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Immunohistochemical response in rats of beta-tricalcium phosphate (TCP) with or without BMP-2 in the production of collagen matrix critical defects

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ABSTRACT

This study aimed to assess the biological response of BMP-2 (bone morphogenetic protein-2) in supplementation with β-tricalcium phosphate (TCP) as a carrier in the bone healing of surgical defects in rats' calvaria. A critical-size defect (5 mm in diameter) was filled with β-TCP alone or added with that plus 5 mg of BMP-2 at 5, 15, and 30 postoperative days. Histomorphometric and immunohistochemical (osteocalcin, collagen type I, and metalloproteinase-9) analysis was performed to assess the features of bone healing. Histological behavior and collagen type I labeling showed increased formation of the collagen matrix, leading to a higher percentage of newly formed bone and biomaterial for tissue and more total mineralization of pure TCP when compared to the other groups. The supplementation with BMP-2 promoted faster TCP remodeling; however, there was no statistically significant difference for the bone formed in both groups ($P > 0.05$). Collagen-matrix formation and new bone formation reached maximum levels when the defects were filled with pure TCP, even exceeding the levels from BMP-2 supplementation.

1. Introduction

The absence of the alveolar bone is one of the major challenges in rehabilitation after dental implants are installed, and in some cases, subsequent surgical procedures are necessary. Autogenous bone remains the gold standard (Boyne and James, 1980; Tatum, 1986; Misch, 1987) for the reconstruction of defects caused by disease and trauma (Tessier et al., 2005), as it enables the placement of implants (Brånemark et al., 1975; Wada et al., 2001; Proussaefs and Lozada, 2005; Esposito et al., 2006). Despite possessing all the desirable properties, autogenous bone has some disadvantages, such as donor site morbidity, limited quantity, and a need for general anesthesia in extraoral grafts.

With the extensive development of tissue engineering, bone substitutes are achieving bone scaffolds with properties similar to those of autogenous bone, especially osteoconductive properties, which favor the process of bone formation (Yao et al., 2005). In this scenario, there is a matrix of β-tricalcium phosphate (TCP), which is carrier bone and is the target of several studies.

Along with the discovered bone scaffolds, the potential osteoinductive bone morphogenetic proteins (BMPs) are produced by osteoblasts, which stimulate the differentiation of undifferentiated mesenchymal cells into chondrocytes (Wozney and Rosen, 1998; Reddi, 1998). Several studies (Wozney and Rosen, 1998) have confirmed the importance of bone morphogenetic protein-2 (BMP-2) in cell differentiation and bone repair.

The study of the cellular and molecular mechanisms involved in bone repair can be better understood and justified by immunohistochemical techniques. In the process of bone formation, osteocalcin signals bone mineralization and collagen type I (Col I). These are products of osteoblasts (the main components of the organic matrix of bone tissue), and they are produced and released during bone formation. The use of extracellular matrix components, such as Col I- and chondroitin sulfate-enhanced osteogenic

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differentiation and bone-matrix accumulation, was found in some studies (Rentsch, 2014).

On the other hand, the answer depends on bone metabolism, such as the bone resorption by osteoclasts that produce controlled proteases. These proteases include the metalloproteinase-9 (MMP-9) enzyme, indicating the degradation of the extracellular matrix, which is replaced by new bone. Matrix metalloproteinases are a family of proteolytic enzymes produced by inflammatory cells, fibroblasts, and endothelial cells. The enzymes cleave to extracellular matrix components and a wide range of bioactive molecules that are involved in wound healing (Carlson et al., 2013).

Considering the dynamics of bone healing and the importance of scientific knowledge of biomaterial, it is necessary and appropriate to carry out histologic and immunohistochemical studies. These studies will further elucidate the performance of bone substitutes, including TCP, which are associated with the use of carriers such as BMP-2 in regenerative techniques and with the expression of bone matrix proteins as markers in the bone process.

Therefore, the present study looks at the biological role of TCP, whether supplemented with BMP-2 or not, in the synthesis of collagen matrices in surgically created cavities within rat calvaria.

2. Material and methods

2.1. Study design and ethics

This present study is in accordance with the principles of laboratory animal care and national laws on animal use, and it was authorized by the Animal Research Ethics Committee of the São Paulo State University, Brazil (protocol #2008-004517). We purchased the 45 Wistar male adult rats (90 days old) used for this study from the Animal Center of São Paulo State University and maintained them at a temperature of 22 °C under a 12 h light/12 h dark cycle, with free access to water and rodent food. A total of 45 calvarial defects (5 mm in diameter) were randomly divided into 3 groups, with a total of 5 defects per treatment group (n = 5), and into 3 evaluation times (5, 15, and 30 days). The treatment groups were established as follows: (1) 500–1000 mm β-TCP (Cerasorb M Curassan Ltd., Germany), (2) TCP plus 5 mg BMP-2 (R & D Systems, Inc., Minneapolis, MN, USA), and (3) the empty control (untreated group). Our group used the same experimental design and evaluation groups in our previous studies (Luvizuto et al., 2011, 2012).

