Effect of bovine bone morphogenetic proteins on radius fracture healing in rabbits[1]

Efeito de proteínas morfogenéticas ósseas de origem bovina na consolidação de fraturas induzidas no rádio de coelhos

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ABSTRACT

Purpose: To investigate the effect of bovine bone morphogenetic proteins (bBMPs) bound to hydroxyapatite plus collagen in the healing of unstable radius fractures. **Methods**: A transverse fracture was induced at the mid of the diaphysis in both radii on 15 Norfolk rabbits with average age of 5.5 months and 3.5kg. A mixture of bBMPs bound to thin powdered hydroxyapatite (bBMP-HA) and bovine collagen as agglutinant was applied to the right radius fracture site. The left radius fracture was considered control and no treatment was used. After 30, 60 and 90 days (5 rabbits/period) the rabbits were euthanized and the radii were collected for histological analysis. **Results**: The descriptive histological analysis revealed that repair was similar for both forelimbs. The histomorphometric analysis showed that the mean area of newly formed bone was 867442.16 mm^2, 938743.00 mm^2 and 779621.06 mm^2 for the control forelimbs, and 841118.47 mm^2, 788038.76mm^2 and 618587.24 mm^2 for the treated forelimbs at 30, 60 and 90 days, respectively. Thus the newly formed bone area was 12.17% larger in the forelimbs treated with bBMP-HA/collagen than in the control forelimbs ($p<0.05$, Tukey test) in the 60-day period after surgery. In both forelimbs the newly formed bone area increased throughout the experimental period until the complete fracture healing. **Conclusion**: Based on the result obtained here we concluded that bBMP-HA/collagen induced a lower but significant improvement in fracture consolidation.

Key words: Bone Morphogenetic Proteins. Fracture Healing. Rabbits.

RESUMO

Objetivo: Investigar a influência de Proteínas Morfogenéticas Ósseas de origem bovina (bBMPs) ligadas a hidroxiapatita mais colágeno na consolidação de fraturas instáveis do rádio. **Métodos**: Em 15 coelhos com aproximadamente 5,5 meses de idade e peso médio de 3,5kg foi realizada uma fratura transversa na porção média da diáfise do rádio de ambos os membros. Na fratura do rádio direito foi aplicada mistura de bBMPs ligadas à hidroxiapatita (bBMP-HA) e colágeno bovino como aglutinante e na do rádio esquerdo, considerada controle, nenhum tratamento foi usado. Os coelhos (cinco por período) foram submetidos à eutanásia aos 30, 60 e 90 dias após a cirurgia para realização do processamento histológico e análise microscópica. **Resultados**: A análise histológica descritiva revelou que a consolidação foi similar para os membros tratado e controle. Pela análise histomorfométrica, a área de novo osso foi em média 867442,16 mm^2, 938743.00 mm^2 e 779621,06 mm^2 para os membros controles e 841118,47 mm^2, 788038,76mm^2 e 618587,24 mm^2 para os membros tratados, aos 30, 60 e 90 dias, respectivamente. Desta forma, aos 60 dias de pós-operatório a área de novo osso foi 12.17% maior no membro tratado com bBMP-HA/colágeno em relação ao membro controle ($p<0.05$, teste de Tukey). Em ambos os membros a área de novo osso aumentou durante o período experimental até a total consolidação da fratura. **Conclusão**: Baseado nos resultados obtidos foi possível concluir que a mistura de bBMP-HA/colágeno induziu pequena, porém significante melhora na consolidação da fratura.

Descritores: Proteínas Morfogenéticas Ósseas. Consolidação da Fratura. Coelhos.

