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Histological and Immunohistochemical Evaluation of Biphasic Calcium Phosphate and a Mineral Trioxide Aggregate for Bone Healing of Rat Calvaria

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Abstract: Eighty Wistar rats were divided into 4 groups and examined over a period of 2 or 8 weeks. First, a surgical defect was created in the calvaria using a 6 mm diameter trephine drill. The cavity was then treated with BCP (biphasic calcium phosphate), MTA, or the combination of the two. In the control, the cavity was allowed to clot. After 2 or 8 weeks, animals were sacrificed and the bones were subjected to a histological evaluation and immunohistochemical staining to evaluate areas of new osteoid tissue and new bone tissue as well as the percentage of labeled cells that utilize anti-BMPR1B antibodies. Twoway ANOVA showed statistical significant diference in all dependent variable (area of new osteoid tissue; area of new bone and % of immunostainning) within group p=0.00000, time p=0.00000 and with the interaction of both p=0.0000. MTA group at 8 weeks showed the highest amount of osteoid tissue (6.25 ± 0.30 mm^2). The same group also exhibited the highest amount of bone tissue formation (3.98 ± 0.14 mm^2). In contrast, both 2-week MTA samples (40.73 ± 2.50%) and 2-week BCP+MTA samples (40.30 ± 1.81%) exhibited the highest percentage of stained cells. Best results in terms of the area of osteoid and bone tissue formation and the percentage of BMPR1B markings were observed for the MTA group, confirming that the combination of BCP and MTA does not result in a significant improvement.

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Introduction

Mineral Trioxide Aggregate (MTA) is a biocompatible material18 and can help in bone repair because its composition provides rapid accession and proliferation cell on its structure[6,10]. There is evidence that MTA promotes a favorable response in osseous environment, with direct bone apposition[30]. It was demonstrated that MTA induces bone morphogenetic protein 2 expression and calcification in human periodontal ligament cells[20] and stimulating human gingival fibroblasts to produce BMP-2[14], is able to promote a favorable formation of mineralized tissue in rat alveolar sockets[15], being characterized by mild inflammatory response and complete bone healing[16]. MTA surfaces also support osteoblast cell attachment, matrix synthesis and RunX2 expression which are essential for osteogenesis[24].

The use of biphasic calcium phosphate (BCP) in orthopaedic and in oral surgeries as an osteoconductive material has proven to be effective in promoting bone healing. In addition, BCP is considered to be a safe biomaterial and exhibits high bio-compatibility.[7,8,26] The structure of biphasic calcium phosphate consists of particles with an average size of 100-500 μm composed of 60% hydroxyapatite and 40% of tricalcium beta-phosphate (b-TCP).[7]

Compounds based on HA exhibit a low or negligible bioabsorbtivity whereas those based on β-TCP are more soluble and can be easily degraded or reabsorbed. At the same time, β-TCP is unpredictable and thus are not an appropriate scaffold for bone growth. The association between HA and β-TCP generates a biomaterial called BCP, which is re-absorbable but also contains a more stable segment (HA) that maintains the stability of the scaffold, the graft and the more soluble component (β-TCP).[7,27]

Immunohistochemical analyses and *in situ* hybridization experiments have been used to reveal that mesenchymal cells, including osteoblasts, express morphogenetic bone proteins (BMP) and receptors (BMPR) during the formation of skeletal and fracture repair bone tissue.[2,17] These glycoproteins are responsible for bringing osteoprogenitor cells to bone formation and repair sites and thus have important implications in cascades of cellular events that regulate bone

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formation and repair. During these processes, mesenchymal cells induce cell proliferation and differentiation, and promote the synthesis of the extracellular matrix.[1,3,17] As a result, BMPs are involved in the differentiation process wherein osteoprogenitor cells transform into mature osteoblasts.[32,33]

The cellular response to BMP depends not only on the expression of type of protein but also of the expression and location of the surface transmembrane receptors BMPR type 1A, 1B, and type 2.[28] In the calvaria, where it is possible to observe intramembranous ossification, the signal for a reparable or formative process is regulated by the BMPR-1B receptor[32] which is involved in the initiation of bone condensation.[2]

Because both calcium phosphate and the aggregate mineral trioxide exhibit features that can help the bone formation process and that there are few data of the use of MTA (used isolated or in association with BCP) on bone formation, our work focused on the process of bone repair of defects in standardized calvaria of Wistar albino rats treated with BCP, MTA, or as a combination of the two. The main analytical techniques used herein were histological and immunohistochemistry analyses

1. Materials and Methods

This study was approved by the Research Ethics Committee for Animal Use (CEUA/PUCPR) under protocol #390/08. 80 animals (Wistar albine rats) with an average weight of 250 g were used in this study. The rats were randomly divided into four groups (n = 20 per group; 10 per group and period); two experiments were performed using two and eight week treatment periods.

