



Biocompatibility and osteopromotor factor of bovine integral bone—a microscopic and histometric analysis

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Background: The objective of this study was to evaluate the biological properties, biocompatibility, and osteopromotor factor of integral bone of bovine origin implanted in critical defects of rat calvaria and the subcutaneous plane of rats.

Methods: The study was divided into two stages. For the first stage, 24 rats were divided into two groups of 12 animals: group GC, in which the critical defect was filled only by clot, and group GO, in which the defect was filled with particulate biomaterial, and the analysis performed at 30 and 60 days postoperatively. For the second stage, 16 rats were divided into two groups of eight animals: the GOP group, in which the biomaterial in its particulate form was inserted in the subcutaneous plane, and the GOB group, in which the block biomaterial was inserted in the subcutaneous; and the analysis performed at 15 and 45 days postoperatively.

Results: The histological and histometric results of the calvaria demonstrated that the biomaterial induced a foreign body reaction over the entire length of the defect and around the particles and was not able to induce bone neoformation. Statistically, no difference was observed for the time, biomaterial, and time × biomaterial parameters ($P > 0.05$). Subcutaneous microscopic examination of the pieces obtained at 15 days showed an inflammatory reaction around the particles of the material with the presence of giant cells and at 45 days, and a reduction in the inflammatory reaction and presence of fibrous connective tissue around the particles was observed with the presence of giant cells, and for the block biomaterial, connective tissue was present in the trabecular spaces. There was no evidence of ectopic bone formation.

Conclusions: The biomaterial, despite being biocompatible, do not promote bone neoformation.

Keywords: Xenogenic; osteopromotor; biocompatibility; integral bone; guided bone regeneration

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Introduction

Biomaterials are frequently used as complementary therapies, especially for the installation of implants, alveolar filling, sinus grafts, or guided bone regeneration (ROG)

(1-3). However, knowing the biological potential of each material is of fundamental importance to indicate their use in different clinical situations; thus, observing their biological behavior in contact with different tissues is

essential (4,5).

In 2008, aimed to unify and expand the concepts of biocompatibility authors proposed that a biomaterial should perform the desired function in relation to medical therapy, without inducing any undesirable local or systemic effects to the beneficiary of the therapy, but generate cellular responses and tissues more beneficial in that specific situation and optimize the clinically relevant responses of that therapy (6). In this context, it can be determined that biocompatibility is a property that materials must possess to enable their use in a biological system without causing adverse reactions or preventing the tissue differentiation characteristic of the implantation site (7). Acute and chronic inflammatory responses and the immune response are among the adverse reactions that can be identified clinically with biomaterials (8,9).

Biomaterials can have different origins and can be heterogeneous or xenogenic in nature, most commonly being obtained from cattle, pigs, or goats (10-12). These biomaterials, except autogenous biomaterials, play a fundamental role in filling the spaces presented by bone defects, without physiological incorporation (13,14). They present only osteoconductive activity; it has also been reported, including by the companies that produce biomaterials or bone substitutes, that their products are only osteoconductors. Additionally, there are some claims that some demineralized biomaterials are also osteoinductive (15). However, there are questions about the osteoinductive function of most bone substitutes.

To resolve the doubts regarding the biological properties of integral bovine bone, the implantation of integral bovine bone was carried out in critical defects in rats calvaria and in the subcutaneous tissue to study the osteoprotomorph potential, osteoinduction capacity and biocompatibility of the biomaterial (16).

We present the following article in accordance with the ARRIVE reporting checklist (available at <https://fomm.amegroups.com/article/view/10.21037/fomm-21-42/rc>) (17).

Methods

Animal model

The research was approved by the Animal Experimentation Ethics Committee of the Araçatuba Dental School - UNESP (CEUA) under the protocol 2011-00893, in compliance with the National Council for the Control of Animal Experimentation (CONCEA), national guidelines

for the care and use of animals.

Biomaterial

OrthoGen (Baummer S.A., Mogi Mirim, Brazil) is produced from bovine origin, a heterogeneous biomaterial, is composed of an organic portion of collagenous proteins (25–30%) and a mineral portion of hydroxyapatite (65–70%). There are two of presentation, particulate and block with a degradation time of approximately six months.

