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Original Article

# Adverse effect of beta-tricalcium phosphate with zeta potential control in repairing critical defects in rats' calvaria☆

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ABSTRACT

*Objective:* To evaluate whether a new biphasic cement composed of calcium sulfate and beta tricalcium phosphate with zeta potential control could induce or lead to bone neoformation in critical defects.

*Methods:* A critical defect of diameter 8 mm was made in the calvaria of forty male Wistar rats. In the Test Group ($n = 20$), the defects were filled with cement. In the Control Group ($n = 20$), the defect was not filled and only coagulum was present. The animals were sacrificed 7, 14, 21 and 42 days after the operation. Calvaria specimens were subjected to microtomography and were then prepared for histological analysis. The analyses included morphological assessment on the histopathology of the repair; comparative morphometric evaluation of the area of formation of bone trabeculae between the groups; and histochemical staining by means of tartrate-resistant phosphatase (TRAP) in order to identify osteoclasts.

*Results:* Microtomographic images of the defects filled by the cement did not show any decrease in area over the course of postoperative evolution. In the Test Group, the material continued to present a foreign-body response until the last observational periods. Histomorphological analysis showed that there were more significant groupings of giant cells in the Test Group and greater maturity of neoformed bone in the Control Group. Exogenous material was also present. Histomorphometric analysis showed that in the Control Group, the total area of bone neoformation was significantly greater ($p = 0.009$) and grew progressively. The giant cells presented a positive reaction to TRAP but no osteoclasts were observed.

☆ Study carried out at the Department of Surgery, Prosthesis and Oral and Maxillofacial Traumatology, Faculdade de Odontologia, Universidade de São Paulo (USP), São Paulo, SP, Brazil.

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*Conclusion:* The ceramic cement did not induce or lead to bone neoformation from the microtomographic or histological point of view.



## Efeito adverso do beta-fosfato tricálcico com controle de potencial zeta no reparo de defeitos críticos em calvária de ratos

*Palavras-chave:*
Regeneração óssea
Ratos Wistar
Materiais biocompatíveis
Potencial zeta

RESUMO

*Objetivo:* Avaliar se um novo cimento bifásico composto por sulfato de cálcio e beta fosfato tricálcico com controle de potencial zeta poderia induzir ou conduzir a neoformação óssea em defeitos críticos.

*Métodos:* Foi feito um defeito crítico de 8 mm de diâmetro na calvária de 40 ratos Wistar machos. No grupo teste (n = 20) os defeitos foram preenchidos pelo cimento. No grupo controle (n = 20) os defeitos não foram preenchidos, permaneceu apenas o coágulo. Os animais sofreram eutanásia em 7, 14, 21 e 42 dias do pós-operatório. Espécimes da calvária foram microtomografados e posteriormente preparados para análise histológica. As análises incluíram a avaliação morfológica da histopatologia do reparo e a avaliação morfométrica da área de formação das trabéculas ósseas comparativamente entre os grupos e coloração histoquímica por meio da fosfatase tartrato-resistente (TRAP) para identificação de osteoclastos.

*Resultados:* As imagens microtomográficas dos defeitos preenchidos pelo cimento não apresentaram diminuição da área de acordo com a progressão dos períodos pós-operatórios. No grupo teste houve permanência do material e resposta corpo estranho até os últimos períodos de observação. A histomorfologia mostrou agrupamentos mais expressivos de células gigantes no grupo teste e osso neoformado mais maduro no grupo controle e comprovou a presença de material exógeno. Na histomorfometria, a área total de neoformação óssea foi significativamente maior (p = 0,009) e crescente no grupo controle. As células gigantes apresentaram expressão histoquímica positiva para TRAP e não foram observados osteoclatos.

*Conclusão:* O cimento cerâmico não induziu ou conduziu a neoformação óssea sob o ponto de vista microtomográfico e histológico.



## Introduction

Autogenous graft is still the material of choice for the reconstruction of bone tissue loss in orthopedic and maxillofacial surgery. However, increase in operation time, surgical trauma and possible complications inherent to the donor area approach does not always make it feasible. New biomaterials and substances that can mimic the characteristics of the autogenous bone tissue have been a constant pursuit of bioengineering.

