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Histological reaction of the subcutaneous tissue of rats following implantation of bone morphogenetic protein (rhBMP-2) in inorganic bone scaffold and stimulation using low-power laser light

Reação histológica do tecido subcutâneo de rato após implante de proteína morfogenética óssea (rhBMP-2) em arcabouço de osso inorgânico e estimulado com luz laser de baixa potência

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Abstract

Objective: The aim of this study was to assess the histological reaction of the subcutaneous tissue of rats after the implantation of natural inorganic mineral scaffold from a calf femur containing recombinant human bone morphogenetic protein (rhBMP-2) and irradiated with low-power laser light. **Material and methods**: Sixteen Wistar rats were incised in the torso in a medial-longitudinal orientation, and the subcutaneous tissue of the left and right sides of the incision was pulled apart for implantation of the inorganic bone scaffold containing rhBMP-2. Diode laser light was applied to the right side implant at a dose of 8 J/cm2 for 3

minutes, forming two groups: G1 (control) and G2 (irradiated with laser). Implants and surrounding tissue were removed from four animals on days 7, 21, 40 and 112 for microscopic study. The histological results were assessed by means of grading (0 = absence, 1 = slight presence, 2 = representative and 3 = very representative), considering the following events: formation of osteoid structure, acute inflammation, chronic inflammation, fibrin deposition, neovascularization, foreign body granuloma and fibrosis. **Results**: The results showed no statistically significant differences in each of the four periods when the two groups were compared (p>0.05 – Mann-Whitney's test). **Conclusion**: The natural inorganic scaffold from a calf femur with rhBMP-2 was a biocompatible combination. Under these conditions, the inductive capacity of rhBMP-2 for cell differentiation was inhibited. A slight hastening of tissue healing was shown in the group that was irradiated with low-power laser light.

Keywords: Inorganic bovine bone scaffold. rhBMP-2. Laser light.

Resumo

Objetivo*: O objetivo deste estudo foi avaliar a reação histológica do tecido subcutâneo de rato após o implante de matriz de osso inorgânico mineral natural de fêmur de vitelo com proteína morfogenética do osso recombinante humana (rhBMP-2) e irradiado com luz laser de baixa potência.* **Material e métodos***: Dezesseis ratos (Wistar) foram incisados no dorso no sentido medio-longitudinal. O tecido subcutâneo do lado direito e esquerdo da incisão foram divulsionados para o implante da matriz de osso inorgânico com rhBMP-2. Na direção do implante do lado direito foi aplicada luz laser, diodo em dose única de 8 J/cm2, por 3 minutos, formando dois grupos: G1 (controle) e G2 (irradiado com laser). Foram removidos implantes com o tecido circundante de quatro animais nos períodos de 7, 21, 40 e 112 dias para estudo microscópico. Os resultados histológicos foram avaliados através de postos (0 = ausência, 1 = discreta presença, 2 = representativo e 3 = muito representativo), considerando os seguintes eventos: formação de estrutura osteoide, inflamação aguda, inflamação crônica, depósito de fibrina, neovacularização, granuloma de corpo estranho e fibrose.* **Resultados***: Os resultados não mostraram diferenças estatísticas significativas nos eventos em cada um dos períodos quando comparados os dois grupos (p>0,05 – teste Mann-Whitney).* **Conclusão***: A matriz de osso inorgânico natural de fêmur de vitelo com rhBMP-2 é um conjunto biocompatível. Nestas condições, a capacidade indutora de diferenciação celular da rhBMP-2 foi inibida. Ficou evidenciado discreto aceleramento na cicatrização tecidual no grupo que foi irradiado com luz laser de baixa potência.*

Palavras-chave*: Osso bovino inorgânico. rhBMP-2. Luz laser.*

Introduction

Dentistry is increasingly utilizing tissue engineering in many of its fields, with the aim of replacing damaged tissues or organs with others that are truly biological and functional. Three strategies are utilized for these purposes: conduction, cell and/or tissue transplantation and induction of cell neoformation (1). These three tissue engineering methods have one feature in common: they are always associated with a scaffold (mesh, carrier, messenger or vehicle) consisting of biological or polymeric materials (collagen, organic or inorganic bone, polylactic and/or polyglycolytic acid etc.). In the conduction method, the scaffold is normally a membrane that impedes negative actions by specific cells in the regenerative process. In the cell transplantation method, the vehicle not only transports the cells or tissue but also serves as a guide for the growth of new tissue. In the induction method, the scaffold is utilized for transporting and sustaining inductive proteins. There are studies in the literature reporting bone inducing and biocompatibility properties of bone morphogenetic proteins (BMPs) and their different possible carriers: matrices that can often be utilized as grafts in bone defects (2-5).

