

Effect of 650 nm low-power laser on bone morphogenetic protein in bone defects induced in rat femors¹

Efeito do laser de baixa potência 650 nm sobre proteínas morfogenéticas ósseas em defeitos ósseos induzidos em fêmores ratos

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ABSTRACT

Purpose: To investigate the influence of 650 nm GaAlAs laser on the action of bone morphogenetic protein (BMP) in bone defects produced in rat femurs. **Methods:** The sample consisted of 12 male albino Wistar rats (*Rattus norvegicus*). The animals were randomly divided into four experimental groups. After undergoing anesthesia, the fur was removed from the lateral face of the right thigh and surgical dissection was performed to view the femur region. A bone defect was created using a spherical diamond-tipped drill bit. In groups 1 and 2, the defect was filled with a paste of Gen-Tech bone-inducing substance. The animals were treated with GaAlAs laser, at a predetermined dose of joules/cm² for 80 seconds, over an area of 1 cm². Groups 2 and 4 were used as controls. Bone samples were removed to perform histological procedures and morphometric analyses on the 7th, 14th and 21st days after the operation. The results obtained were subjected to statistical analysis using ANOVA variance according to two criteria, with four repetitions, followed by the post hoc t test. The rejection level for the nullity hypothesis was 0.05 or 5% ($\alpha \leq 0.05$). **Results:** In comparisons between G1, G2, G3 and G4, $p = 0.024$ was observed. In statistical comparisons using the t test for paired samples, only G1 vs. G4 presented a statistically significant result ($p = 0.021$). **Conclusion:** The association of low-power laser application and Gen-Tech bone-inducing substance achieved a better result than laser application alone or BMP use alone.

Key words: Laser Therapy, Low-Level . Bone remodeling. Bone morphogenetic protein.

RESUMO

Objetivo: Este trabalho tem como objetivo estudar comparativamente a influência do laser AsGaAl de 650nm sobre a ação das proteínas morfogenéticas ósseas (BMP) em defeitos ósseos produzidas em fêmures de ratos. **Metodologia:** Utilizamos uma amostra composta por 12 ratos machos (*Rattus norvegicus*), de linhagem Wistar albino, mantidos confinados em caixas com temperatura ambiente constante e iluminação adequada. Os animais foram divididos aleatoriamente em 4 grupos experimentais. Após o procedimento anestésico, foi realizada a retirada dos pelos da face lateral da coxa direita, seguida de procedimento cirúrgico para permitir a visualização da região. Foi realizado um defeito ósseo empregando brocas diamantadas do tipo esférica. Nos grupos 1 e 2 o defeito foi preenchido com uma pasta formada por substância osteoindutora Gen - Tech. Os animais foram tratados com o Laser GaAlAs, com dose pré-determinada de 4 joules/cm² e tempo de 80 segundos para uma área de 1cm². Os grupos 2 e 4 foram adotados como grupo-controle. As amostras do osso foram retiradas para realização de procedimento histológico e análise morfométrica nos 7^o, 14^o e 21^o dias de pós-operatório. Os resultados obtidos foram submetidos à análise estatística pela variância ANOVA segundo dois critérios, com quatro repetições. Seguido do *post hoc test* de t teste, com nível de rejeição da hipótese de nulidade de 0,05 ou 5% ($\alpha \leq 0,05$). **Resultados:** Na comparação entre G1, G2, G3 e G4, observou-se: ($P = 0,024$) e durante a comparação estatística através do teste “t” para amostras pareadas apenas o cruzamento entre Grupo 1 vs. o Grupo 4 apresentou, resultado estatisticamente significativo com ($p=0,021$). **Conclusão:** Concluímos que a associação da aplicação do laser de baixa potência e a substância osteoindutora Gen Tech alcançou melhor resultado do que do que a aplicação de laser ou apenas o uso das BMPs.

Descritores: Terapia a Laser de Baixa Potência. Remodelação Óssea. Proteínas Morfogenéticas Ósseas.