The procedure began by performing anesthesia on each animal by injecting xylazine 2% hydrochloride (8 mg/Kg of body weight; Anasedan®, Sespo Indústria e Comércio Ltda., Paulinia, São Paulo, Brazil) intraperitoneally in the presence of ketamine 5% hydrochloride (60 mg/Kg of body weight; Vetanarcol®, Laboratory König SA, Avellaneda, Argentina).Having confirmed the efficacy of the anesthetic, a total flap in the form of a "U" was made in the skull of each animal

using a #15 blade by exposing the surface of the calvaria bone. After opening up the flap, a circular osteotomy of 6 mm in diameter was made using a trephine drill attached to an angle using a dental micromotor in the presence of abundant cooling with a saline solution. Prior to the complete removal of the bone fragment, two small osseous incisions in the shape of an 'L' were performed in the antero-posterior skull direction so that the larger base of L coincided with the sagittal suture, which was taken to identify the location of the diameter of the circular defect. The incisions were made using a diamond cylindrical high-rotation drill. The amalgam was then applied to mark the center of the surgical wound.[23]

After carefully removing the incised portion of the calvaria with the aid of a Molt dissector, the BCP was added to the wound of 20 animals (Straumann® BoneCeramic, Institut Straumann AG, Basel, Switzerland) as per the instructions of the manufacturer, filling the entire calvaria cavity (up to the edge of the defect). In 20 other samples, the MTA (MTA - Angelus®, Angelus Industry of dental products S/A, Londrina, Paraná, Brazil) was applied according to the instructions of the manufacturer. The last group of 20 animals was treated with a 1:1 combination of BCP and the MTA in the similar manner as the other groups. The last group allowed for the formation of blood clots in the surgical wound, thus serving as a control in the experiment. In all the animals, the flap was sutured with isolated knots of mononylon 4.0, followed by the surgical procedure. For pain control, sodium dipyrone 500 mg (D-500, Pfizer/Fort Dodge Animal Health Ltda., Campinas, São Paulo, Brazil) was given during three days after the surgery until the day of euthanasia. Subsequently, the animals were sacrificed with a lethal dose of an anesthetic, and during the evaluation periods of 2 weeks (10 animals of each treated group and 10 animals in the control group) and 8 weeks (using the same number of animals).

Histological Development

After performing euthanasia on the animals, the entire calvaria was carefully cut open and soft tissue was gently removed from the bone. All samples were stored in 10% formalin for mounting of

surgical pieces, and hen demineralized in 4.13% EDTA for a period of 75 days.

The material received a routine histological processing for paraffin inclusion, followed by the microtome sagittal incisions using an "L" type marking in rgw amalgam to determine the location of the larger diameter of wound with a 3 µm thick material on the two slides of each piece for a total of 160 slides.

The slides were stained with Masson's trichrome and analyzed using an optical microscope with 40x magnification.

Immunohistochemistry Processing

For immunostaining, serial sections with a thickness of 3 µm thick were deparafinized in xylol at 60 ºC, and hydrated using various concentrations of alcohols (absolute, 95 %, and 80 %). After hydration, the specimens were submitted to antigenic recovery using a 1% solution of trypsin (pH = 7.2) for 45 min at 37 °C in an oven.

The slides containing the histological cuts were washed and then immersed in 3% hydrogen peroxide for 30 minutes to inhibit the activity of the endogenous peroxidase.

After rinsing with distilled water, the specimens were immersed in a buffered saline solution (PBS, pH 7.4) to maintain constant chemical properties of the reaction. Next, sections were incubated overnight with the primary antibody anti-BMPR-1B (200 µg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) using a 1:100 factor dilution in PBS. To detect the primary antibodies, a universal streptavidin-biotin peroxidase kit (Diagnostic BioSystem, Fremont, California, USA) was used according to the instructions of the manufacturer. The immune reaction was monitored using a solution of tetrachloride diaminobenzidine (Diagnostic BioSystem, Fremont, California, USA). Within 2 minutes of administering the drug, a brown-brownish precipitate confirmed the presence of the antigen. The specimens were counter-stained with Harris hematoxylin for 3 minutes.

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For the negative control, we used the poly clonal IgG isotope (2 mg/ml; Abcam) for 10 minutes after incubation at room temperature as the primary antibody of each sample. Three slides were used for the incubation of each antibody.

Analysis of the Images

All specimen images were captured using a digital camera (Samsung, South Korea) coupled to a light microscope (Zeiss, Germany) using the original 200× magnification of each digital image with a resolution of 600 dpi. A virtual image of 117× 80 cm was produced, which made it impossible to catch all aspects of the defect in a single at the magnification used. Accordingly, a digital framework composed of the entire defect was then constructed by combining two smaller images. The resulting images were evaluated in terms of bone tissue deposition and blood vessels by consulting histological references.

All histomorfometrical measurements were performed using Image Tool software 2.00 (Department of Dental Diagnostic Science at The University of Texas Health Science Center, San Antonio, Texas, USA).

