Biochemical Analysis by Calcium Determination on the Newly Formed Bone in Critical Bone Defects Submitted to Different Treatments. A Pilot Study

Análisis Bioquímico para la Determinación de Calcio en Hueso Neoformado con Defectos Críticos Sometidos a Diferentes Tratamientos. Estudio Piloto

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SUMMARY: The aim of this study was to evaluate the calcium dosage of the newly formed bone, in critical bone defects created on the calvarial tissue and filled by different substances, in male Wistar rats. A cylindrical critical bone defect (6x2mm) was created in the posterior portion, left side, on the calvarial bone in nine animals and filled with: animals' coagulum, monoolein gel, poloxamer gel, monoolein gel + 1µg rhBMP-2, monoolein gel + 3µg rhBMP-2, monoolein gel + 7µg rhBMP-2, poloxamer gel + 1µg rhBMP-2, poloxamer gel + 3µg rhBMP-2, poloxamer gel + 7µg rhBMP-2. The results of this study showed a positive correlation between the calcium dosage analysis and the newly formed bone.

KEY WORDS: Bone repair; Biomaterials; rhBMP-2; Carriers; Calcium.

INTRODUCTION

Osteogenic factors, and particularly members of the transforming growth factor (TGF-) superfamily, play a key role in bone formation and repair (Wozney and Rosen, 1998). BMP-2, as the prototype of the bone morphogenetic proteins, induces bone regeneration and ectopic bone formation in adult vertebrates (Reddi, 1997, 1998) and determines important steps during early embryogenesis and limb/wing development in animals (Hogan, 1996). Accordingly, BMP-2 and its close relative BMP-4 have been studied intensively in developmental biology, and these proteins are of interest for therapeutic applications. Although the protein is usually supplemented to the medium in a soluble form in vitro, there are constraints for sustained delivery due to the need to provide bioactive protein for an extended period of time in vivo. An ideal osteoinductive carrier for bone morphogenetic protein-2 (BMP-2) delivery should consist of a biomaterial that is able to deliver protein over the required physiological timeframe, without compromising the biological activity of the molecule or the surrounding tissue upon release. In

addition, the scaffolds should biodegrade at a rate commensurate with new extracellular matrix production and be biocompatible.

Different biomaterials have been used as BMP-2 delivery systems in ectopic bone formation: poly(D,L-lactide) disks (Winn *et al.*, 1999) poly(D,[SCAP]L-lactide-co-glycolide) (PLGA) porous scaffolds (Whang *et al.* 1998) and microparticles entrapped inside calcium-phosphate cement disks (Ruhe *et al.*, 2003), collagen in the form of sponges (Winn *et al.*; Uludag *et al.*, 1999 and Uludag *et al.*, 2001) and gels (Kim & Valentini, 2002), deorganified bovine bone particles (Winn *et al.*), honeycomb-shaped hydroxyapatite (Kuboki *et al.*, 2001) and bioglass in the form of fibers (Mahmood *et al.*, 2001). Additionally, hyaluronic acid scaffolds loaded with BMP-2 induced differentiation of the multipotent cell line C3H10T1/2 (Kim & Valentini). Delivery of BMP-2 has been extensively studied because of its importance in cellular differentiation. Delivery systems

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for other morphogens or cytokines *in vitro* and *in vivo* include poly(D,L-lactide), collagen sponges for BMP-4 and BMP-6 (Uludag *et al.*, 1999), collagen for BMP-7 (Rutherford & Gu, 2000), polyesterurethane for a cocktail containing bone-inducing BMPs (Saad *et al.*, 2000), poly(propylene fumarate) for TGF- β 1 (Vehof *et al.*, 2002). PLGA porous scaffolds for osteoblast stimulating factor-1 (Yang *et al.*, 2003) and tricalcium phosphate and glass-ceramic and PLGA/glass-ceramic composites and human bone for BMP-4, basic fibroblast growth factor (FGF-2), and vascular endothelial growth factor (VEGF) (Ziegler *et al.*, 2002). In all cases, a broader set of biomaterial functional features and control of release profiles of the morphogens or cytokines would be important attributes to expand the benefits of these systems.