Among the alloplastic materials most often used nowadays are bioceramics, mainly hydroxyapatite and beta-tricalcium phosphate (β-TCP). The latter shows a more rapid biodegradation than hydroxyapatite and in some situations this may be a more advantageous characteristic for a biomaterial, mainly when there is no need for mechanical strength. Moreover, beta-tricalcium phosphate has been widely used as a carrier or scaffold in tissue engineering.

Recently, a biphasic ceramic material consisting of calcium sulfate and beta-tricalcium phosphate with a negative surface charge, called zeta potential control, was launched in the international market with the proposal of making beta-tricalcium phosphate an inductive bone substitute and thus, promote bone regeneration.[1]

According to the manufacturer,[2] this ceramic is fully synthetic and has what they called "intelligent porosity", which facilitates cell growth and nutrient distribution in the extracellular matrix internally in the macroporosities of this compound. Some authors have demonstrated intense bone regeneration capacity in vertebral defects in sheep.[1]

The osteogenic potential of this compound, however, has been questioned. Some authors have shown concern about the biological safety of the product.[3] Other researchers[4] discontinued early clinical trials due to the appearance of unexpected adverse effects, such as aseptic inflammation and delayed repair. The literature is scarce on the analysis of the biological behavior of this new biomaterial in bone repair in *in vivo* studies.

In this context, we propose an *in vivo* study to assess, from the microtomographic and histological point of view, whether this bioceramic compound can induce or lead to bone neoformation in critical defect models in rat calvaria.

## Material and methods

### *Experimental procedure*

This research project was approved by the Institutional Ethics Committee on Animal Use (CEUA) under protocol number 006/2014 and in accordance with the ethical principles of animal experimentation adopted by the Brazilian Society for Laboratory Animal Science (SBCAL).

Forty (40) male Wistar rats (*Rattus norvegicus albinus*), weighing between 200 g and 250 g and aged approximately 45 days, were operated on under general anesthesia by intramuscular injection in the right rear paw of each animal of ketamine hydrochloride (Dopalen®, Vetbrands) at a dose of 0.8 mg/kg associated with muscle relaxant xylazine (Rompum®, Bayer) at a dose of 0.3 mg/kg. All animals received antibiotic prophylaxis through intramuscular injection of benzathine benzylpenicillin (Roche®) at a dose of 150,000 IU/kg.

After trichotomy, followed by skin antisepsis with 2% chlorhexidine digluconate, an access was made to the calvaria through a 2 cm-rectilinear incision in the skin of the medial region of the skull, extending from the nasofrontal area to the occipital protuberance. The skin, subcutaneous tissue, temporal muscle and the periosteum were divulsed laterally.

A bone defect was created in the central region of the animal calvaria using an 8 mm-diameter steel trephine drill (Sistemas de Implantes Nacionais – SIN®), adapted to a counter-angle implant motor (Driller® – Carapicuíba – São Paulo) under low speed and constant irrigation with 0.9% saline solution.

The animals were randomly divided into two groups: Test Group ($n = 20$), which received cement consisting of beta tricalcium phosphate (β-TCP) and calcium sulphate at a ratio of 1:1 (Genex – Biocomposites® – Staffordshire – England) to fill the critical defect in the calvaria at the amount of 25 $mm^3$ per defect, and the Control Group ($n = 20$), which remained with the critical defect filled only by a clot.

Synthesis of the skin was performed with 3/0 silk thread (Ethicon®). Throughout the study period, the animals were kept in ventilated polypropylene cages, covered with sterilized wood shavings, with day/night cycles of 12/12 h, and were fed rodent chow (Labina for Rodents, Purina®) and water *ad libitum*.

Five animals from each group were euthanized in a $CO_2$ chamber after 7, 14, 21 and 42 days postoperatively. After euthanasia, the skulls were dissected and representative fragments of the calvaria with the defect area were fixed in 10% buffered formalin.