Introduction

Although most fractures heal, complications such as malunion, delayed union and nonunion have been reported[1,2]. Thus, mechanical, biophysical, and biological methods have been developed to accelerate fracture healing[3]. According to Hulth[4] the discovery of new biochemical mediators, which are released in fractures and in weight bearing and bone load, has provided a better understanding of the biological system involved in fracture healing. Urist[5] was the first to recognize a bone substance capable of inducing bone formation. This researcher observed that the implantation of demineralized bone matrix at a heterotopic site led to the development of an ossicle[5]. The bone matrix protein responsible for this bone induction was called bone morphogenetic protein (BMP)[2,5]. Purified BMPs, alone or in association with inactive demineralized bone matrix, enable osteogenesis in different animal species[6]. The BMP role in a fracture healing seems to be associated with its inductive property promoting mesenchymal cell differentiation in chondroblasts and osteoblasts[7]. Induction of heteretopic bone formation has been consistently demonstrated with the use of rabbit, canine, bovine and human xenogenic demineralized bone matrices implanted in mice. However, rat allogenic matrix or their native BMPs induce ectopic ossification in rats but not in other species[8]. Therefore, restrictions concerning animal species and their responses to BMPs must be considered, and are probably of immunological nature. Also, the BMP preparation procedures and the carriers used affect its osteogenic properties since BMPs easily diffuses through the tissue[9,10]. The aim of this experimental study was to evaluate histologically and histomorphometrically the effect of a commercially available bovine bone morphogenetic protein extract bound to microgranular synthetic hydroxyapatite plus bovine collagen agglutinant in the healing of unstable rabbit radius fractures.

Methods

This study was approved by the Ethics Committee of the School of Veterinary Medicine and Animal Science – Unesp Botucatu. The materials used in this work were Gen-Pro™ and Gen-Col™ produced by Baumer S.A. (Mogi Mirim, Sao Paulo, Brazil). Briefly, the pool of bone morphogenetic proteins (BMP) was extracted from fetal bovine bone-demineralized matrix using caotropic agents. The pool of BMPs was bound to absorbable ultra-thin powdered hydroxyapatite (bBMP-HA). Ultra-thin powdered hydroxyapatite is produced by wet pathway exhibiting high crystallinity, microstructure (50 µm), but not sintered. After BMP extraction collagen was the predominant protein present in the demineralized bovine bone (Gen-Col™).

Animals and surgical procedure

Fifteen male Norfolk rabbits, 5.5 months old and weighing about 3.5 kg were used. Each rabbit was placed in individual 60x60x60cm cage clear of the ground, and received water and commercial chow diet *ad libitum*. The animals were tranquilized with a mixture of acepromazine (0.1mg/kg IV) and butorphanol (0.1mg/kg IV), and 15 minutes later, intranasally anesthetized with a combination of tiletamine and zolazepan at 20mg/kg. The surgical areas of both forelimbs were clipped, prepared, and draped under sterile technique. The left radius (control) was approached craniomedially and a transverse fracture was induced at the mid-diaphysis using a circular saw (2mm). The periosteum around the osteotomy site was not removed. Using a continuous suture apposed to the subcutaneous tissue, the skin was closed with interrupted sutures. The right radius (treated) was submitted to the same procedure except that bBMP-HA and bovine collagen were set inside and around the fracture site. Following the manufacturer's recommendations, one part bBMP-HA was added to 10 parts collagen and dissolved in 0.05 ml of 0.9% saline solution until a creamy consistency was obtained. Two doses of enrofloxacin (5 mg/kg SC) were administered at time of anesthesia induction, and 24 hrs after surgery. Buprenorphine was administered (0.02 mg/kg SC) every 12 hours for five days. The surgical wounds were cleaned daily with saline and povidone-iodine. Skin sutures were removed 10 days after surgery.

Histologic processing and histomorphometric analysis

After 30, 60, and 90 days (5 rabbits/period) the animals were euthanized using sodium pentobarbital (30 mg/kg IV), followed by 20% potassium chloride (1 mL/3 kg IV). The mid-diaphysis of the radius and ulna were collected from both limbs exactly 1 cm on either side of the fracture. The bone specimens were fixed for 7 days in 10% phosphate buffered formaldehyde pH 7.2 and then demineralized in EDTA solution (with 4.13% Tritriplex III *Merck*® and 0.44% sodium hydroxide) at 2 to 8ºC for about 60 days with weekly changes of demineralizing solution. The demineralized specimens were dehydrated in ethanol, clarified in xylol and embedded in Histosec (paraffin + synthetic resin). Semi-serial 5 mm-thick sections were obtained and stained with hematoxilin-eosin. For histomorphometric analysis, the images of 10 microscopic fields/per specimen histological sections were captured using a Leika MR microscope connected to a Sony CCD-IRIS/RGB camera, and transferred to an image analyzer (KS 300; Carl-Zeiss, Hallbergmoos, Germany). The areas of newly formed bone and preexisting bone were determined at 400x magnification, and the percentage of newly formed bone calculated at 30, 60, and 90 days postoperative. A

completely randomized block design was used and the data were submitted to analysis of variance (ANOVA) and Tukey's test for multiple comparison with all tests at probability level of 5%.

