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Collagen fibers evaluation after rhBMP-2 insertion in critical-sized defects

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ABSTRACT

The objective of this investigation was to assess the quantity of collagen fibers with different dosages of recombinant human bone morphogenetic protein, type 2 (rhBMP-2) associated with two different carriers, monoolein and poloxamer gels, in critical bone defects created in the calvaria of Wistar rats. Forty male adult Wistar rats were divided into eight groups of 5 animals each—group I: critical bone defect with application of 1 μg of rhBMP-2 combined with monoolein gel; group II: 3 μg of rhBMP-2 combined with monoolein gel; group III: 7 μg of rhBMP-2 combined with monoolein gel; group IV: 1 μg of rhBMP-2 combined with poloxamer gel; group V: 3 μg of rhBMP-2 combined with poloxamer gel; group VI: 7 μg of rhBMP-2 combined with poloxamer gel; group VII: monoolein gel only and group VIII: poloxamer gel only. A critical-sized defect of 6 mm diameter was produced in the left parietal bone using a surgical round bur and a high-speed micromotor. The bone defects were filled according to the group that animals belonged and after two weeks the rats were perfused and their calvarial bones were removed for histological processing, and collagen fibers quantification. Differences among the eight groups were statistically analyzed by Anova and Bonferroni test ($p < 0.05$). The results did not show statistical difference between the groups, in exception, between the comparisons II and III. According to the experimental methodology used in this research, it was observed, in a general way, a qualitative inverse relationship between collagen fibers presence and rhBMP-2 quantity inserted in the critical bone defect, associated or not to a material carrier.

1. Introduction

One of the primary factors for the development of bone tissue is the production of a number of growth factors by osteoblasts and by their precursors (pro-osteoblasts), or both of them. The osteoblasts secrete several members of the superfamily of bone morphogenetic proteins (BMP), including morphogenetic protein types 2 and 7, which have an important role in the bone repair, whose mechanism is named as osteoinduction.

Nowadays, the BMP carriers are widely tested and they must have some properties such as: not to induce inflammatory or immune reactions, be absorbed during the bone healing process, be free of toxic residues, and supply a sustainable delivery system of the protein. In addition, they should be easily and cost-effectively manufactured for large-scale production, possible to be sterilized and also present appropriate storage conditions and stability (Seeherman and Wozney, 2005).

The collagen is the most representative class of insoluble fibrous protein found in extracellular matrix and connective tissues, and it is responsible for the initial structural conformation of the tissues. Currently, the literature indicates around 18 types of collagens, with their subunits. The collagen types I, II and III are the most abundant in the human body, and as a consequence of this fact, they are the most studied in the literature. The collagen type I is present in skin, tendon and bone, the type II in cartilage and vitreous humor, and type III in skin and muscles.

Structurally, the collagen molecule is represented by a small stick and formed by interlacing in triple helix of three polypeptide chains named alpha chains. Added to this description, the conformation of this protein structure justifies the physical and biological properties of the collagen, which are generally able to provide rigidity, strength and stability.

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Thus, due to the important role of collagen in the bone healing process, the aim of this investigation was to assess the quantity of collagen fibers with different dosages of recombinant human bone morphogenetic protein, type 2 (rhBMP-2) associated with two different carriers, monoolein and poloxamer gels, in critical bone defects created in the calvaria of Wistar rats.

2. Materials and methods

All the aspects of this work were approved by the local ethical committee, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil.

3. Substances

The recombinant human bone morphogenetic protein, type 2 (rhBMP-2) used in this study was obtained from the Theodor-Boveri-Institut für Biowissenschaften, Am Hubland, Würzburg, Germany.

The rhBMP-2 was dissolved in phosphate buffered saline with pH 7.0 and a proportion of 1:1. Monoolein (Myverol 18–99, 98.1% monoglycerides, Naarden, The Netherlands) gel was prepared in a 7:3 (monoolein:water) proportion as described previously (Lara et al., 2005). Briefly, monoolein was weighed, heated to 45 °C and mixed with water at the same temperature. After a resting period, the mixture became a transparent and viscous mass. Aqueous rhBMP-2 solutions of 1, 3 and 7 μg were prepared, added to the mixture and then applied to the critical bone defects. Poloxamer 407 (Pluronic F-127, BASF, Brazil) gels in a 2:1 (polyoxyethylene:polyoxypropylene) proportion containing rhBMP-2 in 1, 3 and 7 μg per volume were applied in the bone defects.

4. Animals

Forty male Wistar rats ($n = 40$) weighing about 300 g were selected and allowed to acclimatize for 1 week prior to surgery. A commercial rat chow was used to feed the animals and they were kept on a 12 h light/12 h dark cycle, lights on at 7 AM, in a temperature controlled room (23 ± 2 °C) with water and food ad libitum. The animals were randomly assigned to eight groups with five animals each ($n = 5$), according to the chosen treatment.