Introduction

It has been estimated that 5.6 million bone fractures occur in the United States every year, of which 5% to 10% evolve to delayed consolidation or pseudoarthrosis. The expenditure on fracture treatment has rarely been studied, but it includes surgery, postoperative care, the cost to the patient and time off work¹. The need for bone restoration, especially as a result of consolidation anomalies, often hinders the work of the clinicians and surgeons responsible for patient rehabilitation². Recent studies have contributed towards consolidating the results from the techniques utilized for bone repair. Among these, there are studies in the field of molecular biology, concerning the discovery of new biomaterials and the elucidation of repair components, particularly with regard to the action of growth factors and their capacity to promote the differentiation of immature osteoprogenitor mesenchymal cells into osteocompetent cells. These have greatly contributed towards consolidating the results relating to techniques utilized in bone repairs^{3,4}. Bone morphogenetic proteins (BMPs) are differentiation factors that have the main function of inducing the transformation of undifferentiated mesenchymal cells into chondroblasts and osteoblasts in a dose-dependent manner⁵. The potential clinical uses for these proteins include the replacement of bone grafts in treating delayed joins, joint failure, poor consolidation, arthrodesis and reconstructive orthopedic procedures, among others⁶. The bone-inducing ability of BMPs has already been demonstrated in a large variety of species, with different carriers, including collagen and polymers, at heterotopic and orthotopic sites. Among the functions of the carrier are to delay the rapid dispersion of the BMP from the implantation site and protect against non-specific lysis⁶. Within this context, with the evolution of biotechnology and medical sciences, low-power lasers have become popular. Today, they form a non-invasive complementary alternative for bone tissue restoration. This has been proven through work by several researchers⁷⁻⁹. The purpose of the present study is to investigate the action of low-power laser on BMPs for repairing bone defects.

Methods

Experimental animals

The sample consisted of 12 adult male albino Wistar rats (*Rattus norvegicus albinus*), with body weight ranging from 230 to 350 grams, coming from the vivarium of the University for Development of the State and the Pantanal Region (UNIDERP), Campo Grande, Mato Grosso do Sul. The animals were confined in cages of 0.15 m² in area, and kept under conditions of alternating 12-hour light and dark periods, constant temperature and humidity (by means of air conditioning), minimum noise and “*ad libitum*” solid food and water provision. They remained under observation for a period of two days, prior to their utilization in the experiment. All the experimental procedures were carried out in conformity with the standards of the Brazilian Collage for Animal Experimentation (COBEA).

Experimental groups

The animals were distributed randomly into four experimental groups named as follows:

Group 1 (N = 3): Bone defect filled with bone-inducing substance, with application of low-power laser.

Group 2 (N = 3): Bone defect filled with bone-inducing substance, without application of low-power laser.

Group 3 (N = 3): Bone defect without filling with bone-inducing substance, with application of low-power laser.

Group 4 (N = 3): Bone defect without any treatment (control group).

All the groups were subdivided such that the resultant subgroups were named: Group 1: A7, A14 and A28; Group 2: B7, B14 and B28; Group 3: C7, C14 and C28; Group 4: D7, D14 and D28.

Production of the bone defects

The animals were anesthetized with a mixture of 10% ketamine and 2% xylazine, in the proportions 1:2, at doses of 0.10-0.15 ml per 100 g of body weight, via intraperitoneal route. Following the anesthesia procedure, trichotomy was performed on the lateral face of the right thigh. The animal was then positioned in ventral decubitus, and the front and hind paws were pinned in an abducted position. The location for the incision was then prepared with antisepsis (iodide alcohol). A direct incision for access to the femur was made using a scalpel. Following incision of the fascia lata, the femoral diaphysis was located and a bone defect was created on the cranial face at around 50 mm from the proximal epiphysis. For this, a No. 1016 spherical diamond-tipped drill bit (KG Sorensen) was utilized, coupled to a dental handpiece (Kawo – Kawo do Brasil) that was operated by an implant motor (Easy Implant – Easy Equipment) at 4,000 rpm. During the procedure, there was constant irrigation with physiological serum. The procedure consisted of drilling through the bone cortex until the medullary canal was reached. In groups 1 and 2, the defect was filled with a paste formed by Gen-Tech bone-inducing substance (Genius Baumer biomaterial division S/A, code 9214.1,6, batch 012991). This consisted of an agglutination of organic bone matrix; inorganic bovine bone; bone morphogenetic proteins; hydroxyapatite and collagen in the proportions 10:1; and 9% saline solution for homogenization. For the biomaterial not to undergo dispersion, the defects were covered with biological membrane consisting of Gen-Derm decalcified bovine bone cortex (Baumer biomaterial division S/A, code 980.S, batch 013310). Following these procedures, the incision was sutured in layers using a needle with 0.30 nylon polyamide thread.