Prior to the processing and histological analysis, a specimen from a rat in the Test Group and from a rat in the Control Group, in each of the study periods, were submitted to microtomography in a 100 kV to 100 μA SkyScan microtomographer in the Department of Animal Physiology of the Biology Institute of Universidade de São Paulo.

The samples were placed at and fixed to the equipment cell, which was set for a resolution of 2000 × 2000 pixels and 16 μm sections. The time of image acquisition was approximately 2 h per sample. Images were analyzed and reconstructed in two and three dimensions, using the InVesalius sofware (Division of Three-Dimensional Technologies of Centro de Tecnologia da Informação de Campinas – São Paulo).

The biomaterial volume was quantified in cubic millimeters ($mm^3$). In the two-dimensional images, the sagittal and axial distances of the bone defect and three-dimensional axial distance were measured in millimeters (mm).

For the histological analysis, the specimens ($n = 5$) from each group and preoperative observation period were immersed in 10% EDTA solution (pH 7.4) for six weeks for bone tissue decalcification. The defect area was sectioned in half, exactly at the median sagittal plane of the rat calvaria, which resulted in two parts of the wound: the left side and the right side (S1 and S2). Each side of the defect was embedded in paraffin and subsequently, four 4.5 cm-thick parasagittal sections were obtained and stained with Masson trichrome. Histological sections were assessed using a conventional light microscope (Olimpus® CH2, Olympus Optical Co. Ltd., Japan) in a blinded fashion by an experienced pathologist. The histomorphological aspects considered the presence of the following repair characteristics: edema, inflammatory infiltrate, granulation tissue, bleeding/blood clot, bone neoformation, foreign body reaction and the presence of exogenous material. The parameters were weighed using an arbitrary scale by the following scores: 0 = absent; 1 = mild; 2 = mild to moderate; 3 = moderate to intense and 4 = intense.

The histomorphometric analysis was used to quantify the neoformed bone tissue area in randomly selected sections on both halves (left and right) in three regions of the wound, the anterior region (close to the nasal bone), the central region and the posterior region (close to the occipital bone). Histological images of each region were obtained with a 40× magnification using a CCD camera (Sony®) coupled to a microscope (Jeol®) and an image capture board (Captivator®).

Then, these images were transferred to a digital morphometry software program (ImageJ, NIH – National Institutes of Health). The formation of neoformed trabecular bone was identified and delimited using a hand free software tool. Measurements were performed in four semi-serial histological sections of each slide.

The total area of bone neoformation was obtained at each field and added to the value obtained on the opposite side of the wound. At the end, mean was calculated for the total area of neoformed bone tissue for each group and period.

In order to identify osteoclastic cells, two randomized cuts on each side of the wound from two animals from each group were randomly chosen and submitted to histochemical staining to demonstrate tartar phosphatase-resistant acid (TRAP). Histological sections were incubated for 3 h at room temperature, in solution containing sodium tartrate, tartaric acid and disodium salt (Sigma™).

After incubation, the sections were rinsed in tap water, allowed to air dry at 25 °C and counterstained with 1% fast green (Fisher Scientific Co.) for approximately 1 min. A mouse femur section (stored paraffin-embedded material) with bone wound repair was used as a positive control for this immunohistochemical reaction. After staining, the slides were kept in dark field to be subsequently assessed under light microscopy.

**Table 1 – Volume of remaining material and microtomographic two-dimensional, three-dimensional measurements of bone defects according to the periods of postoperative observation in the Test and Control Groups.**

| Period | Group | *n* | Volume of biomaterial ($mm^3$) | Two-dimensional distances | | Tridimensional axial distance (mm) |
|---|---|---|---|---|---|---|
| | | | | Axial | Sagittal | |
| 7 days | Control | 1 | | 8.97 | 8.95 | 8.96 |
| | Test | 1 | 25.11 | 8.32 | 8.16 | 9.39 |
| 14 days | Control | 1 | | 9.46 | 9.41 | 9.50 |
| | Test | 1 | 10.96 | 7.72 | 10.81 | 7.34 |
| 21 days | Control | 1 | | 8.41 | 8.58 | 8.15 |
| | Test | 1 | 7.00 | 7.77 | 9.55 | 9.51 |
| 42 days | Control | 1 | | 8.75 | 8.56 | 9.06 |
| | Test | 1 | 11.64 | 9.88 | 11.51 | 11.10 |

Histological data were analyzed by nonparametric inferential statistics using the Mann–Whitney test with the Biostat 5.0 software with a significance level of 5%.