Study design

A total of 40 adult male (3–4 months) rats (*Rattus norvegicus*, Albinus, Wistar) weighing between 300 and 400 g were used. Throughout the experimental period, the animals were provided a solid and balanced food ration (Ração Ativada Produtor, Moinho Primor S.A), with water provided ad libitum in the vivarium of the Araçatuba Dental School – UNESP. Day and night cycles and controlled temperature were maintained. Three animals were housed per cage.

For the first stage of the study, 24 animals were randomly divided into two groups of 12 animals: group GC (clot) in which the bone defect was filled only with clot and group GO (Orthogen) in which the defect was filled with bovine integral bone. All the defects were protected with a bovine bone cortical membrane (GenDerm® Baummer S.A). On the day of the surgery, a critical bone defect of 8 mm in diameter was made in the center of the calvaria in all animals (18). The animals were euthanized at 30 and 60 days after the surgery.

For the second stage of the study, 16 animals were randomly divided into two groups of 8: GOP group (Orthogen particle), in which the bovine integral bone was implanted in a collagen matrix and introduced subcutaneously, and the GOB (Orthogen block) group, in which a block of bovine integral bone was introduced into the subcutaneous tissue. The animals were euthanized at 15 and 45 days after the surgery (19).

Experimental surgical procedure

For the two stages of the experiment, the animals went through a 12-hour preoperative fast and were sedated with ketamine hydrochloride (Francotar-Vibrac do Brasil Ltda, São Paulo, Brazil) associated with xylazine (Rompum-Bayer AS-Animal Health, São Paulo, Brazil), at a dosage of 50 and 5 mg/kg, respectively (4,5,20-22). A strict aseptic protocol

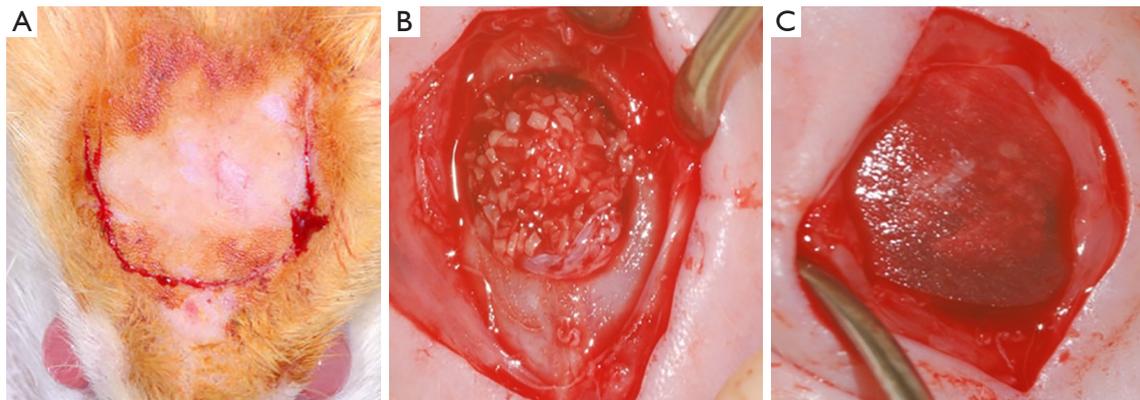


Figure 1 Surgical procedure in rat calvaria. A semilunar incision for access to the calvaria (A); a critical bone defect of diameter 8 mm filled with particulate mineral bovine bone (B); Bovine cortical bone membrane protecting the bone defect, acting as a barrier (C).

was adopted, including sterilization of the instruments used, delimitation of the area to be operated with sterile drapes, and the use of sterile surgical gowns and gloves. All surgical procedures were performed in the operating room of the Vivarium of the Faculty of Dentistry of Araçatuba, UNESP. Prior to each procedure, trichotomy of the region of interest was performed, the region was cleaned with polyvinyl pyrrolidone degenerative iodine (PVPI 10%, Riodeine Degermante, Rioquímica, São José do Rio Preto) and topical PVPI (PVPI 10%, Riodeine, Rioquímica, São José do Rio Preto), and sterile fields were affixed.