Application of low-power laser

A GaAlAs laser made by Laserline® (Inova model) was utilized, with power of 50 mW, beam width of 0.1 cm², and wavelength (λ) of 650 nm. The application was done by means of the transcutaneous point method, with a predetermined dose of 4 joules/cm² and duration of 80 seconds, for an area of 1 cm². Groups II and IV were taken as comparative control groups for the histological analysis.

One animal from each group was sacrificed by means of ethyl ether inhalation on each of the 7th, 14th and 28th days after the operation. Bone samples were then taken and sent for histological procedures and morphometric analysis.

Histological procedures

The specimens for histological evaluation were fixed in 10% formol for 24 hours. Following this, they were decalcified using EDTA and embedded in paraffin blocks. Sections of 5 µm in thickness were cut and slides were prepared containing two sections each. These were stained using hematoxylin-eosin and Masson’s trichrome.

Morphometric analysis

For the morphometric evaluation, the images were digitized and then computer analysis was performed on these images using a specific image processing and analysis program (Image Pro Plus 4.5). To quantify areas that were representative of neoformed bone, five fields were digitized using an Axiolab microscope (Carl Zeiss, 20x objective) coupled to a Sanyo Digital Active BLC image capture camera that was connected to a Pentium IV 1.2 GHz microcomputer equipped with a video board. Prior to the quantification process, all the images were digitized, with standardization of the microscope light intensity and condenser height. The areas of bone neoformation were separated on the image by using the color distribution as a discriminating parameter. For each quantified image, the same color interval was utilized for separating the area to be quantified. The standardized color interval was defined at the start of the experiment. The color bands were adjusted by trial and error until the representative areas of the image had been separated. Subsequently, the same color intervals were utilized to identify the neoformed bone in all the digitized fields. Following this, the area occupied and the quantity of light absorbed by the bone in each of the fields were calculated for each of the fields (Figure 1).

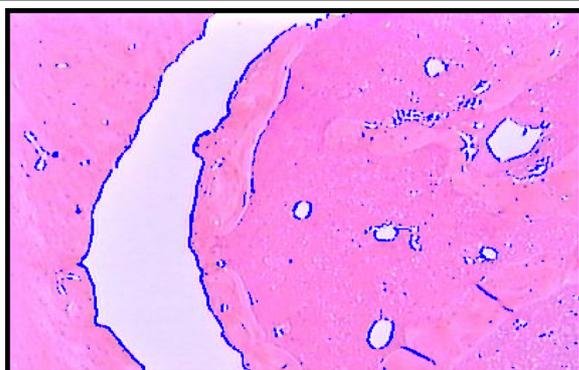


FIGURE 1 - Representation of the histomorphometry process, showing bone neoformation by means of utilizing the Image Pro Plus 4.5 program.

Statistical analysis

The results obtained were subjected to statistical analysis by means of ANOVA variance according to two

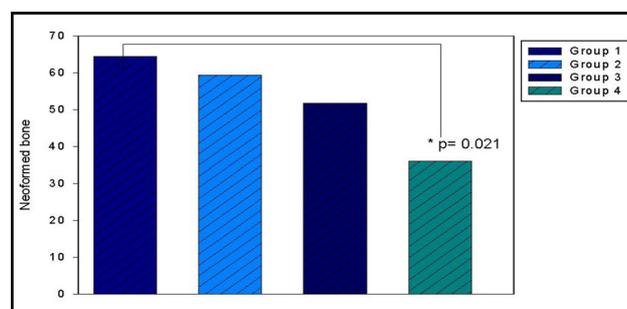
criteria, with four repetitions. This was followed by the post hoc Student t test, considering the nature of the variables studied or the nature of the measurements made. For this, the SigmaStat was utilized, and the rejection level for the nullity hypothesis was set at 0.05 or 5% ($\alpha \leq 0.05$).

