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Research Paper
Bone healing

Histological and immunohistochemical evaluation of biphasic calcium phosphate and a mineral trioxide aggregate for bone healing in rat calvaria

L. G. R. deC.Silva[2], S. H. Kim[2], S. M. Luczyszyn[2], V. Papalexiou[2], A. Giovanini[1], L. E. Almeida[3], V. A. Tramontina[2,*]

[1]School of Dentistry, Positivo University, Curitiba, PR, Brazil; [2]School of Health and Biosciences/Dentistry, Pontifical University of Paraná (PUCPR), Curitiba, PR, Brazil; [3]School of Dentistry, Marquette University, Milwaukee, USA

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Abstract. This work focused on the process of bone repair of defects in standardized calvaria of Wistar rats treated with biphasic calcium phosphate (BCP), mineral trioxide aggregate (MTA), or a combination of the two. Eighty Wistar rats were divided into four treatment groups and were examined at 2 and 8 weeks. A surgical defect was created in the calvaria using a 6-mm diameter trephine drill. The cavity was treated with BCP, MTA, or BCP + MTA; untreated rats with clot formation served as controls. Samples were evaluated histologically and by immunohistochemical staining for areas of new osteoid tissue and new bone tissue, as well as the percentage of labelled cells using anti-bone morphogenetic protein receptor type 1B (anti-BMPR1B) antibodies. Statistically significant differences were found for all dependent variables (area of new osteoid tissue, area of new bone, and percentage immunostaining) by group ($P < 0.0001$) and time ($P < 0.0001$), and for the interaction of the two ($P < 0.0001$). The MTA group at 8 weeks showed the highest amount of osteoid tissue. The same group also exhibited the highest amount of bone tissue formation. The 2-week MTA samples and 2-week BCP + MTA samples exhibited the highest percentages of stained cells. The best results in terms of the area of osteoid and bone tissue formation and the percentage of BMPR1B were observed for the MTA group, confirming that the combination of BCP + MTA does not result in a significant improvement.

Key words: bone regeneration; biomaterials; BMPR1B.

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Mineral trioxide aggregate (MTA) is a biocompatible material[1] that can help in bone repair because its composition allows the rapid adhesion and proliferation of cells on its structure.[2,3] There is evidence that MTA promotes a favourable response in the osseous environment, with direct bone apposition.[4] It has been demonstrated that MTA induces bone morphogenetic protein 2 (BMP2) expression and calcification in human periodontal ligament cells,[5] stimulates human gingival fibroblasts to produce BMP2,[6] and is able to promote a favourable formation of mineralized tissue in rat alveolar sockets,[7] this process being characterized by a mild inflammatory response and complete bone healing.[8] MTA surfaces also support osteoblast cell attachment, matrix synthesis, and RunX2 expression, which are essential for osteogenesis.[9]

The use of biphasic calcium phosphate (BCP) as an osteoconductive material has proven to be effective in promoting bone healing in orthopaedic and in oral surgery. In addition, BCP is considered to be a safe biomaterial and exhibits high biocompatibility.[10–12] The structure of BCP consists of particles with an average size of 100–500 μm composed of 60% hydroxyapatite (HA) and 40% beta-tricalcium phosphate (β-TCP).[10]

Compounds based on HA exhibit a low or negligible bioabsorption, whereas those based on β-TCP are more soluble and can be degraded or reabsorbed easily. At the same time, β-TCP is unpredictable and thus is not an appropriate scaffold for bone growth. The association between HA and β-TCP generates the biomaterial called BCP, which is reabsorbable but also contains a more stable segment (HA) that maintains the stability of the scaffold, the graft, and the more soluble component (β-TCP).[10,13,14]

Immunohistochemical analyses and in situ hybridization experiments have shown that mesenchymal cells, including osteoblasts, express BMPs and their receptors (BMPR) during the formation of skeletal and fracture repair bone tissue.[15,16] These glycoproteins are responsible for bringing osteoprogenitor cells to sites of bone formation and repair and thus have important implications in the cascades of cellular events that regulate bone formation and repair. During these processes, mesenchymal cells induce cell proliferation and differentiation, and promote the synthesis of the extracellular matrix.[16–18] As a result, BMPs are involved in the differentiation process wherein osteoprogenitor cells transform into mature osteoblasts.[19]

