remaining Bio-Oss® particles was 3,364,165  $\mu\text{m}^2$ , representing 26.22% ( $\pm 5.61$ ) of the TMA. In the blood clot samples, the spaces between the bone trabeculae were filled with connective tissue.

## Discussion

Over the last decades, guided bone augmentation has been increasingly used by surgeons. Some researches [1] reported that the best method for guided bone augmentation is to use a stiff occlusive barrier. The stiffness of the barrier used in this study allowed shape maintenance and the creation of a space for graft placement, preventing the collapse of the defect space and achieving the required volume. Although in some cases the stiff barriers may be exposed, we did not encounter this situation in our research. A similar study [14] used rounded and large barrier domes with beta tricalcium phosphate inside in rat's calvaria for 8 weeks and identified bone regeneration with these devices, observing newly formed bone and that the bone augmentation took the same shape as the dome. These results are consistent with the clinical observations of the present study. Thus, this research confirms that the barriers that were used enabled bone-graft and blood-clot stabilization as well as space maintenance, as described in the literature [15, 16].

Although bone formation can be obtained under a barrier with just a clot, it is necessary to combine the clot with a bone graft to augment the bone volume [2]. This statement is not in accordance with the results of this study because both groups exhibited bone augmentation with the barriers, although the difference in the TMA was considerably higher in the Bio-Oss® group.

In this study, the difference between the TMA in the Bio-Oss® and the blood clot groups was significant, with a greater percentage of TMA observed in Bio-Oss® samples. Bio-Oss® provides a hydroxyapatite bone mineral architecture for bone growth, acting as a three-dimensional matrix that allows for rapid clot stabilization and revascularization. Furthermore, Bio-Oss® facilitates osteoblast migration and osteogenesis [11, 17, 18]. The literature reports positive results related to Bio-Oss® and rabbit calvaria defects. Torres et al. [19] concluded that Bio-Oss® achieve a suitable bone volume value. Others authors [20, 21] have reported results consistent with this study as well, observing a significantly greater bone volume at 8 weeks when compared with a control (clot) defects. Similarly, studies in humans [11]

reported that Bio-Oss® exhibited a greater potential to heal in a greater dimension.

Although the Bio-Oss® group exhibited a tendency to increase the NBA, the difference between the blood clot group and the Bio-Oss® group was not statistically significantly. These findings are similar to some researches [22], which reported no differences in new bone formation between the Bio-Oss® graft and a control defect at 4, 6 and 8 weeks sacrifice time.

Histologically, both groups exhibited no significant inflammation, which agreed with previously reported results [19, 21]. Additionally, incomplete graft particle resorption was observed in the Bio-Oss® group, with newly formed bone adhered to the particles; these observations were more common in the center of the defect than in the borders. This histological description is consistent with the findings of some studies that used Bio-Oss® as a bone graft [8, 18, 24]. In addition, the remaining Bio-Oss® particles represented a mean of 26.22% of the total mineralized area, which is close to the 31% reported in the literature [25]. In contrast, the spaces between the bone trabeculae in blood clot samples were observed with connective tissue [21].

Despite approximately the same amount of NBA tissue in both groups, it appears that the remaining bone particles of Bio-Oss® graft were integrated into newly formed bone tissue, filling the spaces and helping to promote a higher TMA when compared with blood clot groups, in which spaces were filled by connective tissue.

Although some authors [23] stated that the decortication of calvaria bone does not result in more bone formation when compared with no cortical removal, we decided to decorticate the calvaria because bone perforation permits progenitor cell migration from the bone marrow to the treated site and facilitates angiogenesis [14].

The technique of guided bone regeneration through barriers may be used for blood clot-filled defects or those with biomaterials, maintaining the space and promoting clot or biomaterial stabilization and cell exclusion [14, 26]. Although a considerable augmentation can be achieved through this technique, the use of a

barrier is advisable to stabilize a particulate bone graft [27]. Thus, in this research, we demonstrated that it is possible to increase the skull bone thickness beyond the skeletal envelope, which agrees with other studies [23, 28].

It is important to note that the developmental pattern of bone formation in the calvaria and jaw bones are intramembranous [29]; thus, the calvaria is a suitable place to test extracortical bone formation in an animal model. Nevertheless, the major limitation of this study was the small number of rabbits used and the single sacrifice period. Thus, further studies with larger samples and other histological techniques could lead to better interpretation of these preliminary results.

In conclusion, the barriers filled with Bio-Oss® exhibited significantly higher TMA than those with only blood clots, and the remaining Bio-Oss® particles were integrated into newly formed bone tissue to fill the spaces and promote a greater volume than the samples from the blood clot groups.

## Disclosure of conflict of interest

None.

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