Results

The data obtained through histomorphometric analyses were compared by means of statistical correlation between Group 1 (bone defect filled with bone-inducing substance, with application of low-power laser), Group 2 (bone defect filled with bone-inducing substance, without application of low-power laser), Group 3 (bone defect without filling with bone-inducing substance, with application of low-power laser) and Group 4 (bone defect without any treatment - control group), and between the sample removal subgroups (7th, 14th and 28th days after creation of the lesions). From the general means for the neoformed bone tissue in the groups, comparisons were made between G1, G2, G3 and G4 by means of analysis of variance (ANOVA) which gave the result of $p = 0.024$. There was a statistical difference between the groups (Table 1). However, among the statistical comparisons using the t test for paired samples, only the correlation between Groups 1 and 4 presented a statistically significant result ($p = 0.021$). The results from the other correlations were Group 1 vs. Group 3 ($p = 0.341$), Group 1 vs. Group 2 ($p = 0.862$), Group 2 vs. Group 4 ($p = 0.061$), Group 2 vs. Group 3 ($p = 0.744$), Group 3 vs. Group 4 ($p = 0.249$) (Figure 2).

TABLE 1 - Resultant means from the histomorphometric analyses on bone neoformation

Days after creation of lesion	Group 1	Group 2	Group 3	Group 4
7	54,2	50,0	42,4	29,3
14	64,7	59,5	52,7	35,9
28	77,3	68,9	60,0	43,1
Mean	64,4	59,4	51,7	36,1



* $p \leq 0.05$

FIGURE 2 - Graph comparing the four groups and their respective standard deviations relating to mean bone neoformation count.

Histopathological analysis

Seven days after creating the lesion

Histological and morphometric analysis of Group 1 (bone defect filled with bone-inducing substance, with application of low-power laser) showed the presence of fibrous conjunctive tissue with neoformation of vessels and areas of neoformed bone tissue, without the presence of inflammatory reaction. There was a large concentration of osteoblasts. Group 2 (bone defect filled with bone-inducing substance, without application of low-power laser) showed the presence of fibrous conjunctive tissue with neoformation of vessels and some rare areas of neoformed bone tissue, with inflammatory reaction involving the biomaterial, which was dispersed in the inflammatory infiltrate. There was presence of osteoblasts. Group 3 (bone defect without filling with bone-inducing substance, with application of low-power laser) presented a medullary area filled with fibrous material and rare areas of neoformed bone tissue. Group 4 (bone defect without any treatment - control group) presented a lesion cavity area with necrotic tissue, severe inflammatory reaction and connective tissue.

Fourteen days after creating the lesion

Group 1 (bone defect filled with bone-inducing substance, with application of low-power laser) presented neoformed bone trabeculae displaying numerous aligned osteoblasts on the periphery and intense presence of vessel neoformation. Group 2 (bone defect filled with bone-inducing substance, without application of low-power laser)

showed the presence of infill tissue, neoformed bone trabeculae, numerous unaligned osteoblasts, giant cells and an area with inflammatory infiltrate. Group 3 (bone defect without filling with bone-inducing substance, with application of low-power laser) showed the presence of well-vascularized conjunctive tissue that was rich in fibroblasts, neoformed bone trabeculae and numerous osteoblasts. Group 4 (bone defect without any treatment - control group) showed an evident cavity lesion with the presence of neovascularization, inflammatory infiltrate and lower osteoblast concentration than in the other groups.

Twenty-eight days after creating the lesion

Histological and morphometric analysis of Group 1 (bone defect filled with bone-inducing substance, with application of low-power laser) showed the presence of dense bone trabeculae with the appearance of lamellar bone and fragments of the biomaterial surrounded by neoformed bone tissue. Reconstitution of the medullary space was observed. Group 2 (bone defect filled with bone-inducing substance, without application of low-power laser) showed the presence of osteoblasts aligned on the periphery and osteocytes aligned inside the trabeculae. A poorly defined medullary canal of small size was seen. Group 3 (bone defect without filling with bone-inducing substance, with application of low-power laser) presented neoformed bone trabeculae with numerous osteoblasts aligned inside the trabeculae. Group 4 (bone defect without any treatment - control group) showed the presence of a cavity area with proliferation of neoformed bone cells and connective tissue (Figures 3 and 4).

