Histological and radiographic evaluation of the muscle tissue of rats after implantation of bone morphogenic protein (rhBMP-2) in a scaffold of inorganic bone and after stimulation with low-power laser light

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ABSTRACT

Objective: The present study histologically and radiologically evaluates the muscle tissue of rats after implantation of bone morphogenic protein (rhBMP-2) in a natural inorganic bone mineral scaffold from a bull calf femur and irradiation with low-power light laser.

Materials and Methods: The right and left hind limbs of 16 rats were shaved and an incision was made in the muscle on the face corresponding to the median portion of the tibia, into which rhBMP-2 in a scaffold of inorganic bone was implanted. Two groups of limbs were formed: control (G1) and laser irradiation (G2). G2 received diode laser light applied in the direction of the implant, at a dose of 8 J/cm2 for three minutes. On the 7th, 21st, 40th and 112th days after implantation, hind limbs of 4 animals were radiographed and their implants removed together with the surrounding tissue for study under the microscope. The histological results were graded as 0=absence, 1=slight presence, 2=representative and 3=very representative, with regard to the following events: formation of osteoid structure, acute inflammation, chronic inflammation, fibrin deposition, neovascularization, foreign-body granuloma and fibrosis.

Results: There were no statistically significant differences in these events at each evaluation times, between the two groups ($P>0.05$; Mann–Whitney test). Nevertheless, it could be concluded that the natural inorganic bone matrix with rhBMP-2, from the femur of a bull calf, is a biocompatible combination.

Conclusions: Under these conditions, the inductive capacity of rhBMP-2 for cell differentiation was inhibited. There was a slight acceleration in tissue healing in the group that received irradiation with low-power laser light.

Key words: Inorganic bone scaffold, rhBMP-2, laser light

Tissue engineering aims to be replacing damaged tissues or organs with others that are truly biological and functional. Three strategies are used for this purpose: conduction, cell and/or tissue transplantation and induction of cell neoformation.[1] These three tissue engineering methods have one characteristic feature in common: they are always associated with the use of a scaffold (matrix, carrier, messenger or vehicle) of biological or polymeric materials (collagen, organic or inorganic bone, polylactic and/or polyglycolic acid, etc.). In the conduction method, the scaffold is usually a membrane that impedes negative action by specific cells during 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 used to transport and sustain the inductive proteins.

Studies in the literature have reported on the osteoinductive and biocompatibility properties of bone morphogenic proteins (BMPs) and their possible different carriers. Scaffolds of this type may often be used as grafts in repairing bone defects.[2-8]

The desirable characteristics for bone replacement material are that it should be biocompatible, predictable and clinically applicable without transoperative risks and with minimal postoperative sequelae, as well as being acceptable to the patient.[9] Although presently there is no material that fulfills all these requirements, there is a variety of options for grafts, and many of these also serve as carriers for osteogenic proteins. Examples of these include materials for lyophilized mineralized or demineralized allogenous grafts, castor oil polymer (*Ricinus communis*) and synthetic materials such as bioactive glass, polymers and synthetic hydroxyapatite.[5-8,10-13]

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According to Torricelli *et al*,[14] xenogenous grafts are one of the most promising organic materials, considering that autogenous materials have limited indication and the use of homogenous materials implies bioethical questions and difficulty in obtaining the material.

Low-power laser has been recognized for its biomodulatory, anti-inflammatory and analgesic potential, and also for its biostimulatory action on tissues. Biostimulation occurs as result of increase in the mitochondrial ATP, which in turn increases cell metabolism, fibroblast proliferation, fibronectin production (an adhesive protein involved in growth and cell differentiation processes) and the expression of collagenic and elastic fibers before and during the healing process.[15-17] A study by Honmura *et al*,[18] using rats demonstrated that irradiation with low-power gallium aluminum arsenide laser inhibited vascular permeability and edema during the acute phase of inflammation, and also granuloma formation during the chronic phase.

Laser radiation has indirect action at the microcirculation level. It acts on the precapillary sphincter with the help of chemical mediators, thereby causing persistent dilatation and increased trophism and speed of cell mitosis. This stimulates the neoformation of vessels and nerves from existing structures, thus accelerating the tissue repair and healing process.

Transportation and sustaining of bone induction factors can be accomplished using a scaffold of cortical bovine bone or deproteinized medulla, which has a morphological structure similar to the human bone structure, similar chemical composition and sufficient porosity to accommodate the proteins.

The present study had the principal aim of evaluating the histological response of muscle tissue in rats after implantation of rhBMP-2 in a natural inorganic bone mineral scaffold from a bull calf femur, with stimulation using low-power laser light.