An image of 1-mm slide was used to calibrate all measurements. The histomorphometric data were counted manually and expressed as areas (mm^2). The perimeters of the deposited histological bone matrix and osteoid tissue areas were carefully traced, all areas were computed into the total histological area. At the same time, the percentage of positive BMPR-1B protein was accounted by automation, following the protocol established by Di Cataldo et al 2010[9]. This automated counting allowed counting only the percentage of protein present in the whole defect; it is not possible to distinguish their specific immunostaining in cells or bone matrix.

Statistical Analysis

The data obtained were analyzed using the statistical software SPSS 18.0 for Windows (SPSS, Inc., Chicago, IL). Normality tests (Kolmogorov-Smirnov and Shapiro-Wilk) were performed to examine

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the data distributions, whilst Levene's test was to examine the homogeneity of variance, also using twoway ANOVA (Group X Time) followed by Games-Howell test for multiple comparisons with a significance level of 5% ($p < 0.05$).

2. RESULTS

Histomorphometrical Analysis

The group that presented the largest amount of osteoid tissue was the 8-week MTA sample (Figure1). This group also exhibited the best results with respect to the amount of bone tissue (Figure2.) formed due to immunostaining. The groups that exhibited the highest percentage of stained cells were the 2-week MTA and 2-week BCP+MTA samples, with no statistically significant difference observed between the two. We also observed a decrease in the amount of immunostained cells from 2 to 8 weeks for groups that were treated with the biomaterials. In the control group, an increase in the immunomarker concentration from 2 to 8 weeks was observed (Figure3.).

Histological Analysis

1. MTA

At two weeks after the operation, it is possible to identify the presence of bone formation (figure 4A) concentrated at the edges of the surgical defect in areas contiguous to the original remaining bone. This area of the bone matrix formation was surrounded by osteoid tissue as well as areas of a significant amount of connective tissue. Very little mature bone was observed in the central region of the defect. This region was composed predominantly of granulation tissue wherein the richly vascularized osteoid formation was concentrated in the peripheral areas of the histological sections.

At eight weeks (figure 4B) after the operation, greater deposition of bone matrix was observed in the areas adjacent to the remaining bone. The body of the defect was filled with a significant formation of osteoid tissue, surrounded by immature medullar tissue.

2. BCP

In the bone repair of the surgical defects filled with BCP, discrete bone formation was observed on the margins of the artificially created surgical defect 2 weeks after the operation (figure 4C). The body of the defect was composed predominantly of dense vascularized connective tissue, resulting in negative images of exogenous material compatible with the particles of the biomaterial. A small amount of giant cells and a specific amount of osteoid tissue were also observed around the biomaterial. Eight weeks after the operation (figure 4D), the histological reparation process similar to that of the second week process was observed. In this case, however, a greater deposition of bone matrix on the edges of the defect and the osteoid matrix near the biomaterial were observed.

3. BCP Combined With the MTA

Two weeks after the operation, a histological healing picture, represented by the moderate quantity of osteoid tissue composing the major part of the defect was observed (figure 4E). At 8 weeks after the operation (figure 4F), the majority of the defect was still composed of osteoid tissue, which was identified by the intense deposition of bone matrix at the margins of the defect in the form of trabeculae that mimic intramembranous ossification.

4. Control (CLOT)

The control group showed a predominance of granulation tissue, along with a diffuse and moderate chronic inflammatory process. Little deposition of bone matrix was identified two weeks after operation in this group (figure 4G); however, areas of osteoid tissue were observed to be concentrated adjacent to the remaining bone tissue or in areas restricted to the body of the defect. At eight weeks after the operation (figure 4H), mild bone formation was identified; however, the foci of bone mineralization were only observed in the thin connective tissue.

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Immunohistochemical Analysis

All groups tested positive for the BMPR-1B antibody. The highest BMPR-1B content was identified as the 2-week MTA group (figure 5A). For this period of time, the immunostaining with BMPR-1B+ occupied nearly the entire area of the defect. In contrast, eight weeks after the operation (figure 5B), the percentage of positive markers decreased as the proportion of osteoid or bone matrix increased. Despite the numerical difference, the overall pattern of immunostaining of the two periods was similar, resulting in a clear identification of the protein both in osteoprogenitor cells and the surrounding extracellular matrix.

A different result was observed for samples treated with BCP alone (figure 5C) and BCP associated with the MTA (figure 5E). In the group treated with BCP, the BMPR-1B+ areas were found in the extracellular fibrous matrix, with an intense deposition of dense and rough fibers mimicking areas of precortical collagen formation of compact bones, involving the grafted biomaterial (5D) .

In BCP/MTA group, the immunostaining of the BMPR-1B was more intense and a greater percentage of immunostaining was observed in comparison to the group in which only the BCP was grafted, both after two (figure 5E) and eight weeks (figure 5F). The presence to BMPR-1B was identified in osteoprogenitor cells and neoformed bone trabeculae in the extracellular matrix.

A smaller amount of cells and BMPR-1B+matrix was found in the control group after both two weeks (5G) and eight weeks (figure 5H). In the control group, the cells are arranged in the area adjacent to the remaining bone tissue or in isolated areas among the connective tissue.