The treatment groups were the following:

Group I: critical bone defect with application of 1 μg of rhBMP-2 combined with monoolein gel.

Group II: critical bone defect with application of 3 μg of rhBMP-2 combined with monoolein gel.

Group III: critical bone defect with application of 7 μg of rhBMP-2 combined with monoolein gel.

Group IV: critical bone defect with application of 1 μg of rhBMP-2 combined with poloxamer gel.

Group V: critical bone defect with application of 3 μg of rhBMP-2 combined with poloxamer gel.

Group VI: critical bone defect with application of 7 μg of rhBMP-2 combined with poloxamer gel.

Group VII: critical bone defect with application of pure monoolein gel.

Group VIII: critical bone defect with application of pure poloxamer gel.

5. Surgical procedure

The rats were anesthetized with an intraperitoneal injection of a solution of ketamine hydrochloride (75–100 mg/kg body wt.) and xylazine (5–10 mg/kg body wt.). After shaving the scalp hair, a longitudinal incision was made in the midline of cranium and the periosteum was elevated to expose the surface of the parietal bones. Under a copious irrigation with saline, a critical bone defect of 6 mm diameter was produced in the left parietal bone using a surgical round bur and a high-speed micromotor. The bone defects were filled according to the above mentioned groups. The periosteum and skin were then repositioned and maintained in place with 4–0 silk sutures (Ethicon, Johnson & Johnson, São José dos Campos-SP, Brazil).

6. Sacrifice and animal perfusion

After two weeks, the animals were anaesthetized with urethane 37.5% (1.5 g/kg body wt.) and submitted to perfusion. The procedure was carried out with a perfusion pump model 550 T2 – Samtronic, involving an intracardiac infusion of PBS (phosphate buffered saline) 0.01 M, pH 7.4 (±200 mL), followed by paraformaldehyde 4% dissolved in a PBS solution 0.1 M, pH 7.4. The rat calvarial bones were removed for histological processing.

7. Histological processing

The calvaria fragments with the critical bone defects were immersed in 4% paraformaldehyde/0.1 M phosphate-buffer solution for 24 h, decalcified in 5% EDTA-Tris for 30 days and neutralized by a 5% sodium sulfate solution. After embedded in paraffin, the specimens were cut into 6 μm thick sections following the sagittal plane and stained by picro-sirius-red stain. The histological sections of collagens fibers were quantified using the Image J free software (Fig. 1). Images processing analyses are composed by the following steps: threshold colour application on the polarized histological images, become in this way, the original image in dark dots. After this sequence, it was used the command of the software "analyze sequence", as showed in Fig. 1, obtaining in the final step of the process, the percentage of collagen fibers in area fraction, which was used for the quantitative analyses of this research.

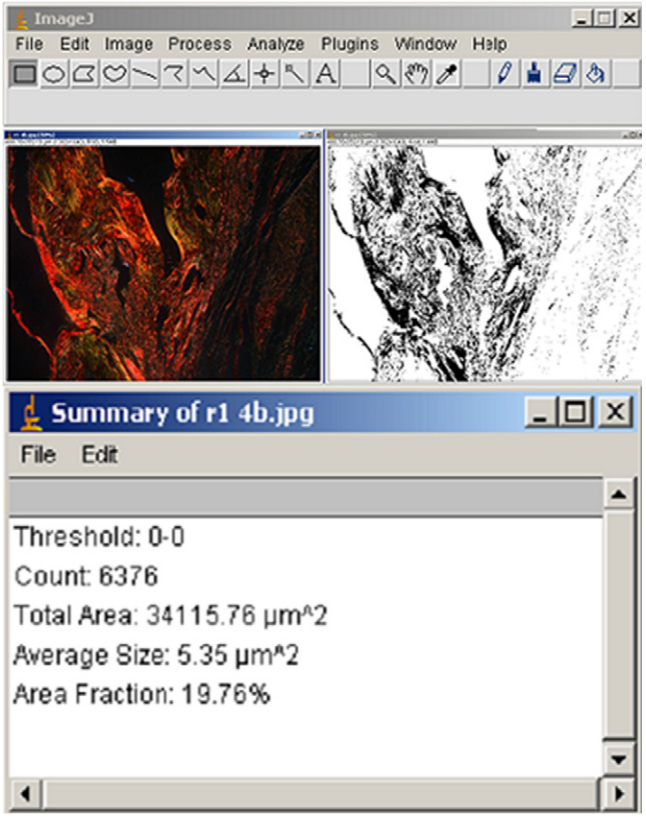

Fig. 1. Methodology used in this research to evaluate the collagen fibers.

