Influência de Proteínas Morfogenéticas Ósseas Bovinas no Processo de Reparo do Alvéolo Dental: Um Estudo Histológico em Ratos

Influence of Bovine Bone Morfogenetic Proteins in Dental Alveolus Healing Process: an Histological Study in Rats

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RESUMO

Introdução: As Proteínas de Morfogenéticas do osso (BMPs) são membros da superfamília dos fatores de cresciemtno transformante beta (TGF-) que participam da fisiologia patologia óssea.

Objetivos: Este trabalho teve por objetivo avaliar o efeito de BMPs purificadas de origem bovina, usando o colágeno tipo I carreador, no processo de reparo do alveolo dental de incisivos de ratos.

Materiais e Métodos: Foram utilizados trinta ratos (Ratus norvegicus albinus, Wistar) divididos em 2 grupos: controle (n=15) e experimental (n=15). Os animais foram sacrificados aos 7, 14 e 21 dias de pós-operatório e os cortes histológicos obtidos foram corados com hematoxilina-eosina. O exame microscópica das dos cortes foi realizado, observando-se 5 parâmetros: quantificação do osteóide, do osso imaturo, do osso maduro, dos osteócitos e dos osteoblastos. Cada espécime foi analisado por 2 observadores diferentes. Os achados microscópicos receberam uma das seguintes contagens: 0=ausência; 1=pouco; 2=moderado; 3=intenso.

Resultados: Os dados foram analisados estatisticamente pelo teste não-paramétrico de U Mann-Whitney. As características avaliadas a respeito do reparo ósseo do alveolo dental mostraram diferenças significativas entre os grupos nos períodos observados.

Conclusão: Nossos resultados sugerem haver uma atividade osteoindutiva das BMPs purificadas de origem bovina, com aceleração do tempo de reparo ósseo, encorajando o uso clínico destas proteínas no alvéolo dental após a extração.

Palavras-chave: Proteínas morfogenéticas ósseas, fator tranformador de crescimento beta, alvéolo dental

SUMMARY

Introduction: Bone Morphogenetic Proteins (BMPs) are members of the transforming growth factor beta (TGF-) superfamily which participate in osseous physiology and pathology.

Objectives: The aim of this work was to evaluate the effect of bovine purified BMPs, by using type I collagen as a carrier, in the healing process of rat incisive dental alveolus.

Methods and Materials: Thirty rats (Ratus norvegicus albinus, Wistar) were used and divided in 2 groups: control (n=15) and experimental (n=15). The animals were sacrificed at 7, 14 and 21 post-operative days and histological slices were obtained and stained with hematoxilin-eosin. Light microscopic examination of the slices was carried out to observe 5 parameters: quantification of osteoid, immature bone, mature bone, osteocytes and osteoblasts. Each specimen was analyzed by 2 different observers. The microscopic findings received one of the following scores: 0=absence; 1=mild; 2=moderate; 3=intense.

Results: Data were analyzed statistically by the non-parametric U Mann-Whitney test. The evaluated features regarding bone healing of the dental alveolus evaluated showed significant differences between the groups in the observed periods.

Conclusion: Our results suggest that purified bovine BMPs present osteoinductive activity with acceleration of the osseous healing time, thus, encouraging the clinical use of these proteins into alveolus after extraction.

Key words: Bone morphogenetic proteins, transforming growth factor beta, dental alveolus.

INTRODUCTION

Bone Morphogenetic Proteins (BMPs) are members of the transforming growth factor *beta* (TGF-β) superfamily with a wide range of functions in vertebrate's embriogenesis (1,2). They were first described by Urist(3), but only during the 80's techniques that permitted purification and isolation from animal bones like dogs, mice, pigs and cattle were developed (4). More recently molecular biology techniques lead to the identification of genes encoding these proteins, making possible the production of recombinant forms of various human BMPs (rhBMPs)(3,5, 6, 7).