Statistical Results

In order to compare if there was difference among mean values of the variables: area of new bone and area of osteoid tissue and % of immunostaining, according to groups (MTA, BCP, MTA+BCP or Control) and period of time (2 or 8 weeks), as n<30, it was initially tested the

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normality of data in each treatment (group X time) using Kolmogorov Smirnov test, that indicated normal distribution for all variables in all treatments, once $p>0.05$. After that, the homogeinity of variances was tested among treatments for each variable using the Levene test that indicated heterogeneity of variances among treatments for all of the three variables as $p<0.05$. Following that, twoway ANOVA (GROUP x TIME) was used indicating the presence of difference among groups and period of time ($p<0.05$), where the observed power was > 0.99. So, the significance of results was finally demonstrated by Games-Howell for multiple comparisons with a significate level of 5% ($p<0.05$). It was possible to observe that MTA group (8 weeks) showed the highest mean value of osteoid tissue mm^2 (6.25 ± 0.30) against control (2 weeks) that showed the lowest mean value (1.92 ± 0.30). When the area of new bone in mm^2 was evaluated, again, MTA (8 weeks) showed the highest mean value (3.98±0.14) and control (2 weeks) had the lowest mean value (1.04±0.13). The % of immunostaining had the highest mean value for MTA in 2 weeks (40.73±2.50) with similar results with BC+MTA at 2 weeks (40.30±1.81), and the lowest mean value was found in control group in 2 weeks (10.02±0.70). (Table.1; Figures .1, .2, .3).

Discussion

Several studies [7, 12, 19, 23, 30, 31] in the literature have described the tissue reactions, both in soft and hard tissues using the materials employed in this research; however, there are only a few works in the literature that compared the use of the MTA and the BCP in isolation or in association for the healing of standardized critical bone defects.

This study aimed to investigate whether the association between the BCP and the MTA could bring some benefits for bone neoformation. The best results in terms of area of osteoid tissue formed (area of bone tissue formed) and the percentage of BMPR1B marking were observed for the MTA group. This may be due to the excellent biocompatibility of material and the ability of osteoblastic cells to connect and spread on its surface.[24]

In addition to stimulating adhesion and cell proliferation[18], the expression of alkaline phosphatase by fibroblasts,[4] osteocalcin and

other interleukins by osteoblasts,[22] MTA was able to induce a greater expression of the BMPR-1B receptor, which is important in the process of osteogenesis, being directly involved in the process of bone condensation.[2]

If only the area of osteoid tissue formed is analyzed, it is possible to observe an increase of this parameter from the period of 2 to 8 weeks in all groups; however, in groups BCP, MTA and BCP+MTA, the increase was more pronounced, giving evidence that the presence of these materials would have stimulated an increased formation of this tissue in the type of critical defect used in this study. These results differ from those found by Frota et al., 2011[13] who surveyed the use of beta tricalcium phosphate, a component of BCP, which did not induce the formation of osteoid tissues in the control group, only in the groups where the biomaterial was present. This effect may be due to the short evaluation time (7, 15 and 30 days).

Still, if one was to compare the control group with the other groups, a greater area of osteoid tissue formed in groups in which the materials were implanted, providing further evidence for the effectiveness of osteoconductive materials used in these critical defects.[13]

The groups that the highest formation of osteoid tissue was MTA and MTA+BCP. No significant difference was observed among all groups, except between the 8-week control and the 2-week MTA, as well as the 2-week MTA and 2-week BCP+MTA. These results demonstrate that in period of 2 weeks, MTA already exhibited a greater area of osteoid tissue the 8-week control sample. The MTA also exhibited more beneficial results than the BCP during the same period of evaluation.

The area of the newly formed bone tissue increased in size from 2 to 8 weeks for the BCP, MTA and BCP+ MTA groups, demonstrating that these materials would continue to stimulate the formation tissue during over this period of time.

If we compare the control group with the other groups, a greater area of bone tissue formation is observed in the biomaterials

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groups. The material with the best results regarding the formation of bone tissue was the MTA. At the same time, when MTA is associated with BCP, the amount of tissue formed was lower than with MTA alone for both the 2 and 8 week-periods. These results indicate that the combination of these materials does not lead to significant benefits. Perhaps the space occupied by the BCP particles, especially if the particles were slowly reabsorbed, complicates the process of repair. However, if we evaluated the bone repair over a longer period of time, perhaps we would observe a higher reabsorption of these particles and perhaps the results would be more similar.