Monoolein is a biodegradable polar lipid that has no marked toxic effects (Ganem-Quintanar *et al.*, 2000). Depending on water content and temperature, several phases can be formed, including reverse cubic and hexagonal phases. The reverse cubic phase of monoolein and water is formed at room temperature, and has been shown to accommodate and sustain the release of drugs with various physical chemical properties, including proteins and peptides (Lee & Kellaway, 2000 a, b; Lara *et al.*, 2005 and Turchiello *et al.*, 2003).

Chitosan is a linear cationic polysaccharide prepared from chitin, found in shells of shrimps, lobsters and crabs. Chitosan consists of *N*-acetyl glucosamine and is classified according to the degree of deacetylation (DDA) (Hejazi & Amiji, 2003). It presents biocompatibility and degradation in biological medium as well as interact with some mucosal membrane due to presents positive charges in physiological pH (Mi *et al.*, 2002).

Poloxamers are non-ionic polyoxyethylene-polyoxypropylene-polyoxyethylene (PEO_n-PPO_n-PEO_n) tri-block copolymers with many pharmaceutical applications. Poloxamer 407 (P407), which has a molecular weight of 12,000 Daltons and a PEO/PPO ratio of 2:1 by weight, has been the most widely used of these copolymers. Solutions of P407 at concentrations of 20% show in situ thermoreversible gelation behavior (Schmolka, 1972; Bohorquez *et al.*, 1999; Moore *et al.*, 2000), which permit them to be administrated in cold liquid form by syringe. After administration, P407 cold solution forms a gel in situ at the site of the injection. The P407 gel is biocompatible with cells and body fluids (Park & Park, 1996). Used in pharmaceutical preparations, its low toxicity and weak immunogenic properties make it a suitable vehicle for drug delivery (Johnston and Miller, 1985; Veyries *et al.*, 1999 and Paavola *et al.*).

The aim of this study was to evaluate the calcium dosage of the newly formed bone, in critical bone defects

created on the calvarial tissue and filled by different substances, in male Wistar rats.

MATERIAL AND METHOD

This study followed the requirements of the Ethics Committee on the Use of Animals in Experimentation at the University of São Paulo, Brazil.

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

Monoolein (Myverol 18-99, 98.1% monoglycerides, Naarden, The Netherlands) gel was obtained in a 7:3 (Monoolein:Water) proportion as described before (Lara *et al.*). Briefly, monoolein was weighed and heated to 45°C. Water in the same temperature was added and the mixture was left to rest until it became a transparent and viscous mass. BMP in water solution (1mg/mL) was added and the mixture homogenized.

Chitosan (Hidagem HCMF, Cognis, Spain) gel was prepared at dispersing 8.3 mg of chitosan in 1.0 mL of rhBMP-2 water solution followed of acidifying with acetic acid (1.0%).

Poloxamer 407 (Pluronic F-127®) was purchased from BASF, Brazil.

Amounts of the gels sufficient to carry 1, 3 and 7mg of rhBMP-2 were applied in the critical bone defects.

Animals. Nine male Wistar rats (n=9), 350g, were selected and allowed to acclimatize for one week prior to surgery. The animals were fed with commercial rat chow and had access to food and water *ad libitum*. A cylindrical critical bone defect (6x2mm) was created in the posterior portion, left side, on the calvarial bone and filled with animals' coagulum (Group 1), monoolein gel (Group 2), poloxamer gel (Group 3), monoolein gel + 1µg rhBMP-2 (Group 4), monoolein gel + 3µg rhBMP-2 (Group 5), monoolein gel + 7µg rhBMP-2 (Group 6), poloxamer gel + 1µg rhBMP-2 (Group 7), poloxamer gel + 3µg rhBMP-2 (Group 8), poloxamer gel + 7µg rhBMP-2 (Group 9).