MATERIALS AND METHODS

Sixteen male Wistar rats were used, with mean initial weight 300-350 g (n=4). They were supplied by the vivarium of the Veterinary School of Universidade Metropolitana de Santos (UNIMES). They were placed in individual cages with water and food available *ad libitum*. All the procedures undertaken were in accordance with the standards of the Ethics Committee of the Dentistry School of UNIMES.

After twelve hours of fasting, the animals were anesthetized with an association of 2% xylazine chlorohydrate and 5% ketamine in the ratio 1:1, in a volume of 0.2 mL per 100 g of body weight, intramuscularly. After shaving the left and right hind limbs of rats, they were positioned in ventral decubitus and antisepsis with iodated alcohol was performed over the area for the surgical procedure. A skin incision was made on the face corresponding to the median portion of the tibia. Next, an incision of approximately 8 mm was made longitudinally and the tissue was pushed aside, thereby opening up a space into which recombinant human bone morphogenic protein (rhBMP-2) in an inorganic scaffold was implanted. The implant measured approximately $3 \times 3 \times 1$ mm, and was developed by Bionnovation Produtos Biomédicos S/A (recombinant human BMP-2, produced in *Escherichia coli* at a concentration of 1 mg/mL). The muscle tissue was rearranged with simple suturing around the implant, and the subcutaneous and cutaneous tissues were closed with simple separate stitches, using No. 3.0 nylon monofilament thread.

Laser light was applied to the right hind limbs, in the direction of the location where the rhBMP-2 in bone scaffold had been implanted, with a single dosage of 8 J/cm^2. The laser was a KC 610 V.R. diode semiconductor of wavelength 670 nm (infrared spectrum) manufactured by Kroman Industria e Comércio Ltda. The left hind limbs thus formed the control group.

The activity of new bone formation was measured by histopathological evaluation of osteoid matrix formation and some others parameters that are present in inflammatory process, for example, acute inflammation, chronic inflammation, fibrin deposition, neovascularization, foreign-body granuloma and fibrosis, were evaluated to observe the biocompatibility of the implant. On the 7th, 21st, 40th and 112th days after implantation, 4 animals were again anesthetized. Before undergoing a new surgical procedure to remove the implants, their hind limb area was radiographed using Kodak film in a Dabi-Atlante X-ray machine using 28 kV, 4 mA for 2 minutes and development in accordance with a standard protocol for radiographic observation of the different groups and times.

Incisions were then made to remove surgical specimens including the implants and a segment of the surrounding fibroadipose and muscle tissue. These samples were fixed in 10% buffered formalin and were processed by means of a routine technique for embedding in paraffin. Sections of thickness 5 μm were cut and stained using the hematoxylin-eosin (HE) technique for evaluation under an optical microscope.

The variation factors were quantified by a calibrated observer using scores as follows: 0=absence, 1=slight presence, 2=representative and 3=very representative. For each variation factor (osteoid matrix, acute inflammation, chronic inflammation, fibrin deposition, neovascularization, foreign-body granuloma and fibrosis) were made statistical comparisons between the times at which the specimens were obtained and groups (Mann–Whitney statistical test).

RESULTS

After reading the slides from the two groups at the different analysis times, we constructed the following Table 1:

Table 1: Quantification of structural elements in the two groups (with and without laser) at each analysis of the experiment, using a grading system

Muscle tissue	Without laser				With laser			
Time (days)/Event	7	21	40	112	7	21	40	112
Osteoid matrix	1	0	0	0	1	1	0	0
Acute inflammation	2	1	0	0	3	1	1	0
Chronic inflammation	1	2	1	1	2	2	1	1
Fibrin deposition	2	0	0	0	2	1	0	0
Neovascularization	2	2	1	1	1	2	1	1
Foreign-body granuloma	1	2	1	1	2	1	1	1
Fibrosis	1	2	2	1	2	3	1	1

There were no statistically significant differences in these events at each evaluation times, between the two groups (P>0.05; Mann–Whitney test).

The radiographic observation of the different groups and times showed reabsorption of the natural inorganic bone mineral scaffold by the organisms of the rats. It can be observed by the increase of radiolucence along the time.

DISCUSSION

This study was carried out on laboratory animals, using the muscle tissue of Wistar rats, into which rhBMP-2 in a natural inorganic bone mineral scaffold from a bull calf femur was implanted. In one group, the region of the implantation was irradiated using low-power laser light, with the aim of observing whether there would be any difference in the quantitative dynamics of any structural elements involved in tissue repair, as seen under an optical microscope.