The BCP group when compared to the control group showed a greater amount of bone tissue formation, and these results were statistically significant. Our results corroborate the findings of Schwarz, et al., 2007 [26] and Cordaro, et al., 2008,[7] which reported microscopic evidence of bone formation in the presence of biphasic calcium phosphate. According to Fleckenstein, et al. 2006 [12] some studies have found some evidence that the ceramic calcium phosphate may have osteoconductive properties[5, 7, 27], while others have discovered that the material becomes encapsulated by connective tissue, without osteoconductive properties.[12]

Do Nascimento, et al., 2008 [11] revealed that the deposition of hard tissue on the MTA is related to sealing ability, biocompatibility, and alkaline pH; the presence of calcium and phosphate of ions in their formulation; and the ability to attract blast cells and promote a favorable environment for cementum formation. The author reported that the osteoconductive effect, stimulation of the proliferation and cell adhesion, stimulation of the expression of alkaline phosphatase by fibroblasts and osteocalcin and other interleukins by osteoblasts, are also characteristics related to this material. These findings may help to explain the higher area of the formation of osteoid tissue and bone in the MTA group and MTA+BCP observed in this work.

According to Singhatanadgit, and Olsen. 2011[28] the endogenous signaling of BMPR1B is required initiate the differentiation of osteoblasts. Thus, we evaluated the percentage of stained cells for BMPR1B, and we observed an increase of this percentage in the 2-week control group relative to the 8-week control group, suggesting

that the beginning of the ossification process in this group occurs during a later period in the absence of biomaterials. For the MTA and BCP+MTA groups, the percentage of stained cells was higher doe the 2-week group than the 8-week group. This finding suggests that there was a decrease in the quantity of osteoprogenitor cells that had free receptors for BMPR1B, indicating that the phase of formation of bone matrix, with greater synthesis activity, occurred during this period prior in the control group. This result differs from the work of Maeda, et al. 2010[20] where different levels of BMPR were observed between the control group and the MTA group. While the findings of the present study confirm a positive effect of MTA, it is necessary to conduct a larger study on the mechanism of this material in bone tissue regeneration as well as that of BCP, which is already used in surgeries. Additionally, the assessment period of two and eight weeks is not enough to consider the possibility of re-absorption of BCP particles.

Within the limits of the present study, it was possible to conclude that the defects treated with MTA exhibited the good results in relation to bone formation and that the use of the MTA study associated with the BCP did not provide any additional benefits. The BCP alone showed better results than the control, but the role of the BCP particles in the material need to be evaluated for longer periods of time.

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Table 1. Results for the study groups (control, MTA, BCP, and BCP + MTA groups) by treatment period (2 and 8 weeks).

Group	Treatment period	Number	Osteoid tissue (mm^2) Mean ± SD	New bone (mm^2) Mean ± SD	% immunostaining Mean ± SD
Control	2 weeks	10	1.92 ± 0.30^{a}	1.04 ± 0.13^{a}	10.02 ± 0.70^{a}
BCP	2 weeks	10	2.34 ± 0.16^{b}	1.51 ± 0.35^{b}	$23.65 \pm 1.85^{b,f}$
MTA	2 weeks	10	3.13 ± 0.14^{c}	2.17 ± 0.13^{c}	40.73 ± 2.50^{c}
BCP + MTA	2 weeks	10	3.23 ± 0.22^{b}	$1.52 \pm 0.14^{b,d}$	$40.30 \pm 1.81^{c,g}$
Control	8 weeks	10	$2.75 \pm 0.32^{a,d}$	2.10 ± 0.16^{c}	14.21 ± 0.78^{e}
BCP	8 weeks	10	4.34 ± 0.20^{e}	3.25 ± 0.16^{e}	22.78 ± 0.83^{f}
MTA	8 weeks	10	6.25 ± 0.30^{f}	3.98 ± 0.14^{g}	31.67 ± 2.77^{g}
BCP + MTA	8 weeks	10	5.67 ± 0.24^{g}	$3.25 \pm 0.16^{e,c}$	$29.63 \pm 2.98^{g,h}$

SD, standard deviation; MTA, mineral trioxide aggregate; BCP, biphasic calcium phosphate. *Note*: The same letters shown within columns indicate no significant difference (Games–Howell multiple comparisons tests; $P > 0.05$).