The desirable characteristics for a bone replacement material are: biocompatibility, predictability and clinical applicability without transoperative risks and minimal postoperative sequelae, in addition to acceptance by the patient (6). Even though

no materials that fulfill all these requirements are yet available, there is a great variety of choices for grafts and many of these serve as carriers for osteogenic proteins. Examples of these include: materials for mineralized or demineralized lyophilized allogenic grafts, coating deproteinized bovine bone with a layer of calcium phosphate, beta-tricalcium phosphate (ß-TCP), adipose stromal cells, monoolein gel and synthetic materials such as bioactive glass, ceramic, polymers, synthetic hydroxyapatite and carbon nanotubes (2,3,7-12). The xenogenous graft materials are one of the most promising organic materials, considering that autogenous materials have limited indication and homogenous materials have implications regarding bioethics and difficulty in obtaining such material (4).

Low-power laser has been recommended because of its biomodeling, anti-inflammatory and analgesic potential. Moreover, it has biostimulating action in tissues, increasing the mitochondrial ATP, which raises cell metabolism, fibroblast proliferation, fibronectin production (an adhesive protein involved in the process of cell growth and differentiation) and collagen and elastic fiber expression before and during the healing process (5,13-15). A study on rats demonstrated that irradiation using a low-power gallium aluminum arsenide laser inhibits vascular permeability and edema during the acute phase of inflammation and inhibits granuloma formation in the chronic phase. Laser radiation has indirect action at the microcirculation level. The laser acts on the pre-capillary sphincter by means of chemical mediators, provoking persistent dilatation and increased trophism and cell mitosis velocity. This stimulates vessel and nerve neoformation from existing structures, thus hastening the tissue repair and healing process (16).

Transportation and sustaining of the bone induction factors can be carried out using a scaffold of deproteinized bovine cortical or medullar bone, which has a morphological structure that is similar to human bone, a similar chemical composition and sufficient porosity for accommodating proteins. The present study had the aim of assess the histological response of the subcutaneous conjunctive tissue of rats following the implantation of recombinant human bone morphogenetic protein (rhBMP-2) in a scaffold of natural denatured bone mineral extracted from a calf femur, with stimulation using low-power laser light.

Materials and methods

Sixteen male Wistar rats of mean initial weight between 300 and 350 grams were utilized. These were supplied by the vivarium of the School of Veterinary Science of the Metropolitan University of Santos. The rats were placed in individual cages with water and food ad libitum. All the procedures undertaken were in accordance with the standards of the Ethics Committee of the Dentistry School of UNIMES. After a 12-hour fast, the animals were anesthetized using an association of 2% xylazine hydrochloride and 5% ketamine in 1:1 proportion, in a volume of 0.2 ml for every 100 g of weight, via intramuscular route. Trichotomy and antisepsis using iodide alcohol were performed on the dorsum. The rats were positioned in ventral decubitus in order to make a medial-longitudinal dorsal incision and pull apart the subcutaneous tissue on both left and right sides of the incision. At these sites, an implant of recombinant human Bone Morphogenetic Protein-2 (rhBMP-2 produced in E. coli at a concentration of 1 mg/ml) in an natural inorganic bone mineral scaffold from a calf femur was made, measuring approximately 3x3x1 mm, which was developed by Bionnovation Produtos Biomédicos S/A, Bauru, São Paulo, Brasil. The margins of the incision were coapted and sutured using monofilament no. 3-0 with nylon thread.