In Table 1, the evolution of the tissue repair process in the two groups can be seen. The acute inflammatory response, which was particularly observed at an early stage, was compatible with a reaction to tissue aggression caused by the surgical procedure. The acute inflammatory infiltrate was shown mainly by the presence of neutrophils and fibrin deposits, and was observed in the samples collected on the 7th day. In the group with laser light irradiation the inflammatory reaction was milder, with greater presence of foreign-body granuloma [Figures 1 and 2]. Over the course of time, the acute exudates diminished due to tissue regeneration, which was characterized by neovascularization and fibroblastic proliferation, and also by the presence of osteoclastic activity for reabsorption of bone lamellae, as shown in the samples removed on the 21st day [Figures 3 and 4]. In the samples from the group with laser light irradiation on the 40th day, there was less angiogenic activity, with smaller bone lamellae separated by fibrotic tissue, thus demonstrating a more advanced stage of repair [Figure 5]. In the samples removed on the 112th day, it could be seen that a large proportion of the implant material had been reabsorbed and had been replaced by neoformed muscle tissue, in both groups [Figure 6].

In the samples removed on the 40th and 112th days, a large proportion of the material was observed to be reabsorbed. This could be confirmed from the sequence of radiographic images made at different times [Figure 7] and the replacement by normal muscle tissue that was characteristic of the area [Figures 5 and 6].

At the end of the experiment, part of the bone scaffold was observed as being reabsorbed and increased numbers of giant cells were noted, sometimes forming foreign-body granuloma. This result is in agreement with the findings of Spector[19] and Batista and Sant'Ana Filho,[20] who declared that they had discovered a process of lyophilized bovine bone reabsorption following implantation in rats.

One notable finding was that some of the samples collected on the 7th day showed slight but suggestive osteoid matrix in the neoformed fibrous tissue. This was shown more evidently in the samples that had been irradiated with laser light [Figure 8]. However, in the sections from the material removed on the 21st day onward, these deposits were no longer present. It could be deduced that the bone morphogenic proteins (rhBMP-2) that were supported by the scaffold had induced the process of differentiation of some cells into osteoblasts, which in turn began to prepare an osteoid matrix, and afterward had been inactivated by some biological signal. This inference may be supported by the findings of Urist and Strates,[21] who stated that the presence of a mineralized bone matrix in the subcutaneous tissue inhibited the inductive capacity of the osteogenic proteins. Osteogenesis is inhibited in the presence of mineralized matrix or matrix associated with demineralized matrix because of the presence of giant cells (resembling osteoclasts) that are rich in organic acids and are probably related to mineral dissolution at the location where the mineralized matrix has been implanted, as highlighted by Reddi and Huggins.[22] It is possible that the mineral ions present in the structure of the mineralized scaffold are responsible for activating the giant cells that inhibit osteogenesis. Another possibility is that these cells act in bone reabsorption with an osteoclastic function.

Considering the advances in the field of biotherapy, which have mainly been through the use of tissue engineering, the search for materials that are ideal for each tissue repair situation has intensified, especially with regard to bone replacement. Undoubtedly, there is a need for research with the aim of clarifying the function and mechanism of each component involved (growth transformation factors, bone morphogenic proteins, scaffold, etc.) in the induction and formation of bone.

CONCLUSIONS

In accordance with the methodology used, and with the results obtained from implanting denatured bovine bone serving as a scaffold for rhBMP-2 in the subcutaneous tissue of rats,

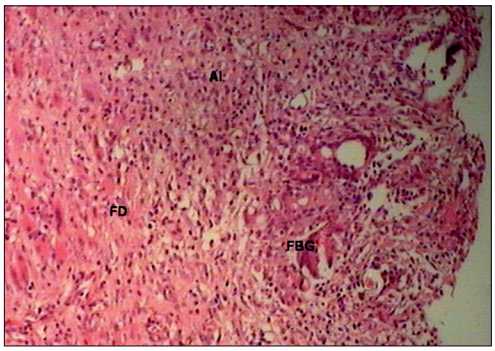

Figure 1: Presence of acute infiltrate (AI), fibrin deposition (FD) and foreign-body granuloma (7th day; laser group; ×100)

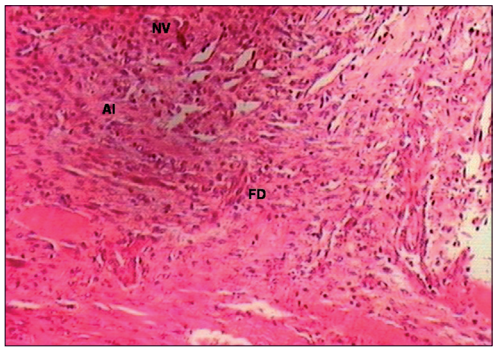

Figure 2: Presence of acute infiltrate (AI), neovascularization (NV) and fibrin deposition (FD) (7th day; non-laser group; ×100)