Surgical critical defect

A semi-lunar incision of approximately 2 cm in the occipitofrontal direction was made and the flap was detached (*Figure 1A*). Subsequently, with the aid of a 7 mm internal diameter trephine drill (3i Implant Innovations, Inc., Palm Beach Gardens, USA) coupled with low rotation under abundant irrigation with 0.9% sodium chloride solution (Darrow, Rio de Janeiro, Brazil), a critical surgical defect of external diameter 8 mm was made in the central portion of the calvaria involving the sagittal suture (18), while maintaining the integrity of the dura mater. Each animal in the GC and GO group received the proposed treatment (*Figure 1B*), and all defects were protected with the bovine cortical bone membrane (GemDerm® Baummer S.A) (*Figure 1C*) (13). At the end of the procedure, the soft tissues were carefully repositioned and sutured in planes.

Subcutaneous implantation

A linear incision of approximately 1 cm was made in the skin of the dorsal region of the animal following the sagittal

line to expose the subcutaneous tissue (*Figure 2A,B*). Subsequently, the particles of the biomaterial (Orthogen) were inserted into a collagen matrix to be implanted into the tissue (*Figure 3A,B*). The block biomaterial was inserted subcutaneously in the other group (*Figure 3C*). At the end of the implantation, a simple interrupted suture was performed using mono nylon 4-0 (Ethicon, Johnson & Johnson).

Each animal received a single intramuscular dose of 0.2 mL of penicillin G-benzathine (Small Veterinary Pentabiotic, Fort Dodge Saúde Animal Ltda., Campinas, SP) in the immediate postoperative period of the two experimental stages. The animals were euthanized with an anesthetic overdose at the respective experimental times. As an exclusion criterion, it was established that any animal with trans or postoperative surgical complication would be excluded from the sample that was being evaluated (4,5,20-22).

Histological analysis

The samples collected from the calvaria and the subcutaneous tissue were fixed in a 10% formaldehyde solution for 48 h, washed in running water for 48 h, decalcified in 20% EDTA for 5 weeks, dehydrated in a sequence of alcohols, and diaphanized. Subsequently, the calvaries were cut in half in the longitudinal direction, separating the bone defect into two equal parts. The pieces obtained from the two stages of the study were embedded in paraffin and cut into semi-series cuts of 6 μm thickness. A total of 10 slides were obtained from each piece and stained with hematoxylin and eosin (H&E) for descriptive histological analysis and

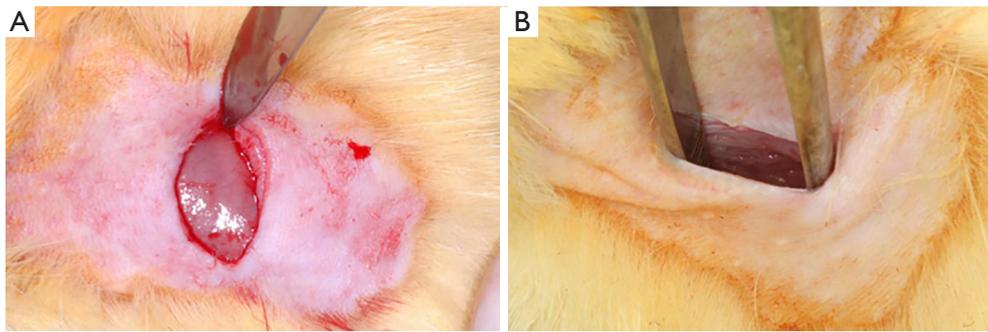


Figure 2 Surgical procedure in the subcutaneous. A linear incision of approximately 1 cm in the skin to access the subcutaneous tissue (A); division of subcutaneous tissue for the implantation of biomaterials (B).



Figure 3 Bovine integral bone in the particulate and block form inserted in the subcutaneous. Particulate bovine integral bone being prepared (A); particulate bovine integral bone inserted in the collagen capsule and implanted in the subcutaneous tissue (B); bovine integral bone block inserted in the subcutaneous tissue (C).

histomorphometric analysis of the calvaria of the newly formed bone tissue. The slides were examined under an Axiokop 2 plus microscope (Carl Zeiss, from Germany) and photographed with the Axio Cam HCR (Carl Zeiss, Brazil). Histometry was performed by overlaying the Merz grid, making it possible to establish the amount of bone neoformation (primary outcome) in the GC and GO groups at the two experimental times, tissue response and cell type present (biocompatibility) in the GOP and GOB groups at the two experimental times (14,23-26).