## Results

The results of microtomographic image analysis are shown in Table 1 and Fig. 1. The intensity in gray levels of the biomaterial was close to that of bone, but the biomaterial showed granularity, allowing it to be differentiated from bone.

Histological examination regarding repair morphology is shown in Table 2 and main histological aspects are shown in Fig. 2. Fig. 3 shows the result of staining for TRAP (tartrate -resistant acid phosphatase) in the Test Group 42 days postoperatively.

Results of the histomorphometric analysis of bone neoformation area are shown in Fig. 4.

## Discussion

The literature describes that calcium phosphates and their derivatives, including beta-tricalcium phosphate, act more directly on osteoblasts and have been extensively studied as bone replacement for more than two decades, as they promote bone neoformation by osteoconduction and have good biocompatibility.[5–7]

Its effects as inorganic scaffold for bone tissue regeneration are already well documented, including dental lesions,[8–11] in spite of still controversial findings, ranging from bone neoformation that is superior to the one observed in autogenous grafts[12] and anorganic bovine bone graft,[13] to robust evidence of insufficient osteogenesis.[14,15]

The biomaterial used in this study (GeneX®) is relatively new and it is a ceramic compound, which has negative surface charge control. Investigations about its efficacy have

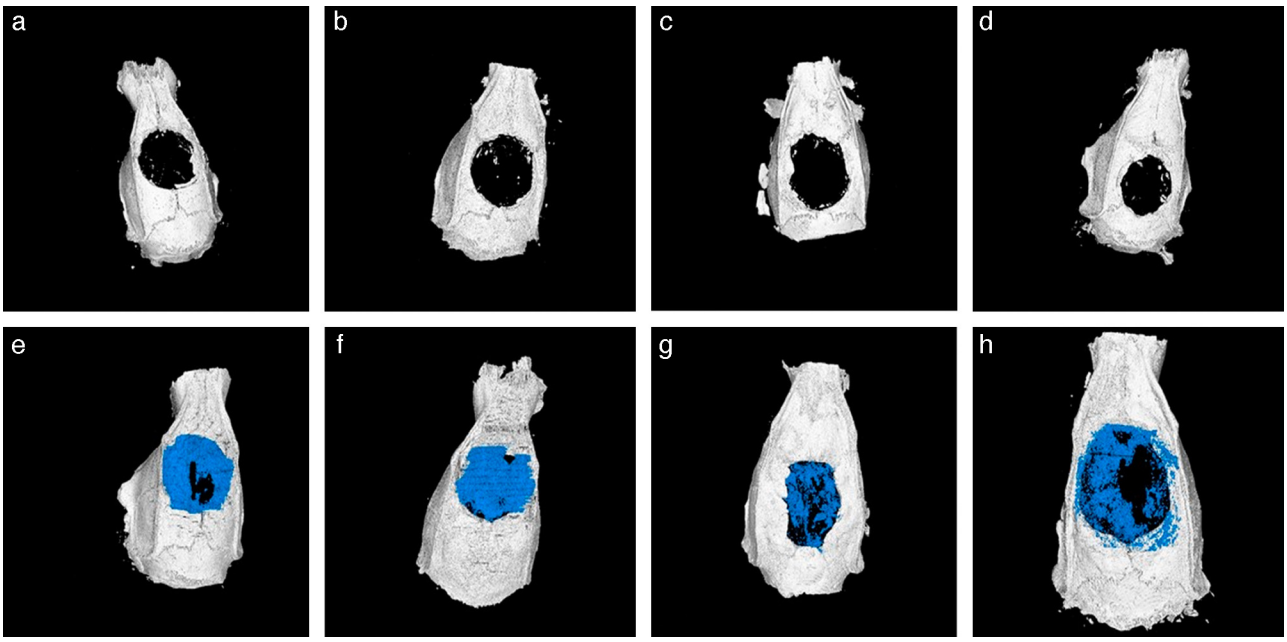


**Fig. 1 – Coronal view of the calvaria defects reconstructed by MicroCT in the control and Test Groups according to the observation periods. At (a) 7 days, (b) 14 days, (c) 21 days and (d) 42 days after surgery the Control Group show the defect image without bone neoformation. At (e) 7 days, (f) 14 days, (g) 14 days and (h) 42 days after surgery, the Test Group shows in blue the remaining volume of βTCP cement.**

**Table 2 – Values of the medians [max–min] of the score intensities for the histological variables of the bone defect.**

| Variables | 7 days | | | 14 days | | | 21 days | | | 42 days | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | Test | *p* | Control | Test | *p* | Control | Test | *p* | Control | Test | *p* |