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Captions for Figures

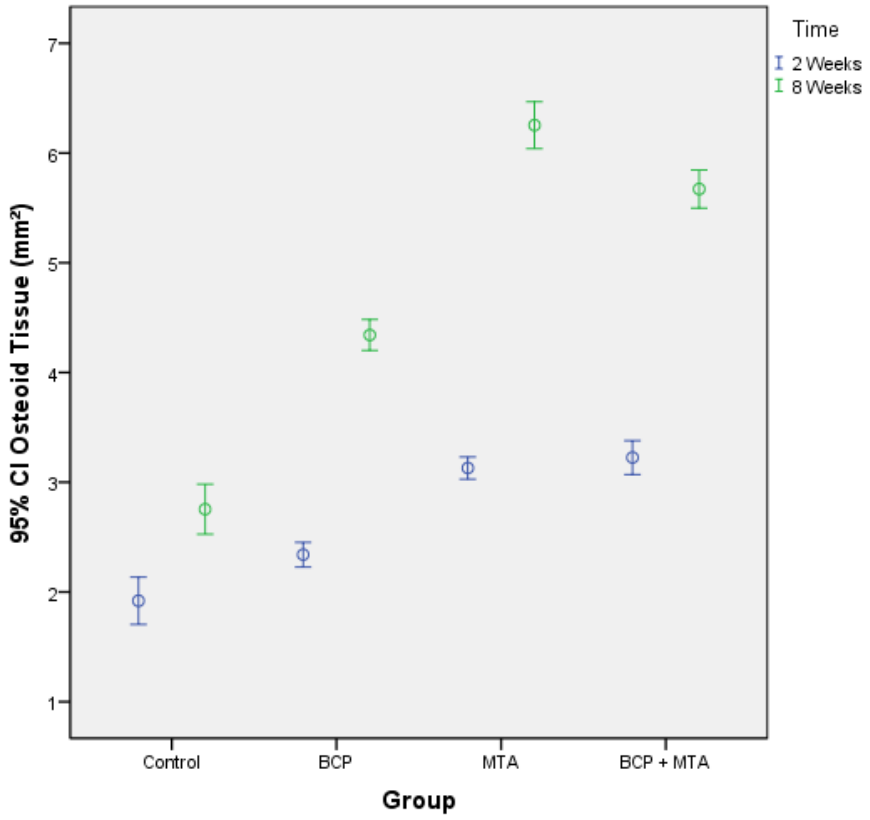

Figure 1. Graph showing the mean (95% confidence interval) of area of osteoidtissue (mm2) in each treatment and period of time.

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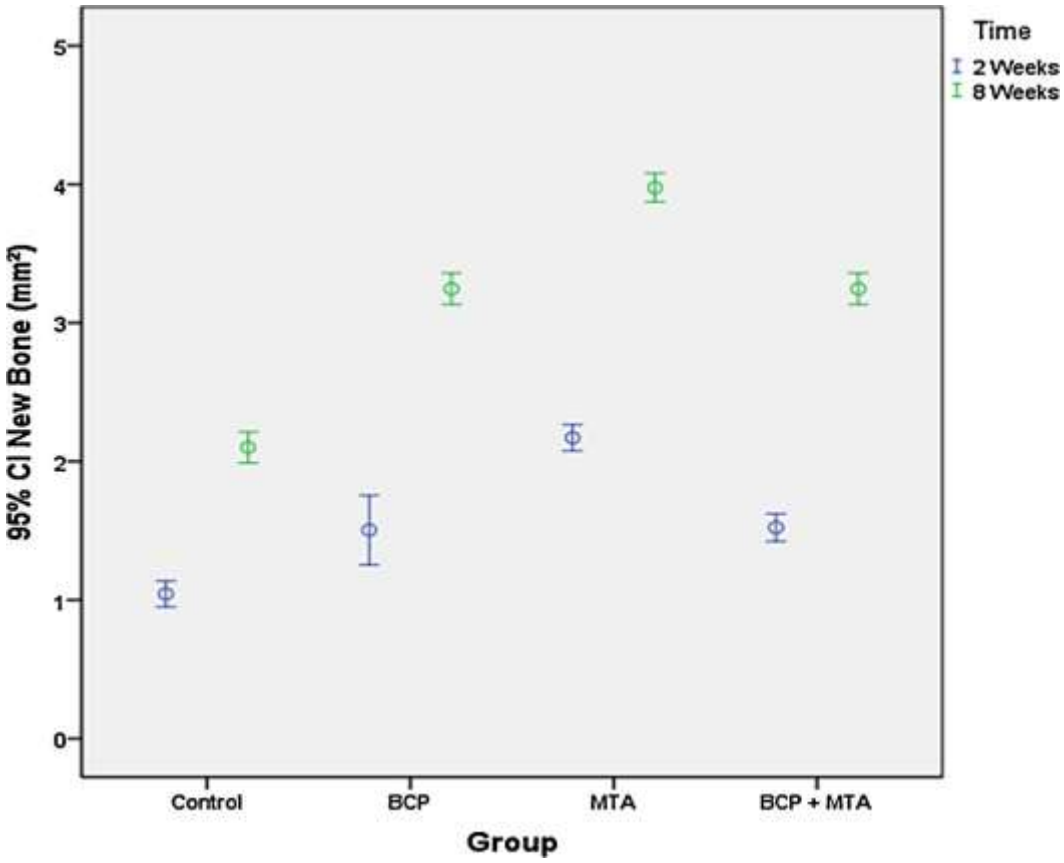

Figure 2. Graph showing the mean (95% confidence interval) of area of new bone (mm2) in each treatment and period of time.