The increasing knowledge regarding BMPs role in osseous physiology and pathology stimulated several researches about its potential clinical use. Both rhBMPS and bovine forms showed efficacy in human and animal studies, mainly in the field of orthopedics, neurosurgery, cranio-facial surgery and dentistry (8,9,10,11). They have been used in a range of procedures, such as acceleration of long bone fracture repair, healing of segmental and critic defects in cranial vault, long bones and periodontal regeneration (6,9,12,13,14).

The aim of this work is to evaluate microscopically the influence of the implantation of bovine purified BMPs, using type I collagen as a carrier, in the alveolus wound healing of rats incisor tooth.

MATERIALS AND METHODS

Sample

A total of 30 male albino rats (*Ratus norvegicus albinus*, Wistar) weighing between 180 to 190g were used in this study. The animals were divided into two groups: experimental (n=15) and control (n=15) according to the implantation or not of the BMPs.

Surgical procedures

The animals were anesthetized with an intraperitoneal injection of an association of ketamine and dihidrothiazine (0,1 ml/kg and 0,05 ml/kg of body weight, respectively). The upper right incisor of all sample was extracted with special adapted instruments as described by OKAMOTO, RUSSO (15). Immediately after tooth extraction, the sockets in the experimental group were filled by bovine BMPs (*Gen pro*®, Baumer SA, Brazil) using bovine type I collagen (*Gen col*®, Baumer SA, Brazil) as a carrier. Both BMPs and carrier were presented as a powder that were mixed with a small volume of saline, obtaining a paste that was placed into the socket with the aid of curettes. In control group the alveolus was until it was filled by the blood clot. In both groups, immediately after this procedure, the gingival mucosa was sutured with 5-0 silk sutures, approximating the borders of the wound.

Five animals of each group were sacrificed by excessive sulfuric ether inhalation after 7, 14 and 21 days. A frontal section was made between middle and posterior thirds of the animal's head. After removing all soft tissue, the right maxilla was separated from the left by a cut along the median sagittal plane.

Laboratorial processing, microscopical and statistical analysis

The right halves were cut tangentially to the distal surface of the last molar and each specimen was fixed for 48 hours in 10% formalin, decalcified in 5% formic acid, dehydrated and embedded in paraffin blocks. Longitudinal semi-serial 6¼m sections were stained with hematoxilin-eosin for histological examination. Light microscopic examination of the slices was carried out to observe the findings related to the dental alveolus healing. Thus, 5 parameters of evaluation were chosen: quantification of osteoid, immature bone, mature bone, osteocytes and osteoblasts. Each specimen was analyzed by 2 different observers. The microscopic findings received one of the following scores: 0=absence; 1=mild; 2=moderate; 3=intense. The histological examination was conducted by two blinded investigators. Data were analyzed statistically by the non-parametric *U* Mann-Whitney test.

RESULTS

Histological findings

Seven days - Control group

The socket was partially filled by connective tissue formed by a great amount of collagen fibers , with eminent newly formed blood vessels . Intense granulation reaction was observed in some areas which was characterized by a connective tissue with intense newly formed vessels and significant number of inflammatory cells. In all specimens an intense chronic linphoplasmocytic inflammatory infiltrate could be observed. Degenerated blood clot was present in some specimens.

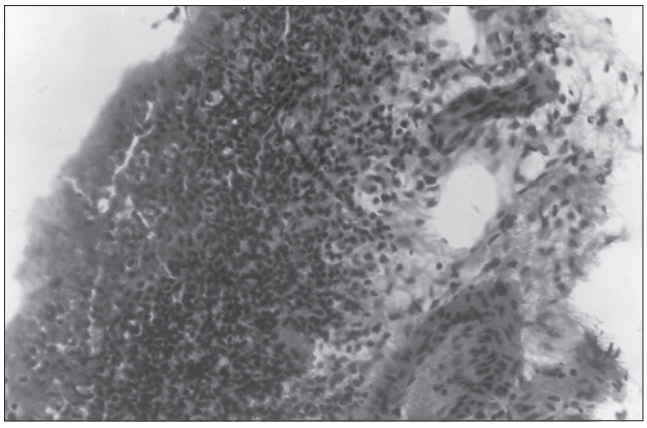

Figure 1. Control Group (Seven days) - Intense granulation reaction showing connective tissue with newly formed vessels and inflammatory cells (HE - 100x).