Surgical procedure. The rats were anaesthetized with standard anesthetic cocktail consisting of ketamine hydrochloride (60mg/Kg) and xylazine (5mg/Kg), administered intraperitoneally. Surgery was performed using aseptic

techniques. A linear incision was made through the skin, and the osteotomy was performed using a high-speed drill, adjusted to 3000rpm, and abundant irrigation.

Animals sacrifice. After two weeks, the animals were anaesthetized with urethane 37.5% (0.4mL/ 100g) and the calvarial bone samples were removed for biochemical analysis, calcium dosage analysis. The samples were inserted into individual beakers and weighed with the objective to determine the fresh weight of the samples (organic component + mineral component) and heated until 700°C, for 15 hours to determine only the mineral compound. After balance determination, the mineral component was diluted in chloridric acid 1N and the calcium dosage was determined by colorimetric method, using a photocolorimeter (Micronau, Brazil) and a comercial kit (Labtest Sistemas Diagnósticos Ltda, Belo Horizonte, Brazil).

RESULTS

Clinical observations. The postoperative process was considered normal, without infection or other complications.

Calcium dosage analysis. Considering the weight of the fresh bone samples, mineral plus organic components, it was obtained the following values (mg) for the nine groups, in sequence: 18.50mg; 2.60mg; 2.70mg; 5.70mg; 10.60; 19.80mg; 10.60mg; 9.80mg; 5.90mg (Fig. 1).

Considering the calcined weight of the bone samples, mineral component, it was obtained the following values (mg) for the nine groups, in sequence: 6.50mg; 1.40mg; 5.40mg; 4.30mg; 7.10mg; 13.30mg; 8.10mg; 11.20mg; 16.50mg (Fig. 2).

Fig. 1. Weight of the fresh bone samples (mg) in the nine studied groups.

Fig. 2. Weight of the calcined bone samples (mg) in the nine studied groups.

Considering the calcium dosage in the calcined bone samples, it was obtained the following values (μg) for the nine groups, in sequence: 128.42 μg ; 60.29 μg ; 149.56 μg ; 21.28 μg ; 328.89 μg ; 822.23 μg ; 328.11 μg ; 399.37 μg ; 743.93 μg (Fig. 3).

Considering the calcium dosage ($\mu\text{g}/\text{mg}$) of the calcined bone samples, it was obtained the following values (μg) for the nine groups, in sequence: 7.78 mg ; 43.06 μg ; 27.69 μg ; 4.95 μg ; 46.32 μg ; 61.82 μg ; 40.50 μg ; 61.44 μg ; 66.42 μg (Fig. 4).

Fig. 3. Calcium dosage (μg) of calcined bone samples in the nine studied groups.

Fig. 4. Calcium dosage (μg) by (mg) of calcined bone samples in the nine studied groups.

DISCUSSION

The aim of this study was to evaluate the calcium dosage by biochemical analysis of the newly formed bone, in critical bone defects created on the calvarial tissue and filled with biomaterials combined or not with rhBMP-2 in different concentrations, in male Wistar rats. It was found in this study a straightly relationship between the newly formed bone, more expressed in the groups that the rhBMP-2 was applied in more quantities, and calcium dosage. Thus, the greatest values for calcium dosage were found when it was inserted 7µg rhBMP-2, independently of the gel carrier used. These results are interesting because they show that the newly formed bone is accompanied by biochemical alteration, represented by the mineralization of this tissue, calcium dosage analysis. Thus, it is possible to relate that besides the rhBMP-2 action to convert mesenchymal cells into osteoblastic and chondroblastic cells, osteoinduction process, the resultant tissue after its action is an organized bone, which it is interesting for clinical application in many fields of the Medical Sciences.

