Int. J. Morphol.,
25(1):125-128, 2007.

Osteoinductive Potential of the rhBMP-2 in Soft Tissues

Potencial Osteoinductor de la rhBMP-2 en Tejidos Blandos

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ISSA, M. J. P.; NASCIMENTO, C.; BARBOSA, S. R. E.; IYOMASA, M. M. & ALBUQUERQUE JR. R. F. Osteoinductive potential of te rhBMP-2 in soft tissues. ***Int. J. Morphol., 25(1)***:125-128, 2007.

SUMMARY: The rhBMP-2 is an osteoinductive protein used in the reconstructive with the objective to create newly formed bone. The aim of this study was to confirm the rhBMP-2 osteoinductive capability, when implanted in soft tissues. The results showed that the protein used in this study is highly osteoinductive.

KEY WORDS: Bone repair; rhBMP-2; Osteoinduction; Soft tissue.

INTRODUCTION

The development of new procedures for bone regeneration has been made possible by advances in molecular biology. Since Urist (1965) demonstrated that demineralized bone matrix could increase the formation of cartilage and bone at ectopic sites, many investigators have attempted to clarify the active component of the matrix. Urist & Strates (1971) identified it as a growth factor protein that induces ectopic bone formation and named it bone morphogenetic protein (BMP). After the BMP genes were cloned by Wozney *et al.* (1988), attempts to use recombinant human bone morphogenetic proteins (rhBMPs) for bone formation have been performed. Wang *et al*. (1988), found that rhBMP-2 formed bone and cartilage when implanted in rat muscles with insoluble bone matrix by inducing muscle cells to differentiate into chondrocytes and osteoblasts. Furthermore, bone defects in the dog (Toriumi *et al.,* 1991) and rat (Yasko *et al.,* 1992) have been repaired by implanting rhBMP-2 with insoluble bone matrix.

The main clinical application of rhBMP-2 is in reconstructive surgery, and it may be applied not only to the heterotopic or orthotopic normal tissues, but also to compromised tissues, such as musculocutaneous flaps, surgical scars, or irradiated tissues. These tissues have compromised blood circulation and are susceptible to infection. Some clinical applications of purified rhBMP have been reported (Sailer & Kolb, 1994; Johnson *et al.,* 1988; Johnson *et al.*, 1990). Up to now, little is known about the clinical applications of rhBMP-2. Among others, Boyne reported the use of rhBMP-2 for reconstruction of extensive bone defects in primates (Boyne, 1996; Boyne *et al.,* 1997, 1998, 1999). However, the activity of rhBMP-2 was less than one tenth that of purified BMP (Bessho *et al*., 1999; Wozney, 1989).

The purpose of present study was to evaluate the osteogenic potential of the complex rhBMP-2/collagen matrix involved in chitosan gel when implanted at abdominal rat muscle.

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.

I- Identification and characterization of the rhBMP-2.

I. A- Electrophorese and Western blot analysis.

It was performed the electrophoretic protein characterization using a polyacrilamyde gel developed by Sigma electrophorese system (St. Louis, MO, USA), according to Laemmli method (1970).

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After electrophoretic transfer of proteins from SDS–PAGE gels to nitrocellulose membranes, these were saturated with 5% skimmed milk in TBS–Tween 0.1% (Tris 20 mM, pH 7.4, NaCl 137 mM, and Tween 20 0.1%) and soaked at room temperature for 3 h. Then they were incubated overnight at 4 °C with rhBMP-2 primary antibodies against (Santa Cruz; dilution 1/1000). After three washes in TBS–Tween–milk solution, the membranes were incubated with the corresponding secondary antibody peroxidase (1/2000) for 1 hour, room temperature. After, they were processed using the Amersham ECL kit following the manufacturer's instructions.

I. B. Spectrophotometer analysis. The purity of rhBMP-2 was assessed by polyacrylamide gel electrophoresis followed by spectrophotometric determinations (Beckman-DU-70, USA) in the stained gel band. The spectrophotometer provides a standard graphic of the electrophoretic gel strain, supplying through the proteins locations and area measurement, the percentage of sample purity.