| Edema | 2 [2-1] | 1 [2-1] | 0.22 | 2 [2-1] | 1 [1-0] | 0.075 | 1 [1-0] | 1 [1-0] | 1 | 0 [2-0] | 0 [1-0] | 0.916 |
| Inflammatory infiltrate | 1 [2-1] | 4 [4-4] | 0.004 | 1 [3-1] | 4 [4-3] | 0.008 | 1 [1-0] | 4 [4-4] | 0.003 | 1 [1-0] | 4 [4-3] | 0.006 |
| Granulation tissue | 3 [4-3] | 3 [3-2] | 0.212 | 2 [3-1] | 4 [4-3] | 0.011 | 1 [1-1] | 2 [3-2] | 0.004 | 1 [2-1] | 1 [1-1] | 0.317 |
| Hemorrhage/thrombus | 3 [3-1] | 2 [3-1] | 0.496 | 2 [3-2] | 1 [4-0] | 0.193 | 3 [3-1] | 0 [4-0] | 0.106 | 2 [3-0] | 1 [3-0] | 0.335 |
| Bone neoformation | 1 [2-1] | 1 [1-1] | 0.317 | 2 [2-1] | 2 [3-1] | 0.605 | 2 [3-1] | 2 [2-1] | 0.339 | 2 [3-1] | 1 [2-1] | 0.418 |
| Foreign body reaction | 0 [1-0] | 4 [4-4] | 0.005 | 1 [2-1] | 4 [4-4] | 0.005 | 0 [2-0] | 4 [4-3] | 0.007 | 1 [2-0] | 4 [4-3] | 0.006 |
| Exogenous material | 0 [0-0] | 4 [4-3] | 0.007 | 0 [0-0] | 4 [4-4] | 0.005 | 0 [0-0] | 2 [3-2] | 0.005 | 0 [0-0] | 3 [4-2] | 0.008 |

0, absent; 1, mild; 2, mild to moderate; 3, moderate to intense; 4, intense.
*P* values for Kruskall–Wallis test. Significant when $p < 0.05$.
Mann–Whitney test is significant when $p < 0.05$.

most often focused on repair defects of the spine or long bones.[16] Yang et al.[1] demonstrated in an *in vivo* study that this ceramic material promoted superior bone formation in vertebral defects in eight weeks when compared with the use of a polymer cement (polymethylmethacrylate). However, in their conclusions, they indicate that the β-TCP ceramic needs to be further assessed.

As the manufacturer's proposal was to add a surface treatment on the ceramic particles that allowed osteoinduction, a critical bone defect model would provide important information if the bone neoformation occurred where it would not be expected.

This type of model is quite often described in the literature and it is, in a way, acknowledged for this type of *in vivo* assay.[17–24] We used the critical defect model in rat calvaria because it is easy to create, reproduce and the fact that an 8 mm-extension defect would allow us to verify the occurrence of osteoconduction or induction of the material in a standardized and reliable manner.