Results

Histological results

In the period of 30 days after surgery, all control and treated forelimbs showed bone cortical reabsorption at the fracture site and intense new formation of woven bone tissue, perpendicular to the cortical axis that fulfilled the medullary canal. This bone tissue deposition was a result of high osteoblastic cell activity from the periosteum and endosteum and from new differentiated cells stimulated by local release of growth and differentiation factors (Figures 1 and 2). Bone fracture edges were joined by a bone callus surrounding totally the stumps, indicating the occurrence of defect stabilization (Figures 1b and 2b). At the periosteal region of the bone facing the epidermis, bone callus was formed by remodeling woven bone tissue and exhibited many osteoclastic reabsorption areas (Figures 1a and 2c). However, the bone callus facing the ulna was larger and showed hyaline cartilaginous tissue that was partially or totally reabsorbed and replaced by cancellous interwoven bone tissue (Figures 1c and 2a). Although the histological picture was similar for both groups, the control forelimbs showed larger and less mature bone calluses than the BMP treated group (Figure 3). Also, control group was characterized by the presence of more hyaline cartilage and thinner trabeculae in newly formed interwoven bone. At 60 days after surgery, in all treated and non-treated defects, the medullary canal was reestablished and the cortical bone facing the epidermis was partially remodeled showing morphology close to original (Figure 3). The cortical bone facing the ulna was thicker than normal ones and in many specimens fused to the ulnar cortical bone forming a single block of cancellous bone undergoing remodeling. At 90 days postoperatively, three out of five defects in the control and also in treated limbs showed reduction of bone callus with morphological reestablishment of the bone and adjacent soft tissues (Figure 4). Histologically the results were similar to that observed at 60 days.

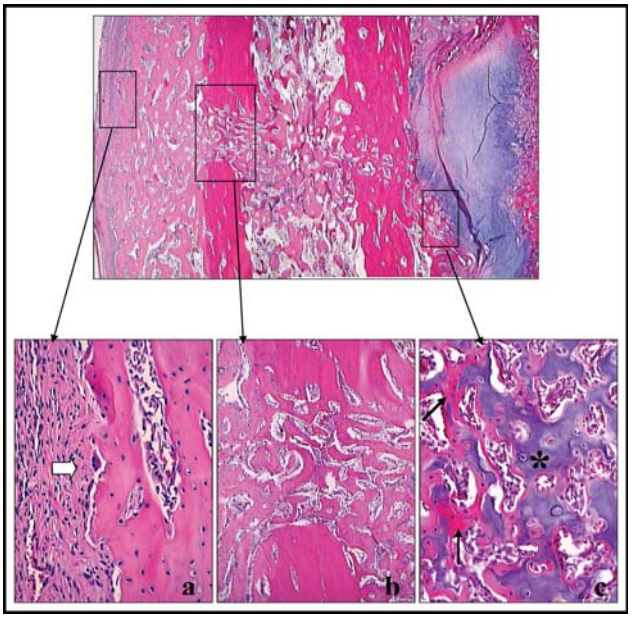

FIGURE 1 - Fracture site of the control forelimb at 30 days postoperative. Observe the presence of bone callus involving the fracture edges (general aspect) and the union of the cortical bone with primary bone tissue (b). At the external region of the bone callus is observed reabsorption of the bone surface by osteoclasts (large arrow) and facing the ulna there is a great amount of cartilaginous tissue (c) (asterisk) being replaced by interwoven bone tissue (arrows). HE, x10, x200, x 40 and x200.