Diode laser light was applied in the direction of the right side implant of rhBMP-2 in natural inorganic bone mineral scaffold. A single dosage of 8 J/cm2 at the wavelength of 670 nm (visible red spectrum) was utilized, produced by the semiconductor laser source KC 610 V.R. (Kroman Indústria e Comércio Ltda., Ribeirão Pires, São Paulo, Brasil). Thus, two groups were formed: a group stimulated by laser (right side) and a control group (left side).

Four animals were again anesthetized and underwent incision to remove specimens containing the implant and a segment of the tissue surrounding this material (fibrous-adipose and muscle tissue) 7, 21, 40 and 112 days after the implantation. These specimens were immediately fixed in 10% buffered formalin, examined macroscopically via sectioning to expose the lesion and processed using the routine technique for embedding in paraffin. A rotating microtome was utilized to obtain histological sections with a 5 µm thickness, and these were stained using the hematoxylin-eosin technique for

histopathological assessment. For each variation factor (osteoid scaffold, acute inflammation, chronic inflammation, fibrin deposition, neovascularization, foreign-body granuloma and fibrosis), comparisons between the times at which the specimens were obtained were made. The variation factors were quantified using scores as follows: 0 = absence, 1 = slight presence, 2 = representative and 3 = very representative.

Results

After reading the slides in the two groups at the different analysis times, Table 1 was made.

The results did not show any statistically significant differences in the events at each of the analysis times between the two groups ($p>0.05$; Mann-Whitney's test),

Table 1 it can be seen through the analysis of structures. The evolution of the process of tissue repair in both groups showed no statistically significant difference between them. The acute inflammatory responses that were particularly observed over the initial periods were compatible with a reaction resulting from aggression to the tissue from the surgical procedure. Acute inflammatory infiltration, which was mainly shown by the presence of neutrophils and fibrins, was observed in the samples collected after seven days, and there was less inflammatory reaction in the group irradiated with laser light (Figures 1 and 2). Over time, there was a decrease in the quantity of exudate because of tissue regeneration. This regeneration was characterized by neovascularization, fibroblast proliferation and osteoclastic activity for reabsorption of the bone lamellae, which was shown in the samples removed from the irradiated group after 21 days (Figure 3)

Table 1 - Graded quantification of the variation factors for the groups at the different analysis times of the experiment

Subcutaneous Issue	Without Laser				With Laser			
Number of days / Event	7	21	40	112	7	21	40	112
Matrix osteoid	1	0	0	0	2	0	0	0
Acute inflammation	3	1	2	0	2	1	2	0
Chronic inflammation	2	2	2	2	2	2	3	2
Fibrin deposition	2	1	1	0	2	1	1	0
Neovascularization	3	3	3	2	3	3	2	2
Foreign body granuloma	2	2	2	2	2	2	2	2
Fibrosis	3	3	3	2	2	3	3	3

Discussion

This study was conducted on laboratory animals, with the utilization of subcutaneous tissue from Wistar rats, into which rhBMP-2 in a natural inorganic bone mineral scaffold from a calf femur was implanted. For one group, the region of the implant was irradiated with low-power laser light with the aim of observing whether there would be any difference in the quantitative dynamics of any of the structural elements involved in the tissue repair, by means of optical microscopy.

and 40 days. In these, the angiogenic activity was lower and the bone lamellae diminished and were separated by fibrotic tissue, thus demonstrating a more advanced stage of repair (Figures 4 and 5). In the samples removed after 112 days, it could be seen that a large proportion of the implant material had been reabsorbed and was being replaced by the normal subcutaneous conjunctive tissue that is characteristic of this area, with minimal collagen deposition for both groups (Figure 6).