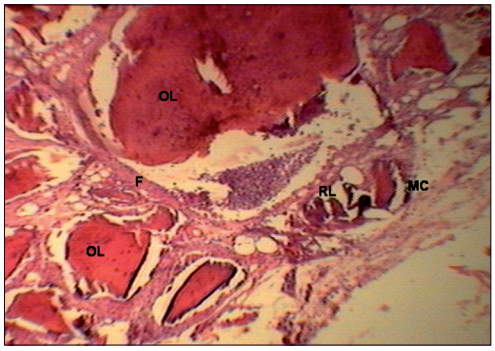

Figure 3: Presence of bone lamella (OL), fibrosis (F), bone lamellae undergoing reabsorption RL) and multinucleated cells with osteoclastic activity (CM) (21st day; laser group; ×100)

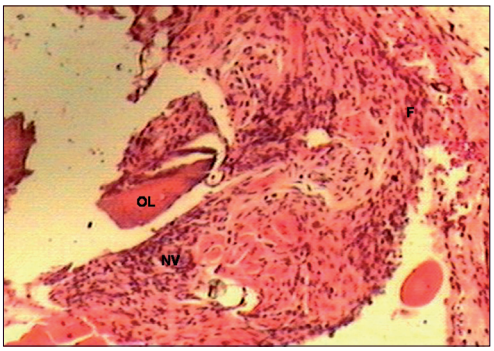

Figure 4: Presence of bone lamellae (OL), fibrosis (F) and neovascularization (NV) (21 day; laser group; ×100)

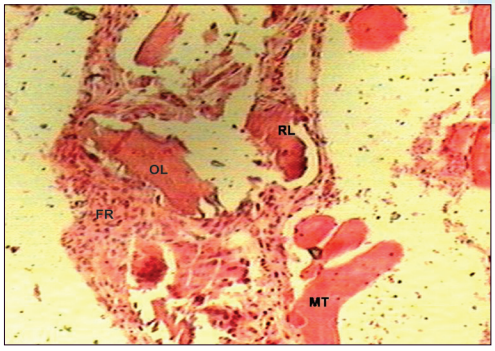

Figure 5: Presence of bone lamellae (OL), repair fibrosis (RF), bone lamellae undergoing reabsorption (RL) and muscle tissue (MT) (40th day; laser group; ×100)

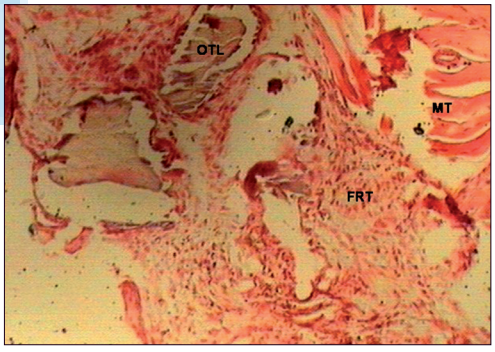

Figure 6: Presence of tenuous bone lamellae (OTL), fibrotic repair tissue (FRT) and muscle tissue (MT) (112th day; non-laser group; ×100)

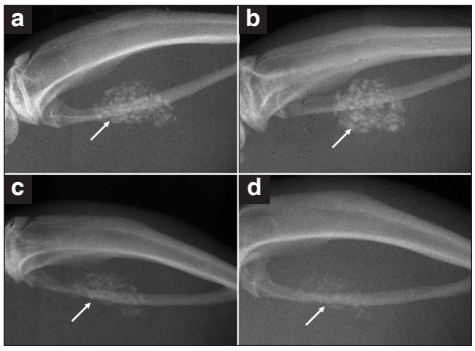

Figure 7: Radiographic images of implants of rbBMP-2 in a framework of inorganic bone: (a) on 7th day, showing start of reabsorption; (b) on 21st day, slight increase in reabsorption; (c) on 40th day, more evident reabsorption; and (d) on 112th day, almost completely reabsorbed

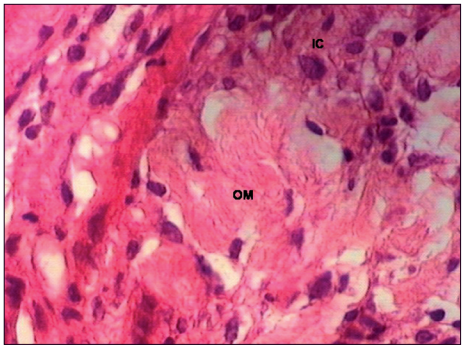

Figure 8: Presence of inflammatory cells (IC) and osteoid matrix (OM) (7th day; laser group; ×400)

and with irradiation of low-power laser light, the following conclusions can we arrived:

- There was a slight acceleration in the repair of rat muscle tissue in the region of the implant, following irradiation with low-power laser light.
- The combination of inorganic bone scaffold and rhBMP-2 is biocompatible, as it did not promote tissue necrosis or microabscess.
- The presence of an inorganic bone scaffold in muscle tissue inhibited the inductive capacity of rhBMP-2 for cell differentiation.
- The combination of inorganic bone scaffold and rhBMP-2 was gradually reabsorbed radiographically and histologically, while not causing alterations to the adjacent structures.

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