Statistical analysis

The data obtained during the histomorphometric analyses for the amount of newly formed bone of the sample averages were subjected to a normality test to assess the distribution of the samples (Shapiro-Wilk, $P > 0.05$). After confirming the normal distribution of the samples, for histomorphometric analyses ($P = 0.156$), the 2-factor analysis of variance was used to compare the time, biomaterial, and

time \times biomaterial variants. For the analysis of the amount of biomaterial at 30 and 60 days, the normality test was non-parametric; therefore, the Mann-Whitney test ($P = 0.0167$) was used.

Results

Histological analysis of the calvarias

After 30 days of bone repair, new bone formation was observed in the bone stumps of the GC and GO groups. Notably, areas of neoformed bone tissue (AON) with immature bone characteristics were observed in the center of the defect in the GC group, surrounded by an organized connective tissue (CT) that occupied the central region of the defect and membrane fragments (M) (Figure 4A). For the GO group, microscopic sections were examined to reveal the presence of the membrane (M) of young connective tissue (CT), containing macrophages, lymphocytes, vascular neoformation, fibroblasts, and scarce extracellular matrix filling the space between the

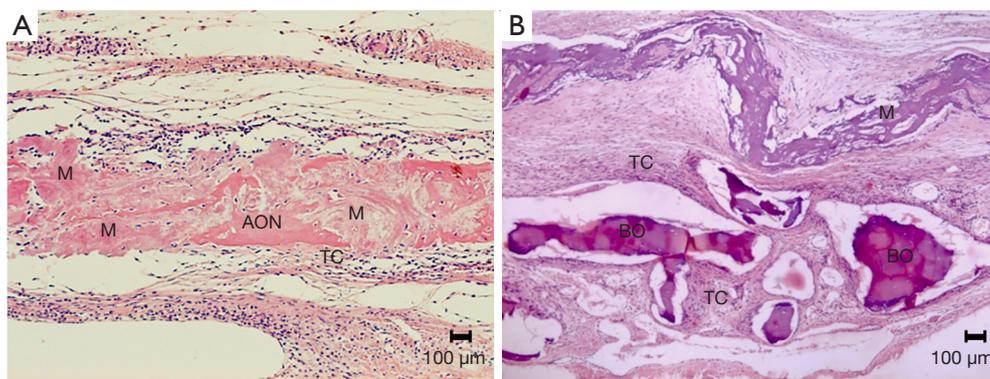


Figure 4 Center of the critical defect at 30 days in the CG group showing areas of immature neoformed bone tissue (AON), surrounded by connective tissue (CT) (A); Center of the critical defect at 30 days in the GO group showing particles of the biomaterial (BO) with varying morphology and sizes and occasionally forming right angles (B). H&E stain. Magnification of 12.5×.

Table 1 Group OG (orthogen)—microscopic analysis

Experimental times	30 days						60 days					
	1	2	3	4	5	T	1	2	3	4	5	T
Specimens histological	1	2	3	4	5	T	1	2	3	4	5	T
Granulation tissue	+	+	+	+	+	5	+	+	+	+	+	5
Blood vessels	+	+	+	+	+	5	+	+	+	+	+	5
Fibroblasts	+	+	+	+	+	5	+	+	+	+	+	5
Osteoblasts/MBM	-	-	-	-	-	0	-	-	-	-	-	0
Granuloma FB	+	+	+	+	+	5	+	+	+	+	+	5
Macrophages	+	+	+	+	+	5	+	+	+	+	+	5
IMGC	+	+	+	+	+	5	+	+	+	+	+	5

T, total; MBM, mineral bone matrix; FB, foreign body; IMGC, Inflammatory multinucleated giant cells; +, presence; -, absence.

particles of the biomaterial (BO) both in the central region of the wound as well as at the edge of the bone defect. The identifiable particles of the material are basophilic and polyhedral with varying morphologies and sizes, occasionally forming right angles.

A large concentration of macrophages and inflammatory multinucleated giant cells (IMGC) as well as delicate collagen fibers and fibroblasts were observed circumscribing the surface of each particle, characterized as foreign body granulomas. There was no bone neoformation related to the material; additionally, bone neoformation was only noticed in the stumps of the surgical wound, due to the normal process, which was absent in the central region (Figure 4B and Table 1).