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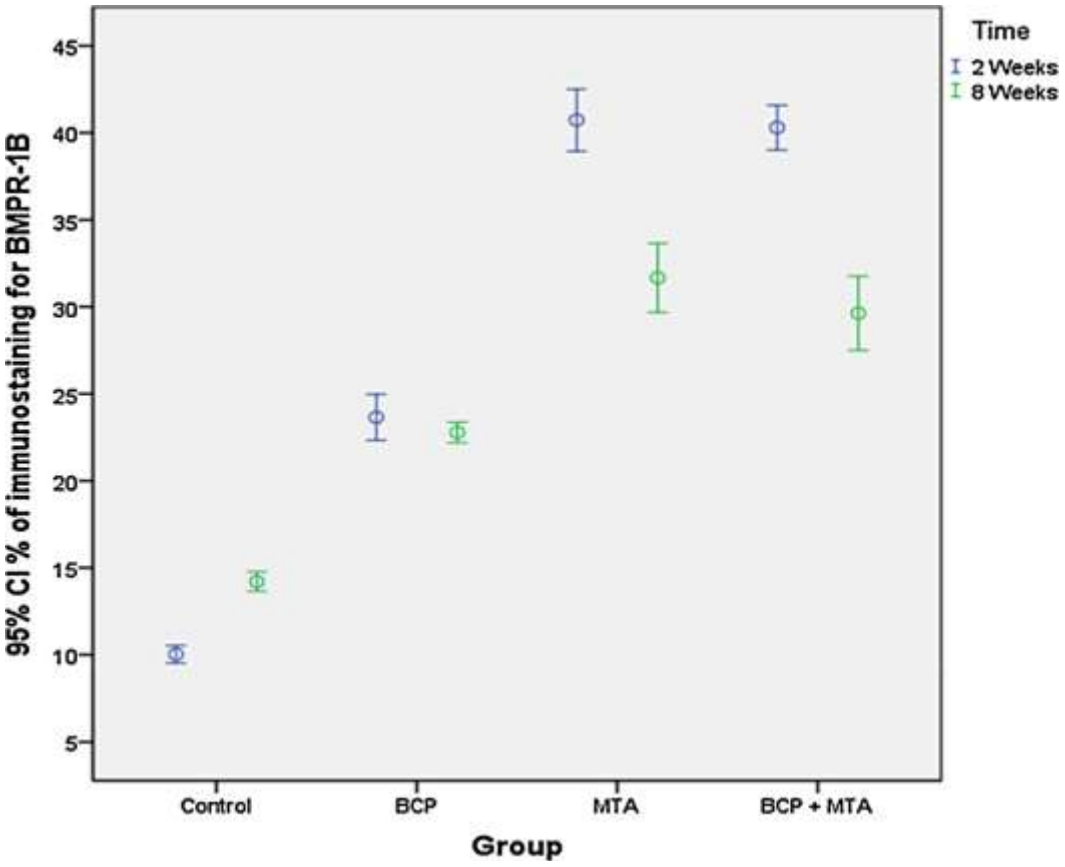

Figure 3. Graph showing the mean (95% confidence interval) of % of immunostaining in each treatment and period of time.

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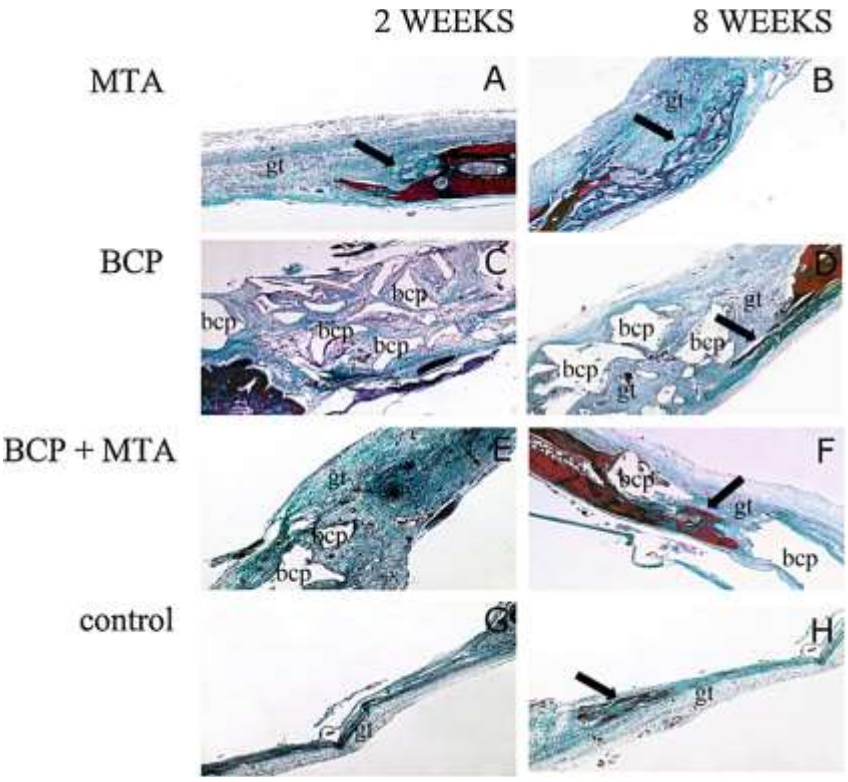

Figure 4. Reveal histological aspects for each group in all time period. Micrographs A and B demonstrate the reparative frame in the calvária treated with MTA on 2nd and 8th weeks post surgery respectively. Verify the granulation tissue (gt) and the new fone formation (arrow) in the group. Figures C and D show the group treated with BCP on 2nd and 8th week respectively. Verify the granulation tissue (gt) and BCP particles in all time period, however few new bone formation was verified in these groups. Micrographs E and F reveal the characteristics histological of regenerative development in the group BCP mixed to MTA. Verify the intensive bone deposition (Arrow) in these group on the 8th week (F) when compared to group treated only with BCP. Control group is demonstrated in the micrographs G and H, where may be seen the intensive granulation tissue in all time period analyzed. gt= granulation tissue, Arrow = bone tissue deposition. (All micrograph stained with Masson's trichrome staining, original magnification 40x).