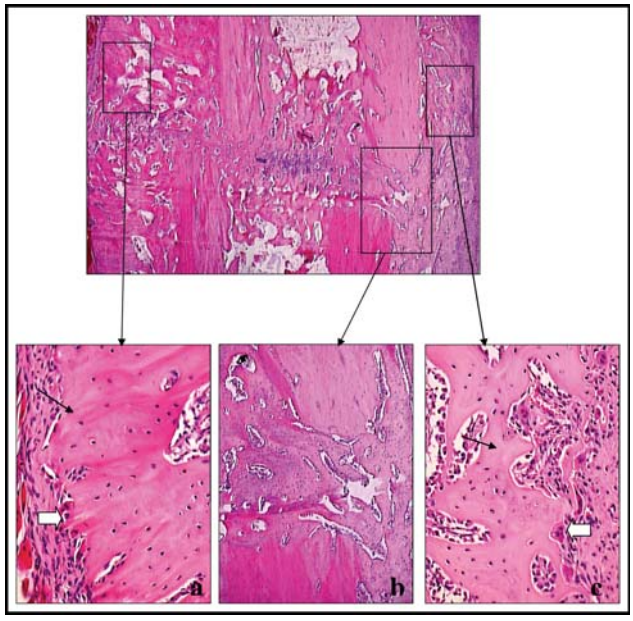

FIGURE 2 - Fracture site of the treated forelimb at 30 days postoperative. Observe the presence of bone callus involving the fracture edges (general aspect) and the union of the cortical bone with bone tissue more compact and mature in relation to control limb (b). The region of the bone callus facing the epidermis (c) as well as the bone callus facing the ulna (a) shows bone tissue (arrows) in reabsorption by osteoclasts (large arrows). HE, x10, x200, x40 and x200

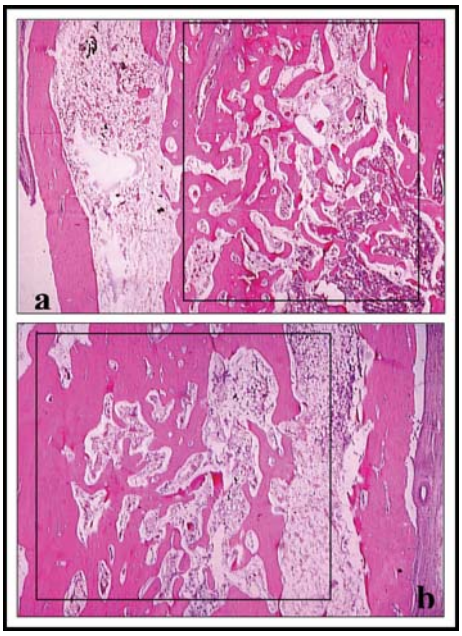

FIGURE 3 - Newly formed bone tissue at 60 days postoperative. Observe bone tissue more immature and thinner trabeculae in the control limb (a) compared to the treated limb (b). HE, x300.

FIGURE 4 - Fracture site of the treated (a) and control (b) forelimb at 90 days postoperative. Observe the decrease of bone callous, repair of cortical (large arrows) and medullary canal (asterisk). HE, x 300

Histomorphometric results and statistical analyses

The histomorphometric data are presented in Figure 5 and Table 1, and shows that only at 60 days did more new bone formation occur in the bBMP-treated forelimbs (12.17%) than that in the controls ($p<0.05$).

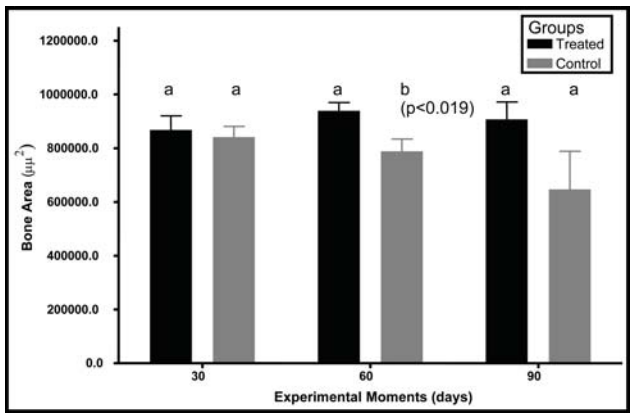

FIGURE 5 - Mean of area of newly formed bone and preexisting bone in the control and treated forelimbs at 30, 60 and 90 days postoperative

TABLE 1 - Mean of new bone area formation (μm2) and standard deviation of the treated and control limbs

Postoperative days	Control	Treated
30	867442.16 (±117507.54) a	841118.47 (±89433.97) a
60	938743.00 (±68719.04) a	788038.76 (±102116.69) b*
90	779621.06 (±399887.62) a	618587.24 (±314934.52) a