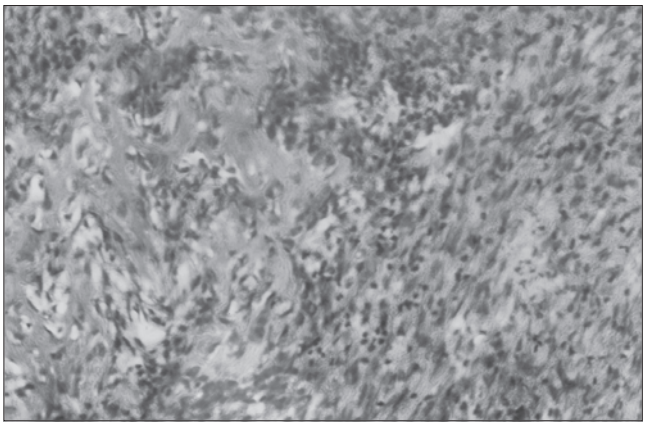

Figure 2. Experimental Group (Seven days) - Deposition of osteoid matrix indicating bone neoformation (arrow) (HE - 100x).

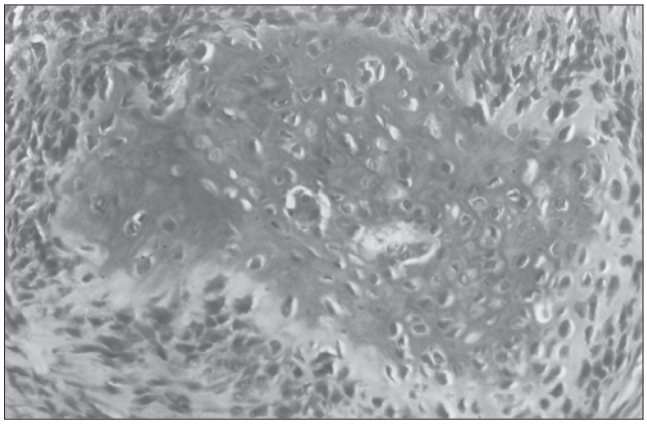

Figure 3. Control Group (Fourteen days) - Trabeculae of immature bone (HE - 200x).

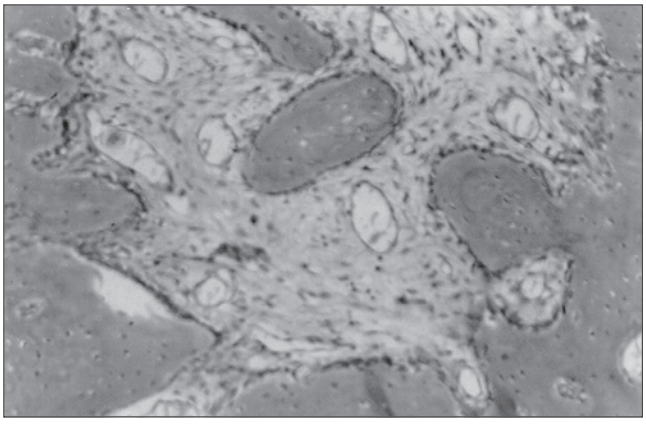

Figure 4. Experimental Group (Fourteen days) - Mature bone and fibrous connective tissue with mild quantity of newly formed vessels. (HE - 100x)

Seven days - Experimental group

More organized connective tissue was present into the socket when compared with the control group. Granulation reaction areas were uncommon, with a chronic mononuclear inflammatory reaction ranging from mild to moderate. Newly formed blood vessels were found in all specimens. Implanted material was found as an amorphous, brown stained region in variable quantity. The connective tissue in contact or near the BMPs showed a clearly different pattern in some specimens, with condensation of the fibroblasts at these areas. In some regions, cell differentiation into osteoblasts and, consequently, deposition of osteoid matrix was identified. Trabeculae of immature bone in variable quantity could be observed in all specimens.