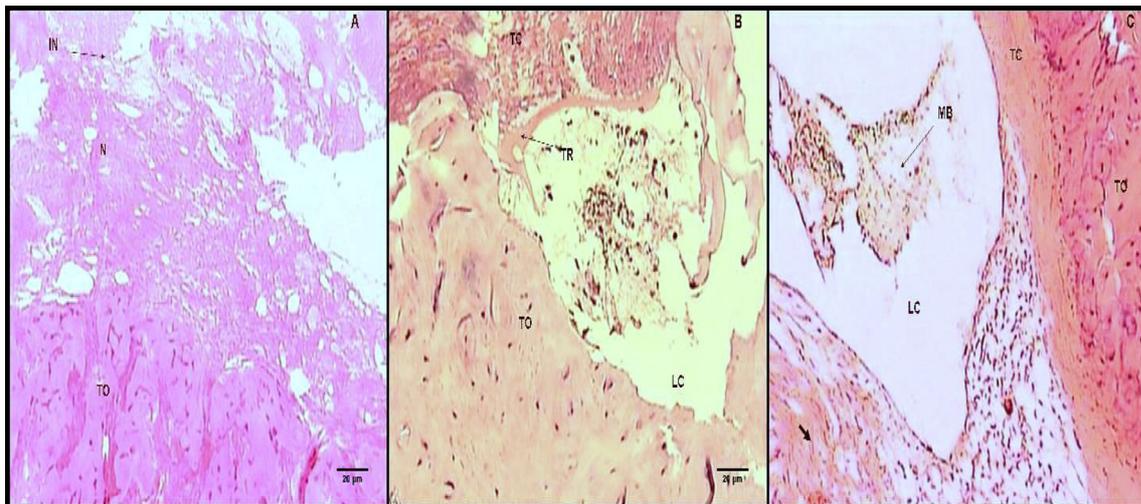


FIGURE 3 - Montage of photomicrographs of bone tissue sections from femurs of animals in Group 4, stained with hematoxylin-eosin, at 100x magnification. In **A**, group irradiated with laser and associated with BMP on the seventh day, with dose of 4 J/cm, showing neoformed bone (TR) and area of tissue necrosis (N). In **B**, group irradiated with laser and associated with BMP on the fourteenth day, showing bone neoformation (TR), cavity lesion (LC), presence of biological material (MB) and inflammatory cells (IN). In **C**, group irradiated with later and associated with BMP on the twenty-eighth day, showing presence of conjunctive tissue (TC), neoformed bone tissue (TO), biological material (MB) and cavity lesion. Scale bar = 20 μ m

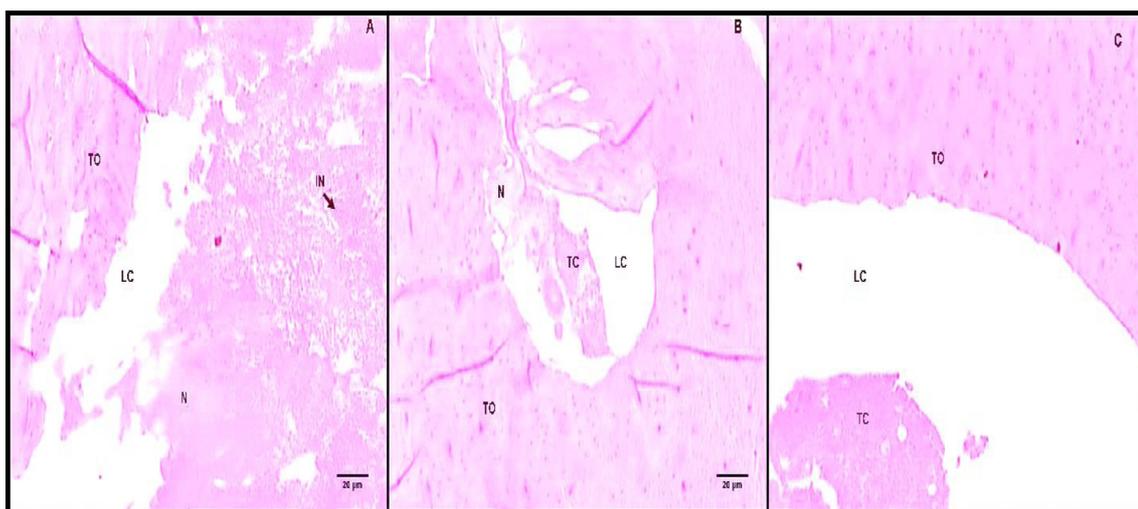


FIGURE 4 - Montage of photomicrographs of bone tissue sections from femurs of animals in Group 1 stained with hematoxylin-eosin, at 100x magnification. In **A**, control group on the seventh day after creating the lesion, showing details of primary bone (TO), area of inflammatory infiltrate (IN), area of tissue necrosis (N) and cavity lesion (LC). In **B**, control group on the fourteenth day after creating the lesion, showing details of primary bone (TO), area of tissue necrosis (N), cavity lesion (LC) and presence of conjunctive tissue. In **C**, control group on the twenty-eighth day after creating the lesion, showing primary bone (TO), area of tissue necrosis (N), cavity lesion (LC) and presence of conjunctive tissue. Scale bar = 20 µm.