* $p<0.019$

Discussion

Despite the different phases of bone healing potentially to be subjected to different regulating factors[11], in the present investigation the association of bBMP-HA/collagen was used in a single dose immediately after osteotomy. According to Critchlow et al.[12] the bone healing induction agents should be used in the first cell proliferation and differentiation phase of the bone repair and not in the late remodeling phase where they interfere in a negative way. Here, the application of a bBMP set inside and around the fracture site. In this respect, Welch et al.[13] reported that the use of rhBMP-2 bound to collagen sponge on the fracture site in a circumferential way or on the anteromedial area did not influence formation of periosteal callus. Histological evaluation 30 days after surgery showed in both treated and control groups, woven bone tissue formation characterizing the hard callus stage of the fracture repair, according to Cornell and Lane[14]. In addition, the control radii showed larger and less mature calluses with more hyaline cartilage and thinner bone trabeculae compared to the treated radii. Bax et al.[15] reported, in the same model that rhBMP-2 accelerated cortical union and callus development rate, but not increased the amount of new bone and cartilage. In the present study was observed the predominance of periosteal callus in the lateral cortical bone in relation to the medial region; this fact can be associated with the action of the bBMP product used and/or with the preservation of the periosteum in this site since the surgical approach was craniomedial. Similarly, Bouxsein et al.[16] observed, after bilateral ulnar mid-portion osteotomies in rabbits, different histological repair patterns on their palmar and dorsal surfaces, suggesting that these differences were associated, probably, with the surgical approach and implant placement. Different from histological aspects, only at 60 days post-surgery period, the morphometric analysis showed more new bone formation in treated radii than that in the controls, but no differences were seen between groups in subsequent period. These results showed that the latter 90 days period corresponds in the rabbits to final fracture healing stage[14], when a significant bone callus reduction and reestablishment of the morphofunctional bone structure already had occurred. However, it should be pointed out that the induction of bone formation here obtained, was less intense than observed in other studies[2,17,18,19]. One possibility explanation for this mild osteoinduction response could be related to the origin of BMP. There are several sources and methods for isolating native BMPs[8], in purified or semipurified forms. The bBMP used in this experiment was a pool of BMPs extracted with guanidine hydrochloride from cortical bones of bovine fetuses without any kind of chromatographic purification. The identity and concentration of each BMP present in the pool was neither determined nor available according to the manufacturer's recommendations. The identity and concentration of each BMP present in the pool was neither determined nor available by the manufacturer's product catalog. As the effect of BMP is dose dependent the knowledge of the correct concentration of BMP available is important to set up the amount of biomaterial to be used[8,17,20]. On the other hand, BMPs extract could contain other hydrophobic noncollagenous proteins that may provoke associated immunological reactions sufficient to interfere with bone repair. There are several carrier types used to delay BMP dispersion and to protect it from lyses on the tissues consequently favoring bone formation[2,6,8,21]. The carrier used in this study was microgranular synthetic hydroxyapatite that allows BMP adsorption. As this hydroxyapatite is constituted by solid micro-particles (about 50 mm) the paste prepared to fill the bone defect could be so dense that it acts as a barrier for cell migration, delaying bone repair. Critical size defects in rat skulls treated with the same product showed no bone repair, probably due to a granulomatous foreign-body reaction induced by aggregates of hydroxyapatites[22]. The authors concluded that this type of hydroxyapatite is not the best carrier for BMPs. According to Kuboki et al.[23], solid particles of hydroxyapatite inhibit bone formation whereas porous particles or tablets stimulate it, suggesting that the geometry of the carriers controls the phenotypic expression of cells in BMP-induced osteogenesis. Based on the results obtained here, it was possible to conclude that fracture healing route was similar between bBMPs-treated and control forelimbs, but the bBMP set accelerated new bone formation in the 60-day period after surgery.

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Conflict of interest: none
Financial source: CAPES

Received: March 21, 2007
Review: May 15, 2007
Accepted: June 14, 2007

How to cite this article
Lima AFM, Rahal SC, Volpi RS, Granjeiro JM, Taga R, Cestari TM. Effect of bovine bone morphogenetic proteins on radius fracture healing in rabbits. Acta Cir Bras. [serial on the Internet] 2007 July-Aug;22(4). Available from URL: http://www.scielo.br/acb

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