At 60 days, the specimens were observed to have small and few spaces containing connective tissue (CT), with mature bone tissue found throughout the defect (TO) in the control group; however, complete defect closure was not observed in any specimen (Figure 5A), and the bone tissue that was observed was thin. The GO group, as in the previous period, continued to be filled with numerous particles of the biomaterial (BO), following the same microscopic pattern already described; however, the foreign body granuloma reaction observed on the surface of the particles was intensified, with granulomas of the type with foreign bodies being much more established with a more organized fibrous capsule surrounding each particle. Among the particles, the mature connective tissue (CT),

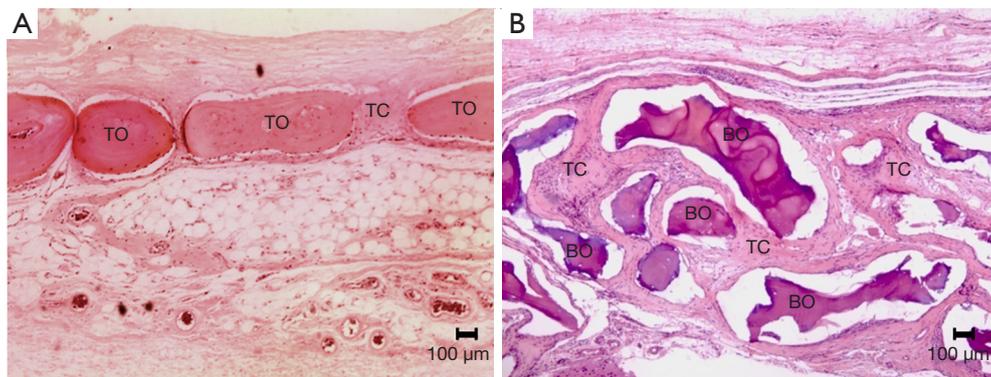


Figure 5 Center of the critical defect at 60 days in the CG group showing mature bone tissue (TO) in all extensions of the defect, except in the center (A); center of the critical defect at 60 days for the GO group showing numerous particles of the biomaterial, with an intensification of inflammatory foreign body reactions, with boundaries of the capsules surrounding the particles of the biomaterial (BO) (B). H&E stain. Magnification of 12.5 \times .

containing macrophages, lymphocytes, a lesser amount of vascular neoformation, fibroblasts, and neoformed collagen fibers, was observed. As in the previous period, there was no bone neoformation related to the material; additionally, bone neoformation was only noticed in the surgical wound stumps, due to the normal process, which was absent in the central region (*Figure 5B* and *Table 1*).

Histometric analysis

From the data obtained from the histological analysis of the amount of newly formed bone, it can be observed that the time-variant was not statistically significant ($P=0.305$), and neither was the presence of the biomaterial ($P=0.052$). In a comparative analysis between the two variants, duration, and biomaterial, there was no statistically significant difference ($P=0.736$).

Regarding the amount of biomaterial present at 30 and 60 days, no statistically significant difference was observed, demonstrating that time did not interfere with the amount of biomaterial in the bone defect during the evolution of the repair.

Histological analysis of the subcutaneous defect

No remnants of the empty collagen capsules implanted on the backs of the animals were observed at 15 and 45 days.

At 15 days, the particulate biomaterial (BOP) implanted in the muscle plane with cellularized connective tissue (CT) in its vicinity was observed in the GOP group. Connective tissue was observed to be richly vascularized (V) and

close to the particles; there was a predominance of loose connective tissue (CF), which is characterized by a greater number of fibroblasts. At a distance from the particles, the connective tissue was fibrous, characterized by the presence of bundles of collagen fibers oriented parallel to the particles (*Figure 6A*).

For the GOB group, a biomaterial material (BO) was implanted in the muscle plane with loose connective tissue (CF) in the trabecular spaces, and the remaining clot was observed, with an absence of inflammatory infiltrate and loss of connective tissue with vascular proliferation (V) invading the trabecular spaces (ET) in the vascular tissues. In some areas, the development of fibrous connective tissue was observed (*Figure 6B*).