Fourteen days - Control group

The alveolus was filled in almost all extension with well arranged connective tissue rich in collagen fibers and fibroblasts. At the medium and apical thirds of the socket was observed the presence of osteoid and immature bone with great osteoblastic activity. A mild to moderate chronic inflammatory infiltrate was present in all specimens as well as variable quantity of newly formed blood vessels.

Fourteen days - Experimental group

Near total filling of the socket by immature bone was observed with great amount of active osteoblasts depositing osteoid matrix and many lacunae with osteocytes inside. In some specimens, mature bone in variable quantity could be identified. In some areas, mainly in the cervical region of the socket, there was a presence of fibrous connective tissue with mild quantity of newly formed vessels and rare mononuclear inflammatory infiltrate. Remanescents of the implanted material was observed with no apparent reaction of the organism.

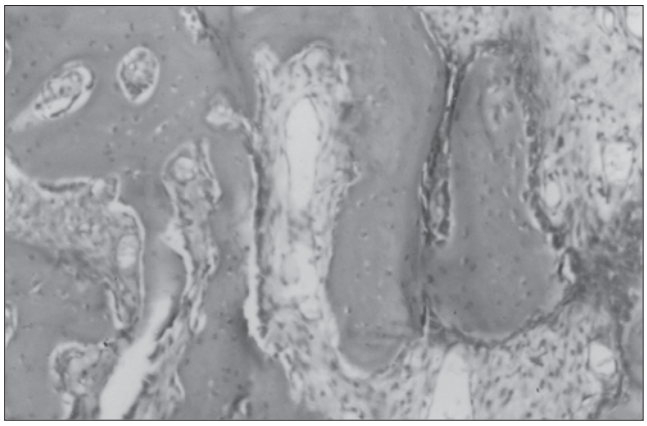

Figure 5. Control Group (Twenty-one days) - Trabecular bone filling the dental alveolus (HE-100x).

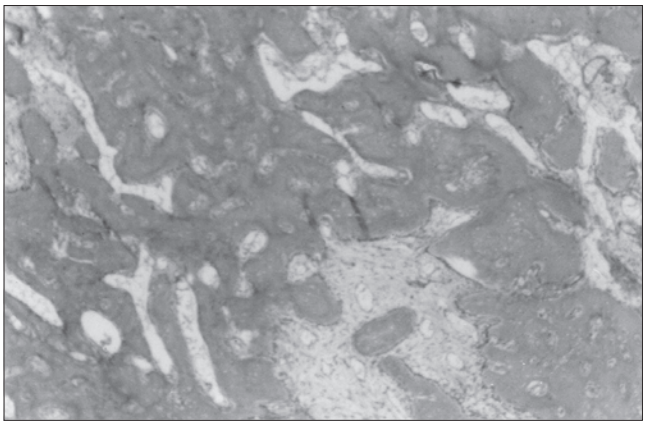

Figure 6. Experimental Group (Twenty-one days) - Dense lamellar bone observed into the socket (HE-100x).

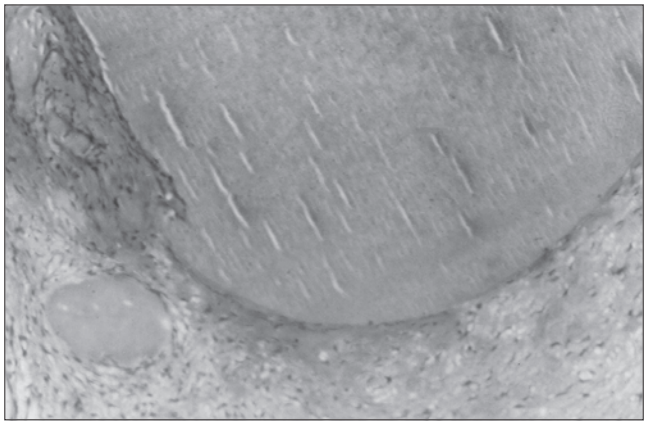

Figure 7. Experimental Group (Twenty-one days) - Remanescents of the implanted material with no inflammatory reaction (HE - 200x).