Discussion

The proposal to analyze bone repair in rats was based on the vast literature that reports the difficulties in consolidation caused by loss of bone integrity.^{1,2,4-7,9} Studies¹⁰ have revealed that biomaterials (BMPs) promote slow and gradual bone neoformation and that associating these with the use of low-power laser brings forward bone repair because there is stimulation of tissue cells and microcirculation. Thus, the present study was conducted with the objective of investigating and comparing the bone neoformation results from four groups as follows: Group 1 (laser with BMPs); Group 2 (BMPs alone); Group 3 (laser without BMPs); and Group 4 (absence of treatment). In this study, we utilized Wistar rats as the experimental model, considering the positive bone tissue response characteristics in this mammal, which resemble those found in humans, and also the ease of acquiring and handling these animals.^{7,8,10,11} When the animals in this study were analyzed on the seventh day after creating the bone defects, it was observed that Group 1 (treated with laser associated with BMPs) was at a more advanced stage of repair than the other groups studied, presenting an area of vessel neoformation, neoformed bone tissue with a large concentration of osteoblasts and absence of inflammatory reaction. On the other hand, in the other groups the presence of inflammatory reaction and necrosed tissue (Group 4 only) was very evident. These data are similar to the findings from other studies,^{12,13} although the authors of these studies utilized the tibia as the lesion site and evaluated the results after five days. In our study we utilized the femur, collected the first samples after seven days and utilized laser applications. The analysis of the results obtained after 14 days for Group 1 (treated with laser in association with filling in with BMPs) presented neoformed bone trabeculae with osteoblasts aligned on the periphery. In contrast, Group 2 presented unaligned osteoblasts with the

presence of inflammatory infiltrate. Group 4 (control) was at an earlier stage of repair than the other groups, with a cavity lesion, lower osteoblast concentration and more inflammatory infiltrate. Another study¹⁴ presented similar results on the 15th day after creating the lesion, in a group treated with laser and biomaterials (BMP), with the presence of large numbers of osteoblasts, cells, blood capillaries and a bed filled with the biomaterials that was in contact with the conjunctive tissue. In the control group for that study, there was no sign of bone joining or maturation of the collagen fibers. The inflammatory process still persisted, the conjunctive tissue was disorganized and the cells were undergoing a regeneration process. In our results from the samples collected on the 28th day after creating the bone lesion, Group 1 was distinguished by the presence of reconstruction of the medullary canal. Nonetheless, Groups 2 and 3 also presented favorable results, with the presence of bone trabeculae and osteoblasts aligned within these, and the presence of a poorly defined medullary canal of small dimensions can be highlighted in Group 2. These results confirm other studies^{7,8,15-17} in which low-power laser in association with filling the bone defects with BMPs accelerated bone neoformation. Our study also corroborates studies^{4,15} that presented favorable results from grafts using BMPs, in which bone incorporation occurred more rapidly and the repair took place with intense bone neoformation. An investigation using AsGaAl laser (650-680 nm) with a density of 5 J/cm² for a guided bone regeneration process in rabbits¹⁰ showed results similar to those achieved in the present study, given that the histological analysis showed the early formation of immature bone, thereby accelerating cell activity. From analysis of the morphometry results, we observed that Group 1 (bone defect filled with bone-inducing substance, with application of low-power laser) presented a greater general mean for the quantity of neoformed bone that was statistically significant, in relation to Group 4 (control). Comparing Group 1 with Groups 2 and 3,

the analyses demonstrated that there was no significant difference, although the numerical results from Groups 2 and 3 were lower than for Group 1. The same could be observed in the histopathological analysis, and these results are affirmed by similar studies. Another study comparing different biomaterials concluded that, although devitalized bovine bone presented greater inflammatory reaction with the presence of giant cells of foreign body type, formation of cystic cavities containing implant residues and slower pore invasion by bone tissue, in comparison with other implants, this bone material showed osteoconductive properties in bone defect repairs¹⁷. In the present study, the results indicate that there was an inflammatory reaction of lesser intensity and duration in the groups that were subjected to irradiation using low-power laser, and this is corroborated by the results achieved by other studies using laser.¹⁵⁻¹⁶

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