At 45 days, for the GOP group, the images were similar when compared to the presence of the biomaterial (BO) in the muscular plane; however, the connective tissue (CT) surrounding the implanted material presented with more fibrous connective tissue in the vicinity, a greater amount of multinucleated cells, and areas that suggest resorption of the material (*Figure 7A*). In the GOB group, the implanted material was found in the muscular plane, presenting its trabecular spaces (ET) filled with loose connective tissue (CF), with a significant decrease in residual bone (thinner trabeculae). Fibrous connective tissue can be observed in the space between the muscular plane and block of the implanted material. Additionally, the presence of multinucleated giant cells in the vicinity of the implanted material is notable; furthermore, resorption is suggested at the periphery of the material (*Figure 7B*).

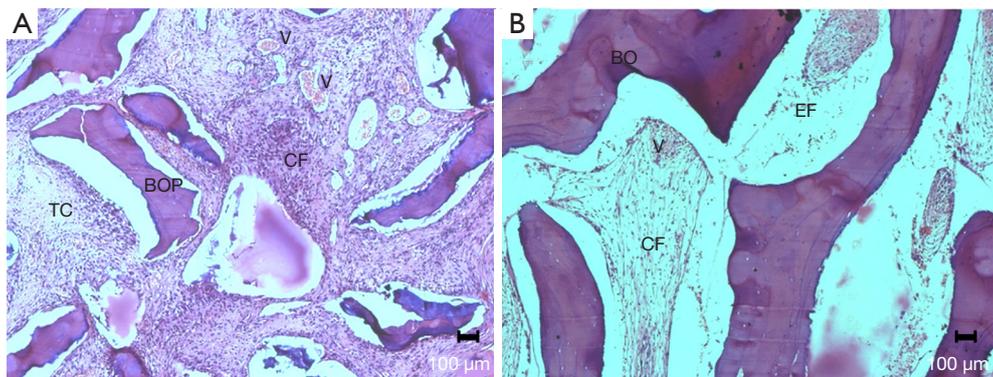


Figure 6 Subcutaneous tissue at 15 days showing particulate biomaterial (BOP) surrounded by connective tissue (CT) (A), and block biomaterial (BO) showing the trabecular spaces (ET) filled with loose connective tissue (CF) (B). H&E stain. Magnification of 12.5 \times .

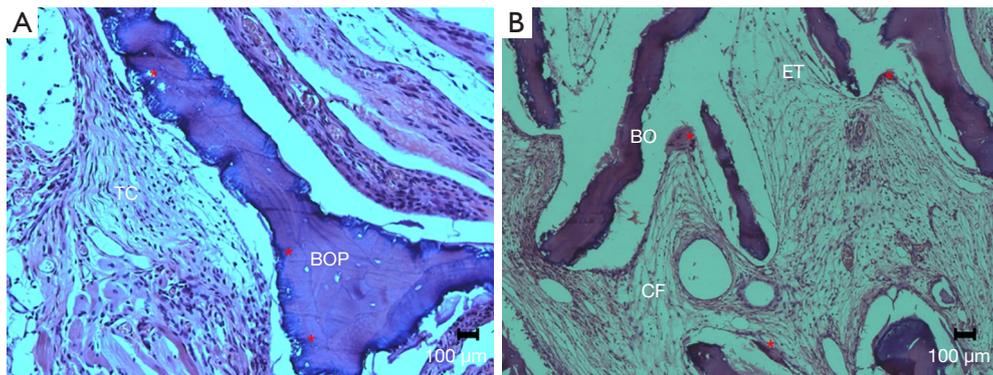


Figure 7 Subcutaneous tissue at 45 days showing fibrous connective tissue (CT) involving particulate biomaterial (BOP) and areas on its surface that suggest partial resorption (red asterisk); block biomaterial (BO) presenting its trabecular spaces (ET) filled with loose connective tissue (CF), and the presence of multinucleated giant cells in the vicinity of the implanted material (red asterisk) (B). H&E stain. Magnification of 12.5 \times .

Discussion

The methodology used in this study aimed to characterize the integral bone (Orthogen, Baumer S/A) regarding its biological activity, biocompatibility and osteopromotor potential. For this purpose, a critical defect was made in the animal calvaria to evaluate the osteopromotor potential; additionally, the subcutaneous or muscular plane, which is characterized by being a non-skeletal site, was used to evaluate the osteoinduction activity of the biomaterial (19,27).