Table 1. Seven days. Mean score and standard deviation of the microscopical parameters.

Microscopical parameters	Groups		*p*value
	Control	Experimental	
Osteoid	0.0 ±0.0	1.9 ±0.6	0.000380*
Immature bone	0.0 ±0.0	1.9 ±0.6	0.000380*
Mature bone	0.0 ±0.0	0.0 ±0.0	1.000000
Osteocytes	0.0 ±0.0	1.7 ±0.5	0.000380*
Osteoblasts	0.0 ±0.0	1.8 ±0.4	0.000380*

*Statistically significant

Twenty-one days - Control group

Almost all the socket was filled with immature bone but variable amount of mature bone could be seen. Significant number of active osteoblasts was found. At the cervical area of the socket the presence of fibrous connective tissue was observed with poor chronic inflammatory infiltrate and rare newly formed blood vessels.

Twenty-one days - Experimental group

The socket was totally filled with mature and immature bone. In some specimens, dense lamellar bone was present with few osteoblasts in the periphery and osteocytes lacunae. It was possible to observe the implanted material predominantly in the apical region of the socket. At these areas there was no bone tissue present, being the material encapsulated by fibrous connective tissue, with the presence of mild mononuclear inflammatory infiltrate.

Statistical results

The mean scores and standard deviation values of the 5 histologic parameters evaluated for alveolar osseous healing in the control and experimental groups on each observation time point (7, 14 and 21 days) are presented in Tables 1, 2 and 3, as well as the statistical differences.

Discussion

The alveolar healing process in rats is well documented by several studies (15, 16,17). Furthermore, this experimental animal model has been used successfully

Table 2. Fourteen days. Mean score and standard deviation of the microscopical parameters.

Microscopical parameters	Groups Control	Experimental	*p*value
Osteoid	1.8 ±0.8	2.0 ±0.9	0.496297
Immature bone	1.9 ±0.8	2.7 ±0.5	0.049375*
Mature bone	0.1 ±0.0	1.2 ±1.1	0.017263*
Osteocytes	1.6 ±0.7	2.8 ±0.4	0.002499*
Osteoblasts	1.7 ±0.7	2.7 ±0.7	0.009113*

*Statistically significant

Table 3. Twenty-one days. Mean score and standard deviation of the microscopical parameters.

Microscopical parameters	Control	Groups Experimental	*p*value
Osteoid	1.5 ±0.5	1.1 ±0.6	0.293629
Immature bone	2.5 ±0.5	2.6 ±0.5	0.674427
Mature bone	1.0 ±0.7	2.7 ±0.4	0.001631*
Osteocytes	2.3 ±0.5	2.7 ±0.4	0.207588
Osteoblasts	2.6 ±0.7	3.0 ±0.0	0.400820

*Statistically significant

in other studies that investigated de effect of local factors on the healing of rat's dental alveolus (6,18).

The morphological events are basically the same occurring in human dental alveolus healing process, differing basically in the timing of their occurrence. The first step is the proliferation phase, when the blood clot is invaded by fibroblasts originated by mitosis of pre-existent fibroblasts and adventice cell differentiation, both present at the periodontal ligament attached to the alveolar wall. After the substitution of the clot by connective tissue, gradual substitution of this tissue by immature bone occurs, being the maturation of the bone the final phase of the process (15,16). Rat alveolar healing process takes about one third of the time of human healing process (21 days for rat dental alveolus repair against 64 days in man) (15). This healing time allows the realization of a complete study in small time period. Furthermore, this animal model has the advantage of being relatively easy to handle and to maintain and has low cost.

BMPs are extra-cellular signaling proteins and members of the transforming growth -2superfamily acting by stimulation of cell proliferation, as well as potentializing or inhibiting the response of most cells to other growth factors (6). Depending on the cellular type, BMPs actions are: inhibition or stimulation of cellular proliferation, extracellular matrix synthesis and bone formation stimulation and chemotactic cell attraction (1). BMPs osteoinductive activity is well documented in the literature. This activity was evaluated by *in vitro* and *in vivo* animal and clinical human studies, specially in long bones and craniofacial region (2,10,11,12,14,17,18,19).