2.2. Surgical procedures

After general anesthesia with xylazine (0.03 mL/100 g body weight [bw]/intraperitoneal [ip]; Dopaser Laboratories Calier SA, Barcelona, Spain) and ketamine (7 μL/kg bw/ip; Fort Dodge Saúde Animal Ltd., Brazil), trichotomies of the animals' skulls were completed, disinfecting with polyvinylpyrrolidone iodide (PVPI 10%, Riodeine Degermante, Rioquímica, São José do Rio Preto, SP, Brazil). Using aseptic techniques, an incision was made through the skin and periosteum of the skull, and a full-thickness flap was obtained. A defect of 5 mm diameter was prepared in each animal's parietal region with a bone trephine drill (3i Implant Innovations Inc., Palm Beach Gardens, USA) under copious saline irrigation.

The defect was treated as described above prior to the repositioning of the periosteum and sutured with polyglactin 910 (Vycril 5.0, Ethicon, Johnson Prod., São José dos Campos, Brazil). The skin was sutured with nylon (Nylon 5.0, Mononylon, Ethicon). All animals received a single dose of 20,000 IU of benzathine penicillin (Pentabiotic, Veterinário Pequeno Forte, Fort Dodge Animal Health Ltd., Campinas, Brazil) intramuscularly. The rats were euthanized with anesthetic overdose (Sodic Thiopental, 150 mg/kg) after 5, 15, or 30 postoperative days.

2.3. Sample processing

The skull parts in the study were fixed in formalin solution, underwent decalcification in EDTA (18%), and were then dehydrated using a series of alcohols. After these steps, they were cleared with xylene, embedded in paraffin, and cut to obtain thicknesses of 5 μm for mounting on slides. The pair slides were stained with hematoxylin and eosin (HE) (Merck & Co., Inc.), and the odd slides were then prepared for immunohistochemistry analysis.

2.4. Histomorphometric analysis

The measurements were performed using an optical microscope coupled with a camera image capture (JVC TK 1270 Color Video Camera) lens (with 4× increase); the microscope was connected to a computer that had a software analyzer to scan images (Leica Qwin Color/RGB). The scanned images were saved as JPEG files, analyzed, and projected on a monitor (SyncMaster 3NE, 15 inches). All slides were encoded, and the observer performing the evaluation did not know which group the blades belonged to. There were 3 fields in each histological section. These fields corresponded to the areas of bone tissue present in the central region (1 field) and the peripheral regions (2 fields) of the bone defects. The fields were standardized through a toll straight from the RGB software. First, the images from the defect were measured in all dimensions (from one side to another side). Thus, the images were divided into 3 parts to show the 2 peripheral regions and the central region.

The data obtained in this study were transformed into absolute values of pixels to get the relative percentage values so as to minimize the interference of the size difference. The data obtained were (1) the total volume of new bone, (2) the total volume of biomaterial, and (3) the total volume of mineralized tissue. Each was expressed as a percentage of the total volume of tissue.

2.5. Statistical analysis

The data were analyzed through multiple comparisons of the means using a 2-way ANOVA and a Tukey posttest. The difference was attributed to a $P < 0.05$.

2.6. Immunohistochemical analysis

Immunoperoxidase was used for detection. The endogenous peroxidase activity was inhibited with hydrogen peroxide. Following this, the blades passed though the stage of antigen retrieval, with a citrate phosphate buffer (pH 6.0). The primary polyclonal antibodies produced in goats were those that act against osteocalcin (OCN; SC-365797, Santa Cruz Biotechnology, Dallas, TX, USA), Col I (SC-8785), and MMP-9 (SC-6840), with the aim of analyzing the formation and degradation of the organic matrix. We used a polyclonal biotinylated secondary goat antibody that is produced in rabbits (Pierce Biotechnology, Waltham, MA, USA) and an Avidin and Biotin amplifier kit (Vector Laboratories, Burlingame, CA, USA). The chromogen used was diaminobenzidine (Dako, Santa Clara, CA, USA), and the end of the reaction was carried out against the cut-staining with the Harris hematoxylin. For each of the antibodies used, we evaluated the expression of these proteins semiquantitatively by assigning scores. The analysis was performed under a light microscope (Leica DMLB, Heerbrugg, Switzerland). Negative controls were performed to assess the specificity of the antibodies. Absence of immunostaining was observed when the primary antibody was substituted with the serum of the host species, acting as a negative control for the secondary antibody. All lab processing was standardized based on previous studies (Luvizuto et al., 2011, 2012).

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