Critical defect

The experimental model of the critical bone defect created in the skull cap of rats allows the evaluation of the

specific biocompatibility of the implanted material, which constitutes a prerequisite for the safety and efficacy of the clinical indication of a product (18).

In the first part of this study, no bone neoformation activity was observed when evaluating a bone cavity, such as the calvary of rats that shows the presence of pre-osteogenic cells and osteoblasts. It is suggested that there will be bone neoformation with a greater or lower speed of neoformation depending on the use of biomaterials (28,29) and on the functioning of the endocrine disorders (30,31).

The results obtained in this study corroborate with others studies in the literature in which biomaterials of xenogenic origin were used in critical defects, and despite showing interaction with the repair process, they did not

induce bone neoformation (32). However, he disagrees with the results found by Santos Kotake *et al.* (33) who demonstrated that xenogenous biomaterials can play a positive role to new bone formation.

The repair process did not show histological changes from the first to the second observation period, with no newly formed bone tissue and growth of fibrous connective tissue involving the remaining particles.

As regards histometric analysis, the results of this work were different from those obtained by Mendes *et al.* (34), who suggested the osteoconductive activity of the material, as it did not allow the development of bone tissue along the surface of the biomaterial; therefore, the clinical application must be such that the technique used and the indications for use be optimal for the material, fact also concluded in a comparative study between xenogenous biomaterials and synthetic nano-hydroxyapatite/beta-tricalcium phosphate composite (28,29,35).

The optimization in the use of a bone substitute aims for the following: the material must be manipulated to fill cavities, it must be stable, since the micro-movement can induce the formation of fibrous connective tissue in its surroundings or cause foreign body reaction, which may have been a limitation of the study, and it must be protected by the membrane to prevent the proliferation of unwanted cells (3,36,37).

Subcutaneous plane

The possibility that a lyophilized, demineralized, homogeneous, or heterogeneous material has osteoinductive potential has been suggested by some authors (38-40) and refuted by others (41,42). These opposing results suggest that depending on the origin, the industrial process, and the age of the donor, active growth factors may be present that would cause ectopic bone formation. The results obtained in this study did not show ectopic bone formation on postoperative days 15 and 45.

Regarding the cellular events observed in this study, moderate inflammatory infiltrate was observed at day 15 in the vicinity of the implanted composite bone particles, associated with the surgical procedure and the presence of foreign material in the subcutaneous plane, confirming results obtained in other studies that evaluated the biocompatibility of biomaterials and also concluded that, although bone substitutes have biocompatibility properties, ectopic bone formation was not found (41,42).

In the groups of particulate or block of integral bone, a

difference was observed between the tissues that involved the implanted materials at day 15. In the particulate Orthogen group, there was an acute inflammatory reaction of mild intensity and, predominantly, there was loose connective tissue surrounding the particles, while in the Orthogen block group, no acute inflammatory infiltrates were observed. The justification for this difference may be linked to the fact that the particles implanted in the muscular plane would have lesser stability on muscular movements than the material implanted in the form of a block. This suggestion is based on the statement by Dahlin *et al.* (43) that micro-movements of the implanted material can prevent tissue organization.

At 45 days, in the composite bone group, there was a decrease in the polymorphonuclear neutrophils and a greater amount of collagenous matrix deposited around the material particles, indicating encapsulation, even though there were still a large number of multinucleated cells around the inorganic materials. There were no particles of organic material, suggesting its reabsorption.

Although the methodology of this study did not enable measurement of the volume of the implanted materials, it was possible to observe that in the 45-day group, there was partial reabsorption both in the particulate material and in the block. This fact requires proof in the bone cavity sites because if these occurrences persist, it is inferred that the grafts may undergo volume changes, especially when used in sinus grafts, alveolar cavities (particulate matter), or for horizontal or vertical enlargement with fixation (block material).

These results are similar to those presented by Sanada *et al.* (41) and Greggi *et al.* (44), who did not find ectopic bone neoformation activity with inorganic or organic materials, respectively. Nevertheless, a gradual decrease in the inflammatory cells and formation of fibrous connective tissue around the implanted materials indicates that the material has good biological acceptance.

Conclusions

Based on the methodology and results obtained in this study, it can be concluded that the integral bone of bovine origin is a biocompatible material but do not demonstrate potential to induce the bone neoformation, being contraindicate for GBR procedures.

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Footnote

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