The cellular response to BMP depends not only on the expression of the type of protein but also on the expression and location of the surface transmembrane receptors BMPR types 1A, 1B, and 2.[20] In the calvaria, where it is possible to observe intramembranous ossification, the signal for the repair or formative process is regulated by the BMPR1B receptor,[19] which is involved in the initiation of bone condensation.[15]

As both BCP and the MTA exhibit features that can help in the bone formation process and there are few data on the use of MTA in bone formation (in isolation or in association with BCP), the aim of this study was to focus on the process of bone repair of defects in standardized calvaria of Wistar albino rats treated with BCP, MTA, or a combination of the two. The main analytical techniques used in this study were histology and immunohistochemistry.

Materials and methods

This study was approved by the institutional research ethics committee for animal use. Eighty Wistar albino rats with an average weight of 250 g were used in this study. The rats were divided randomly into four groups; two experiments were performed, using treatment periods of 2 and 8 weeks ($n = 20$ per treatment group; $n = 10$ per time period).

Each animal was anaesthetized by injection of 2% xylazine hydrochloride intraperitoneally (8 mg/kg of body weight) (Anasedan; Ceva Saúde Animal Ltda, Paulinia, São Paulo, Brazil) in the presence of 5% ketamine hydrochloride (60 mg/kg of body weight) (Vetanarcol; Laboratory König SA, Avellaneda, Argentina). Having confirmed the efficacy of the anaesthetic, a total flap in the form of a 'U' was made in the skull of each animal using a number 15 blade, exposing the surface of the calvaria bone. After opening the flap, a circular osteotomy of 6 mm in diameter was made using a trephine drill attached at an angle to 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 anteroposterior skull direction so that the larger base of the 'L' coincided with the sagittal suture; this was done to identify the location of the circular defect. The incisions were made using a diamond cylindrical high-rotation drill. Amalgam was then applied to mark the centre of the surgical wound.[21]

After carefully removing the incised portion of the calvaria with the aid of a Molt dissector, BCP was added to the wound of 20 animals (Straumann Bone-Ceramic; Institut Straumann AG, Basel, Switzerland) as per the manufacturer's instructions, filling the entire calvaria cavity (up to the edge of the defect). In another 20 animals, MTA (MTA Angelus; Angelus Indústria de Produtos Odontológicos S/A, Londrina, Paraná, Brazil) was applied in accordance with the manufacturer's instructions. A further group of 20 animals was treated with a 1:1 combination of BCP and MTA in a similar manner to the other groups. The formation of blood clots in the surgical wound was allowed to occur in the final group, which thus served as the control for the experiment. In all animals, the flap was sutured with isolated knots of Mono-Nylon 4–0 following the surgical procedure. For pain control, sodium dipyrone 500 mg (D-500; Pfizer/Fort Dodge Animal Health Ltda, Campinas, São Paulo, Brazil) was given for 3 days after the surgery. Subsequently, the animals were sacrificed with a lethal dose of anaesthetic at either 2 or 8 weeks (10 animals in each treatment group and 10 animals in the control group).

Histology

Following euthanasia, the entire calvaria was carefully cut open and the soft tissue gently removed from the bone. All samples were stored in 10% formalin prior to mounting of the surgical specimens, and then demineralized in 4.13% ethylenediaminetetraacetic acid (EDTA) for a period of 75 days.

The material underwent routine histological processing for paraffin embedding; a microtome was used to cut 3-μm-thick sagittal sections using the 'L' marking in the amalgam to determine the location of the largest wound diameter. Two slides were made for each piece of material, for a total of 160 slides. The slides were stained with Masson's trichrome and analysed using an optical microscope at 40× magnification.

Immunohistochemistry processing

For immunostaining, serial sections of 3 μm in thickness were deparaffinized in xylol at 60 °C and hydrated using various concentrations of alcohol (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.