There are few studies on the use of BMPs in dental alveolus. Cochran *et al.* (20) reached good results with the implantation of rhBMP-2 in post-extraction alveolus previously to implant placement in humans. However, it must be emphasized that the size sample was small and no control group was used in the study to compare the healing and osseointegration of implants in areas that did not receive BMPs. Reddi *et al.* (2) also used bovine BMPs in a single case report in which a trephine biopsy revealed better repair in the alveolus treated with the BMPs when compared to other alveolus that not received the proteins. In the present study, the analysis of the 5 parameters chosen as indicative of osteoinduction showed statistically significant differences between the studied groups in the observed intervals. At 7 days period, it was possible to observe well organized bone trabeculate in the experimental group, which could not be found in the control group. The quantity of osteoid, immature bone, osteocytes and osteoblasts were significantly higher in the experimental group. Giving support to these data, our histological findings of control group are according to other studies that evaluated the normal healing process of rat alveolus (15,16). Taken together, our findings are indicatives of acceleration of the healing process due to the implanted BMPs in the experimental group.

In the 14 days period, the microscopic features of control group are compatible with the normal healing process of dental alveolus in rats at this time period. In the experimental group, more organized bone tissue was observed and the mature bone quantity was significantly higher in the experimental group showing that the implanted material was still promoting acceleration of bone repair.

At 21 days, no statistical differences between control and experimental groups were observed in the parameters related to osteoid, immature bone and osteocytes. This may be explained because at 21 days, the normal alveolar repair process is almost finished, with near total filling of the socket by bone, as described previously (15,16). However, presence of mature bone was significantly higher in the experimental group clearly showing that the healing process was in a more advanced stage.

Some comments are to be made regarding the choice of the BMPs used in this experiment. Because the concerns regarding the antigenicity and possible transmission of diseases by the bovine derived BMPs,

several studies suggest that the use of recombinant human forms is preferable. However, its synthesis demands complex technology and high financial costs (5,11,21). The purified bovine BMPs have well documented osteoinductive activity even more potent than the recombinant human form (9). This osteoinductive activity is not species specific (6). The technology to obtain purified bovine BMPs is easier and cheaper than the correspondent recombinant form. Chemical processing, sterilization and the size of the proteins reduces its antigenicity and the risks of transmission of diseases (13).

The use of an appropriate material acting as a carrier to promote gradual release of the BMPs into the implantation site is a crucial point since these proteins are readily solubilized and eliminated from the tissues after implantation (4,22,23,24,25,26). Many synthetic and natural materials have been used with this aim, including demineralized bone matrix, collagen and various synthetic polymers. Type I collagen is the main component of bone organic matrix and also the most studied carrier material both *in vitro* as well as *in vivo*. Although of xenogenic origin, purification processing of type I collagen and its associated low molecular weight lessens its antigenicity. Type I collagen is also readily resorbed by the organism. The release of BMPs by the collagen carrier is very slow, with a half-life time of 3 to 5 days (4,13). In our work, Type I bovine collagen presented as granules was used. When mixed to BMPs with saline, an easy to handle paste was obtained which was easily inserted into the socket. The carrier was efficient in releasing BMPs at the implantation site with minimal inflammatory response in the examined specimens. Although in small quantities, the presence of the material at later observation times may indicate a delay in the carrier's resorption which can be unfavorable to the later stages of bone healing process. The material was encapsulated by a fibrous connective tissue with no evidence of foreign reaction. Other studies should be performed to observe if in later periods of healing the material could be resorbed and replaced by bone.

Conclusion

Under the conditions of this experiment, our results clearly showed that the purified bovine BMPs, using type I collagen as a carrier, presented osteoinductive activity when implanted into the dental alveolus of rats after incisor tooth extraction, accelerating the osseous healing time. These findings are encouraging regarding the future clinical use of this BMPs into alveolus after extraction, accelerating healing period and allowing the placement of osseointegrated implants in a small period of time.

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