The Wistar rat experimental model used in this study is an excellent animal experimental model with some positive characteristics, a rapid healing period, animals easily lodged and fed, resistance to climatic variations, low cost, besides being routinely used in other experimental conditions involving bone reconstruction. The calvarial bone tissue was chosen to evaluate the bone repair because it shows favorable bone structure, trabecular and cortical bone, in addition, adequate volume to observe the healing bone process in sagittal, frontal and transversal planes.

The biomaterials used in this study, monoolein, chitosan and poloxamer gels, are already being used as sustained release media of several drugs and medicines including proteins and peptides (Geraghty *et al.*, 1997; Nielsen *et al.*, 1998; Lee and Kellaway, 2000b; Paavola *et al.*, 2000; Lee *et al.*, 2001; Bonacucina *et al.*, 2005; Bhattarai *et al.*, 2005; Kofuji *et al.*, 2005 and Kao *et al.*, 2006). The

gels physical and chemical properties such as consistency, viscosity, biocompatibility to neighboring tissues are adequate for implantation, allowing rhBMP-2 to remain in situ and to adhere to the bone defect walls. Furthermore, they are of low cost and easily synthesized from carbohydrates and fats (Nylander *et al.*, 1996; Geraghty *et al.*; Lee and Kellaway, 2000b; Shah *et al.*, 2001; Lee *et al.*, 2005 and Boyd *et al.*, 2006).

The protein purity grade is an important factor directly influencing indexes of new bone formation. In this study, gel electrophoretic analysis followed by spectrophotometric determinations indicated that rhBMP-2 was highly pure, 86% of purity, explaining the large quantity of newly formed bone found in animal groups where it was applied and a positive correlation between these newly formed bone and calcium dosage analysis.

The effects of growth factor dose are intensely investigated not only in terms of efficiency but also cost. In this study bone defects with 6x2mm were treated with 1, 3 and 7µg of rhBMP-2 during 2 weeks before animals sacrifice and analysis. Other authors, like Yasko *et al.*, 1992, reported bone regeneration after creation a critical bone defect with 5mm of extension in rat femurs and insertion of 11 µg rhBMP-2, by mechanical, radiographic and histological evidence while a much smaller dose, 1.4µg rhBMP-2, produced new bone tissue, but not sufficient to close the defect. Higher dosages, as 20mg of rhBMP-2 applied to supra-alveolar bone defects, 5mm of height, in Beagle dogs showed substantial bone regeneration after 8 weeks (Sigurdsson *et al.*, 1995). On the other hand, extremely high dosages as 50 µg rhBMP-2 combined with collagen matrix and applied to 4mm defects in rat skulls were capable to produce new bone formation (Inoda *et al.*, 2004), but did not show advantages in relation to smaller and sustained release dosages as shown by Wang *et al.* (1990) who tested 0.5 to 115 µg dosages of rhBMP-2.

The results of this study showed a positive correlation between the calcium dosage analysis and the newly formed bone.

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RESUMEN: El propósito de este estudio fue evaluar el contenido de calcio del hueso neoformado, en defectos óseos críticos provocados en la bóveda craneana y rellenados con diferentes sustancias, en ratas Wistar machos. Un defecto crítico cilíndrico (6x2mm) fue provocado en el lado izquierdo de la porción posterior del hueso de la bóveda craneana, en nueve animales y rellenado con coágulo sanguíneo del propio animal, gel de monooleína, gel de poloxamer, gel de monooleína + 1µg de rhBMP-2, gel de monooleína + 3µg rhBMP-2, gel de monooleína + 7µg rhBMP-2, gel de poloxamer + 1µg rhBMP-2, gel de poloxamer + 3µg rhBMP-2, gel de poloxamer + 7µg rhBMP-2. Los resultados de este estudio mostraron una correlación positiva entre el contenido de calcio y el hueso neoformado.

PALABRAS CLAVE: Reparación ósea; Biomateriales; rhBMP-2; Transportadores; Calcio.

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