Contrary to the favorable outcome described by German researchers,[25] our results did not show bone neoformation after insertion of this ceramic compound in filling defects. Cement with zeta potential control did not stimulate bone neoformation in critical defects created in rat calvaria or showed rapid absorption by the volume of exogenous material observed in microtomographic images in all study periods.

Recently, Saadoun et al.[3] and Friesenbichler et al.[4] showed significant complications of this material on tissues when

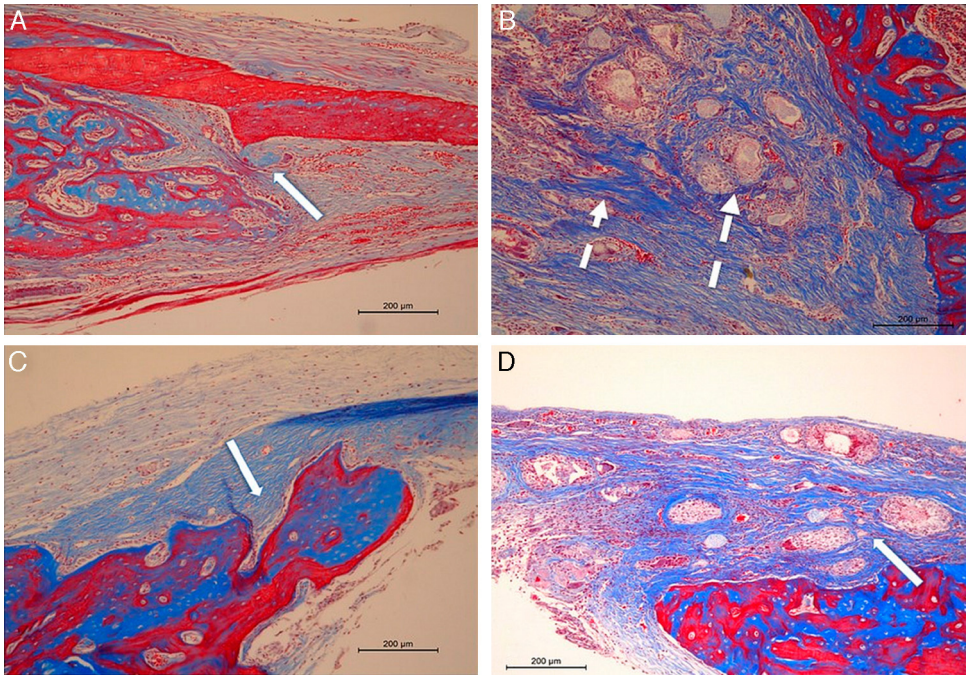


**Fig. 2 – Histological sections representative of critical defects in each group in the periods of 21 and 42 days postoperatively. In (A) Control Group – 21 days, shows deposition of collagen and osteoid matrix adjacent to the defect border (arrow). In (B) Test Group – 21 days, shows exogenous material cluster (long arrow) encapsulated by intense deposition of fibrous connective tissue (short arrow). In (C) Control Group – 42 days, shows bone deposition of mature and immature bone on the defect borders (arrow). In (D) intense deposition of fibrous connective tissue and clusters of exogenous material and nests of adjacent giant cells (arrow) (Masson staining, magnification 2.5×).**

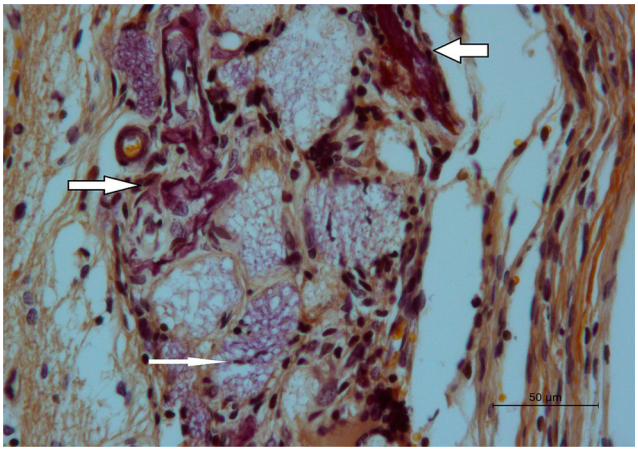


**Fig. 3 – Positive expression of tartrate-resistant acid phosphatase (TRAP) of multinucleated giant cells (large arrows) adjacent to the exogenous material clusters (thin arrows) – Test Group period of 42 days (TRAP staining, magnification 40×).**

used to fill bone defects in clinical trials, although Laycock and Cooper[26] attributed these adverse effects to the still inadequate use of this biomaterial.