An observation made that should be registered was that in some of the samples collected on the

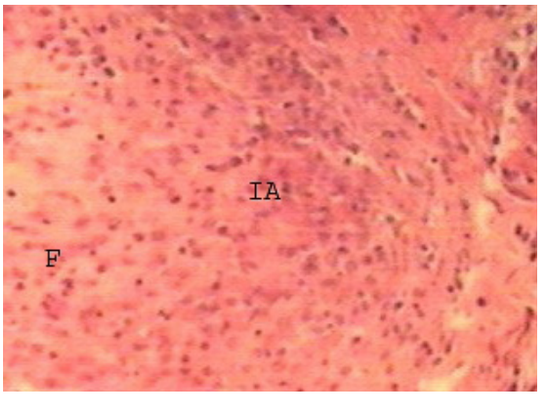

Figure 1 - 7th day; with laser; 100x – Less intense acute infiltrate (AI), fibrins deposition (FD)

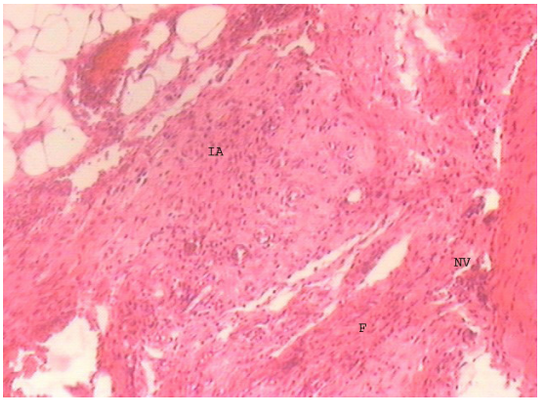

Figure 2 - 7th day; 100x – Acute infiltrate (AI), neovascularization (NV), fibrins deposition FD)

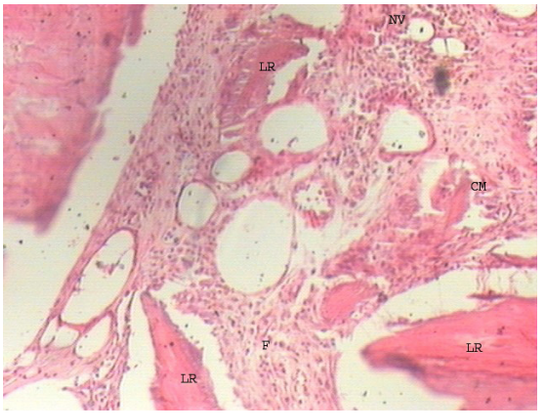

Figure 3 - 21st day – Presence of fibrosis (F), neovascularization (NV), bone lamellae undergoing reabsorption (LR), multinucleated cells with osteoclastic activity (MC)

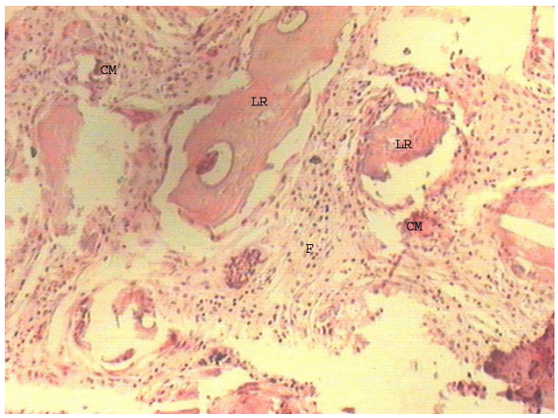

Figure 4 - 40th day; with laser; 100x – Repair fibrosis (RF), bone lamellae undergoing reabsorption (LR), multinucleated cells (MC).

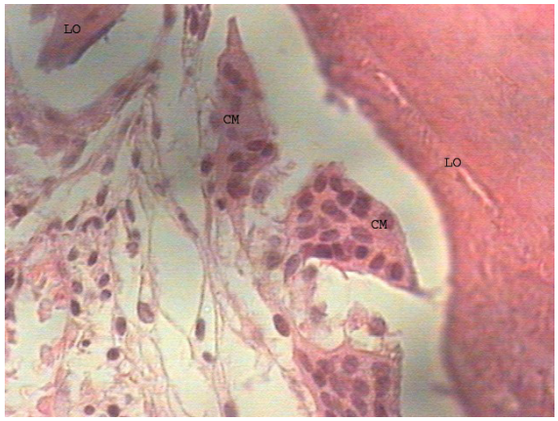

Figure 5 - 40th day; with laser; 400x – Note bone lamella (OL) undergoing reabsorption by multinucleated cell (MC)