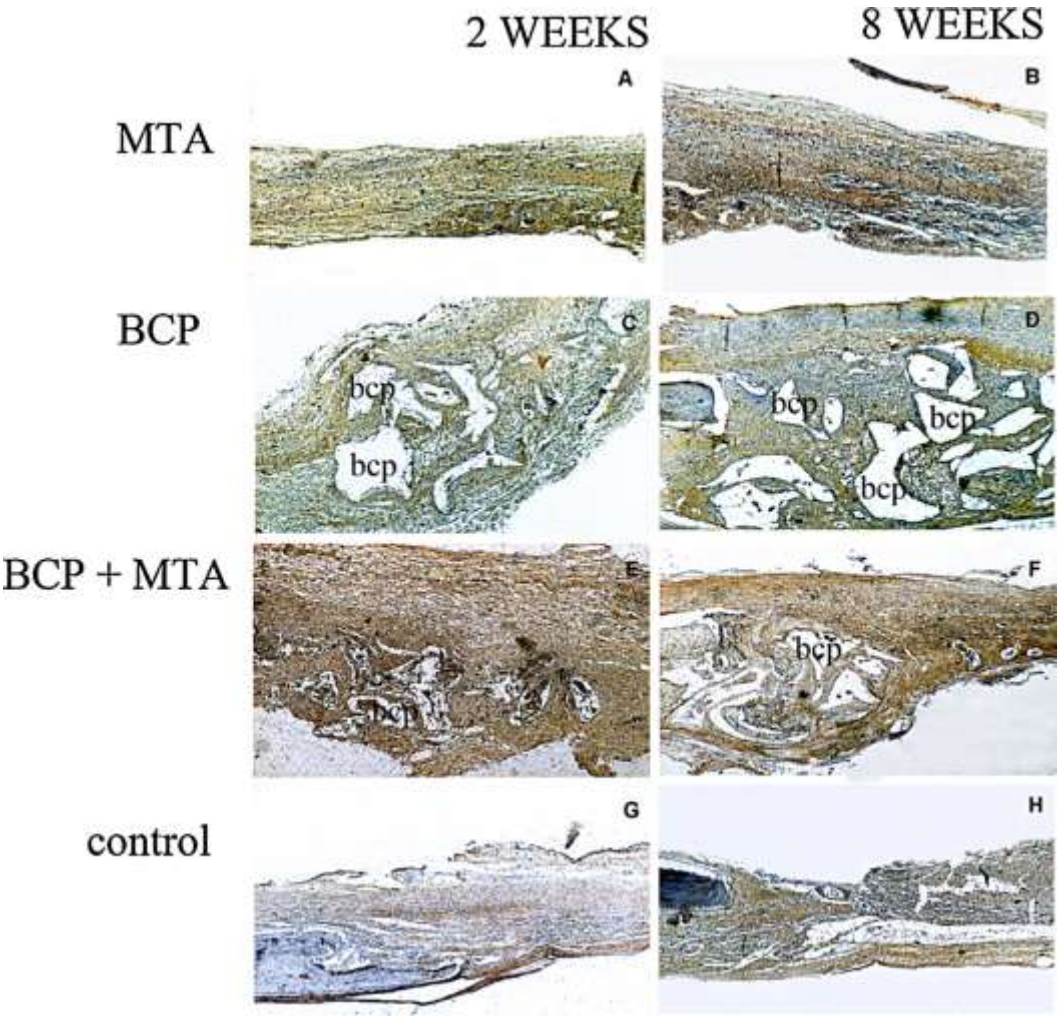

Figure 5. Shows the immunohistochemical positivity for BMPR1B (Brownish color) among the groups. A and B reveals the intensive presence of positive cells among the granulation tissue respectively on 2 and 8 week post surgery. Micrographs C and D show the groups treated with BCP. Verify the BMPR1B positivity in cells and fibers that compound the granulation tissue only surrounding the BCP particles both on 2nd and 8th week post surgery. Micrographs E and F reveal the intensive presence of BMPR1B in the group BCP mixed to MTA on 2nd and 8th week post surgery. Figures G and H demonstrate the positivity for control group. Verify the scarce positivity for BMPR1B, except on 8th week post surgery where the positivy was restrict to border of de defect (Original magnification 40x)

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