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

It was used a collagen sponge sufficient to carry 15µg of rhBMP-2 or without this protein, in sequence this material was involved in a chitosan capsule.

III. Animals. It was used 6 rats, divided into 2 equal groups:

Group 1: Collagen sponge with 15µg of rhBMP-2 involved in a chitosan capsule;
Group 2: Collagen sponge, without rhBMP-2, involved in a chitosan capsule;

The capsules were implanted at the abdominal region, between the muscular and cutaneous tissue, in these rats.

The animals were fed with commercial rat chow and had access to food and water *ad libitum*.

III. A. 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. An incision was made through the skin, subcutaneous tissues and the abdominal muscle were exposed. The chitosan capsule was inserted between these tissues.

III. B. Sacrifice and animals´ perfusion. After two weeks, the animals were anaesthetized with urethane 37.5% (0.4mL/100g) and submitted to perfusion. This procedure involves an intracardiac infusion of saline solution (100mL) followed by 10% formalin and paraformaldehyde 4% in phosphate buffer 0.2M (100mL). The soft tissue, muscular and cutaneous tissues were removed for histological processing (Fig. 1A).

III. C. Histological processing. The soft tissues were immersed in 4% paraformaldehyde/0.1M phosphate-buffer solution for 24 h, and neutralized by a 5% sodium sulphate solution. After imbedded in paraffin, the specimens were cut into 6 µm thick sections and stained with hematoxylin-eosin and Trichrome-Masson. The histological sections were evaluated with the objective to verify if that is possible to observe new formed bone in this tissue. It was used an optical microscope (Leica MZ125 connected to a digital camera, Germany).

RESULTS AND DISCUSSION

It was performed the Western blot analysis with the objective to confirm, by a laboratorial method, if this sample was really the recombinant human bone morphogenetic protein, type 2 (rhBMP-2) and the spectrophotometer analysis with the aim to verify the purity of grade in this sample. The data indicated that this sample is really rhBMP-2, by Western blot analysis (Fig. 1B), and it has 86% of purity grade, by spectrophotometer analysis.

It was observed that the rhBMP-2 used in this study, seems to be able to induce formation of new bone tissue (Figs. 1C and 1D), this substance is an osteoinduction protein that acts chemotactically in the differentiation of mesenchymal cells into bone synthesizing cells (Lee 1997; Ripamonti *et al*., 1997; Wozney, 1998; Ripamonti & Duneas, 1998; Ducy & Karsenty, 2000; Schilephake, 2002; Arosarena & Collins, 2005). Although the bone morphogenetic protein, type 2, is able to promote osteoinduction by itself (Desilets *et al.,* 1990), many other studies about bone repair have shown that the process is optimized by association of the protein to a sustained release carrier (Issa *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, thus explaining the large concentration of newly formed bone. According to Wang *et al*., active principle purity affects not only indexes of new tissue formation but also the presence or absence of specific cellular types.

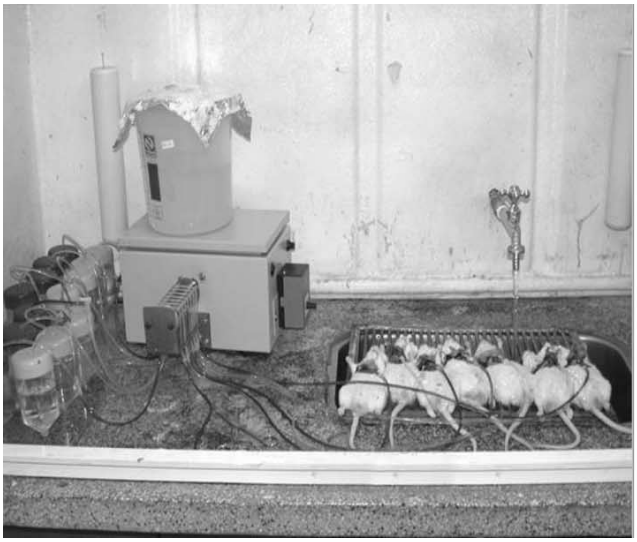

Fig. 1A. Representative photomicrograph showing the animals´ perfusion.