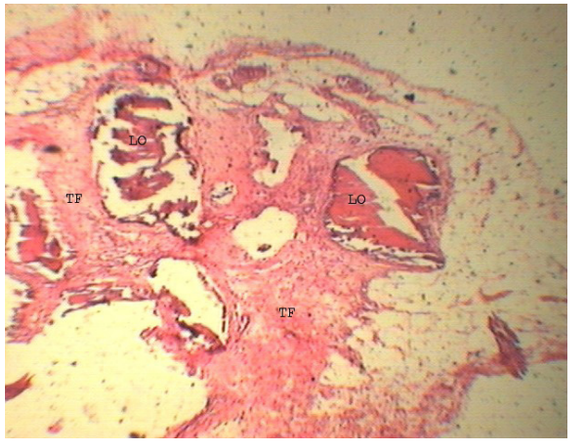

Figure 6 - 112th day; 100x – Tenuous bone lamellae (OL), fibrotic repair tissue (FT)

seventh day the neoformed fibrous tissue presented features slightly suggestive of osteoid matrix. This was more consistent and evident in the samples that had been irradiated with laser light (Figure 7). However, in the sections from the material removed on the 21st day, these deposits were not presented. It may be possible to deduce from this that the rhBMP-2 that was sustained by the scaffold had begun the process of inducing the differentiation of some cells into osteoblasts, which had started to produce osteoid scaffold and then had been inactivated by some biological signal. This inference may be supported by the affirmation that the presence of mineralized bone matrix in subcutaneous tissue inhibits the inductive capacity of the osteogenic proteins (17). Osteogenesis is inhibited in the presence of mineralized scaffold because of the presence of giant cells (similar to osteoclasts) at the location where the mineralized scaffold is implanted, which are rich in organic acids and probably related to mineral dissolution (18). It is possible that the mineral ions present in the structure of the mineralized scaffold are responsible for activating the giant cells, which are osteogenesis inhibitors. Another possibility is that these cells have an osteoclastic function in their bone reabsorption activity.

At the later observation times of the experiment (Figures 3, 4 and 6), it could be seen that a proportion of the bone scaffold was being reabsorbed and that the number of giant cells had increased. Sometimes these were seen to form foreign body granulomas, and these results corroborate researches that emphasize the presence of the absorption of lyophilized bovine bone when implanted in rats (19,20).

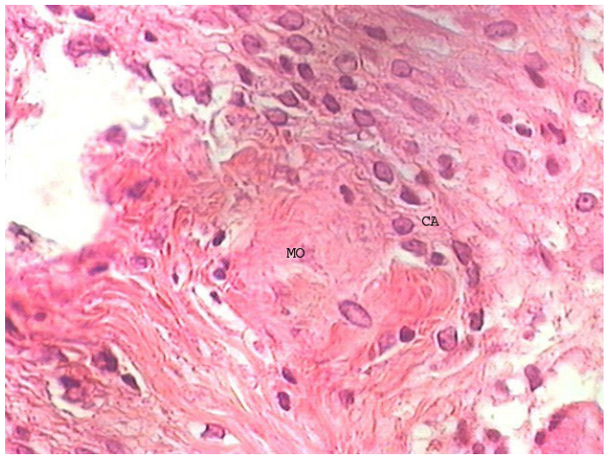

Figure 7 - 7th day; with laser; 400x – Central region with amorphous eosinophilic material surrounded by aligned cells (AC), suggesting osteoid matrix (OM)

Considering that the advances in the field of biotherapy are coming principally through the utilization of tissue engineering, the search for materials that are ideal for every tissue repair situation has been intensifying, especially with regarding bone replacement. There is no doubt that researches need to be conducted with the objective of clarifying the function and mechanism of action of each component involved (growth transformation factors, bone morphogenetic proteins, matrices etc.) in bone induction and formation.

Conclusions

In accordance with the methodology utilized and the results obtained, it could be concluded that the natural inorganic scaffold from calf femur with rhBMP-2 was a biocompatible combination, considering that it did not cause tissue necrosis or the formation of microabscesses. Under these conditions, the inductive capacity of rhBMP-2 for cell differentiation was inhibited. A slight hastening of tissue healing was shown in the group that was irradiated with low-power laser light.

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