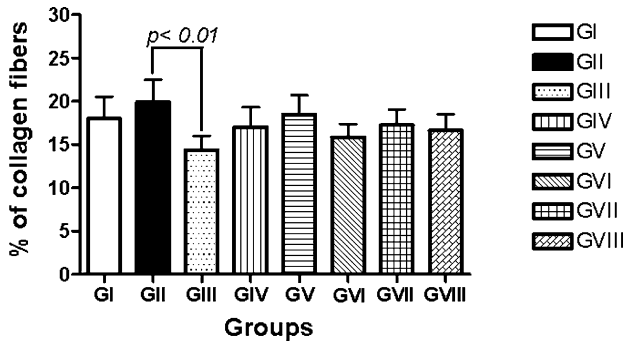

Fig. 2. Graph showing the quantitative data obtained.

8. Statistical analysis

One-way Analysis of Variance (ANOVA) was used and in sequence, the Bonferroni Multiple Comparisons Test ($p < 0.05$ was considered significantly different).

9. Results

The quantitative data did not show statistical difference between the groups, except between the groups II (critical bone defect with application of 3 μg of rhBMP-2 combined with monoolein gel) and III (critical bone defect with application of 7 μg of rhBMP-2 combined with monoolein gel) $p < 0.05$ (Fig. 2).

10. Discussion

This study evaluated the bone healing process with quantification of collagen fibers grown in critical bone defects after two weeks, using the bone morphogenetic protein type 2 (rhBMP-2), associated with two different carriers, in three different dosages.

According to the statistical method used in this work, significant difference was not observed between the groups, except in comparisons between the II and III groups ($p < 0.05$). In an overview, it was observed a delay in the bone repair in groups that were applied with the lowest doses of rhBMP-2, which can be demonstrated by the increased amount of collagen fibers observed. According to Guastaldi (2004), the initial delay in the bone healing process can be explained by a natural physiological reaction of the tissue due to the presence of bone graft particles or biomaterial used in the study.

In the present study, it was not considered groups were it was only performed the bone defect, without any material insertion. Bone deposition around the inserted material in the bone defect is slower and directly proportional to the absorption of this substance, promoting a reparative equivalence only in the final chronological periods of the bone repair, which is affected by the age and animal species, analyzed area, and physico-chemical characteristics of the material studied, being possible to achieve significant level of newly formed bone in control animals were it was only performed the critical sized defect (Pinto et al., 2003; Artzi et al., 2000).

In this research, the recombinant human bone morphogenetic protein type 2, rhBMP-2 was used, in three different dosages associated with two different carriers. The literature indicates a positive correlation between the bone morphogenetic protein type 2 and osteogenesis (Issa et al., 2008a,b), especially by the osteoinduction process, when the mesenchymal cells are able to transform into osteoblastic cells due to chemotactic action. Thus, there is a correlation between this type of protein and collagen fibers that are present in the early stages of bone regeneration. At cellular level, the collagens are synthesized as pro-collagens. After the secretion of these molecules, their terminal fragments are cleaved by extracellular enzymes named collagenase and released into the bloodstream (Prockop et al., 1979). These molecules are formed from collagen that polymerizes to form collagen fibrils, which, in turn, are added to form collagen fibers. Different cell types can synthesize collagen, depending on each tissue that it is being studied. The collagen type I is the main product of osteoblast secretion, which is responsible for the organic bone matrix synthesis. Approximately 90% of the bone matrix consists of collagen type I, and the remaining 10% of non-collagenous proteins such as osteocalcin, osteonectin, and other some growth factors, including the bone morphogenetic protein type 2 (Triffit, 1987).

The collagen fibers that were analyzed in this study are described in literature as structures presented in the early stages of the bone healing process, which are released in the bloodstream as pro-collagen molecules (during the synthesis process) and as collagen (during resorption process) that, currently, can be dosed in serum, plasma or urine and by immunoassay methods. Structurally, in the bone tissue, collagen molecules are united by three hydroxylysine amino acid residues, lysine or its derivatives, allowing that two collagen molecules will be joined between them by a fluorescent cyclic structure named pyridinoline (Risteli et al., 1993). Thus, in this bone healing process four basic requirements are present: a morphogenetic signal, host cells that respond to the signal, an appropriate signal carrier that can deliver it to specific places and serve as a support for growth of cells responsive to host and a viable and well vascularized bed (Burg et al., 2000).

It is concluded in this study based on the methodology and the experimental model used that there exists in a general way, a qualitative inverse relationship between collagen fibers presence and rhBMP-2 quantity inserted in the critical bone defect, associated or not to a material carrier.

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