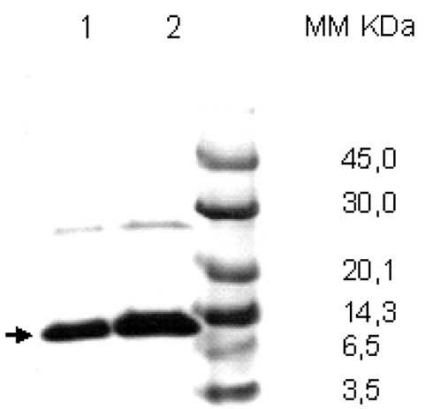

Figure 1B. Representative photomicrograph of the Western blot analysis, showing the molecular weight (10.5 KDa) of the rhBMP-2 (arrow).

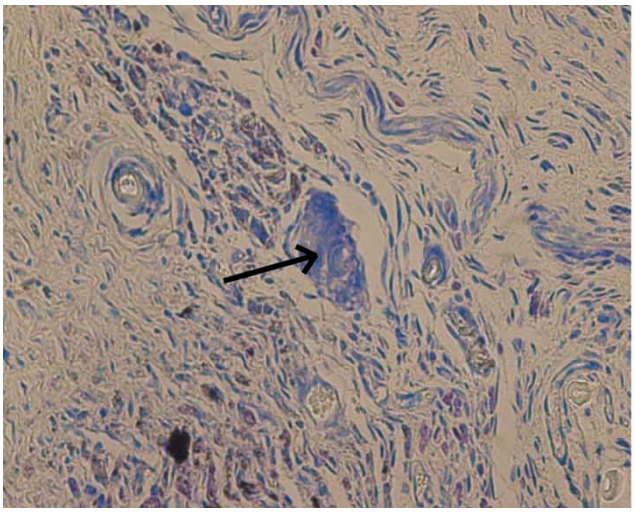

Fig. 1. C. Representative photomicrograph showing the new formed bone (arrow) in the soft tissue (500X of original magnification, Trichrome-Masson stain).

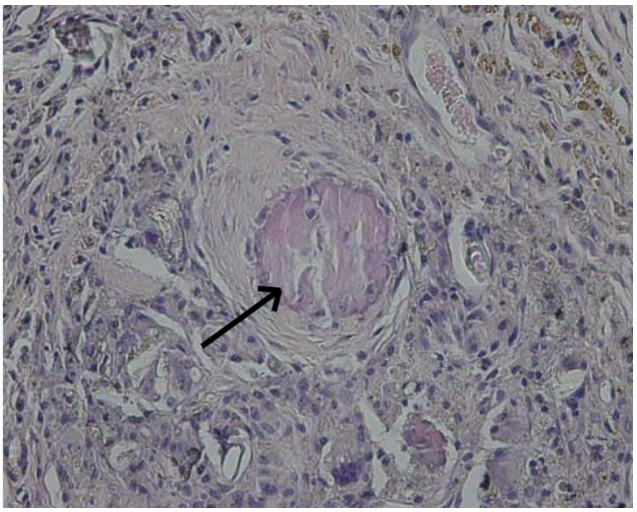

Fig. 1. D. Representative photomicrograph showing the new formed bone (arrow) in the soft tissue (500X of original magnification, H-E stain).

The method used in this study showed that, rhBMP-2 incorporated with collagen sponge and involved in chitosan gel was able to produce new formed bone over the soft tissues when was compared with the group that the rhBMP-2 was not present.

Thus, it is possible to use this sequential methodology, in soft tissues, with the objective to verify the osteoinduction potential of the rhBMP-2.

Acknowledgements. We are grateful to FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) for financial support (04/12013-0).

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RESUMEN: La proteína oseoinductora rhBMP-2 es usada en cirugías reconstructoras, con la finalidad de crear tejido óseo neoformado. El objetivo de este estudio fue confirmar la capacidad oseoinductora de la rhBMP-2, cuando fue implantada en tejidos blandos. Los resultados mostraron que la proteína usada en este estudio es altamente oseoinductora.

PALABRAS CLAVE: Reparación ósea; rhBMP-2; Oseoinducción; Tejido blando.

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Received: 14-10-2006
Accepetd: 22-12-2006