Obviously, the presence of a significantly higher amount of inflammatory infiltrate in the Test Group is directly related to the greater response to the presence of a foreign body when compared to the Control Group. The fact that the foreign body response remained for the entire duration of the experiment can lead to the formation of material clusters, which should be expelled from the wound area in the encapsulated form. This fact may help to explain the description of "soft tissue cysts" made by Friesenbichler et al.[4]

The clinical relevance of this study should be emphasized from the histopathological point of view, because there were

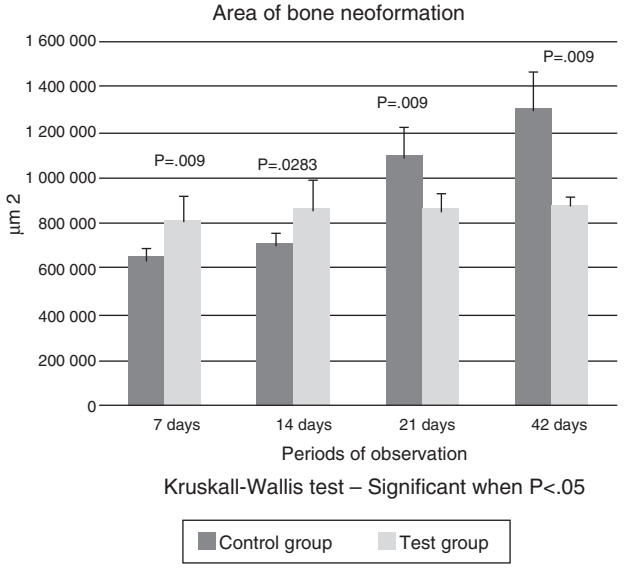


**Fig. 4 – Histomorphometric analysis for the area ($\mu m^2$) of bone neoformation related to the groups and periods. Means (± standard deviation).**

no standardized experimental preclinical studies to disclose the behavior of this material on the repair of healthy bone tissue yet.

According to the histomorphometric findings, the proposal of a more significant bone neoformation in the Test Group was not demonstrated either. The results show that bone formation was more intense and significantly higher in the Control Group and denied our hypothesis of trying to demonstrate the osteoinductive potential promised by the manufacturer.

Results showed that the superiority in bone formation in controls led us to believe that this ceramic composite prevented the small bone formation that would occur in the defect.

Our results are also in accordance with those from other authors,[27,28] according to whom the application of other types of beta-tricalcium phosphate for bone loss repair, in minor defects and crossover studies in the same animal did not influence the amount of bone formation.

To assess whether there was increased osteoclastogenesis and increased bone resorption, a histochemical reaction was carried out by TRAP staining in the wound area. However, the foreign body response giant cells were the ones that showed positivity for this biochemical marker. This may lead to the hypothesis that the material in the tissue might possibly have induced specific biochemical signals.

Thus, giant cells, probably in an attempt to absorb exogenous material, might also have resorbed bone. Studies demonstrating the expression of other specific markers related to repair and induction of bone tissue formation could be carried out to better explain this fact.

By the time this study was planned, we expected to find beneficial effects of Genex® in bone regeneration, especially considering that this material had favorable reports in clinical use. Finally, our results are relevant, as they confirm that this type of ceramic must be reconsidered as a bone substitute.

## Conclusion

Within the limitations of this study, the biphasic ceramic cement consisting of calcium sulfate and beta-tricalcium phosphate and zeta potential did not induce or resulted in bone neoformation of 8 mm critical bone defects created in rat calvaria from the microtomographic and histological points of view.

## Conflicts of interest

The authors declare no conflicts of interest.

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