Photomedicine and Laser Surgery
Volume 26, Number 4, 2008

Pp. 371–377
DOI: 10.1089/pho.2007.2172

Does the Use of Laser Photobiomodulation, Bone Morphogenetic Proteins, and Guided Bone Regeneration Improve the Outcome of Autologous Bone Grafts? An *in Vivo* Study in a Rodent Model

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Abstract

Objective: The aim of the present investigation was to histologically assess the effect of laser photobiomodulation (LBPM) on the repair of autologous bone grafts in a rodent model.
Background Data: A major problem in modern dentistry is the recovery of bone defects caused by trauma, surgical procedures, or pathologies. Several types of biomaterials have been used to improve the repair of these defects. These materials are often associated with procedures of guided bone regeneration (GBR).
Materials and Methods: Twenty four animals were divided into four groups: group I (control); group II (LPBM of the bone graft); group III (bone morphogenetic proteins [BMPs] + bone graft); and group IV (LPBM of the bed and the bone graft + BMPs). When appropriate the bed was filled with lyophilized bovine bone and BMPs used with or without GBR. The animals in the irradiated groups received 10 J/cm^2 per session divided over four points around the defect (4 J/cm^2), with the first irradiation immediately after surgery, and then repeated seven times every other day. The animals were humanely killed after 40 d.
Results: The results showed that in all treatment groups, new bone formation was greater and qualitatively better than the untreated subjects. Control specimens showed a less advanced repair after 40 d, and this was characterized by the presence of medullary tissue, a small amount of bone trabeculi, and some cortical repair.
Conclusion: We conclude that LPBM has a positive biomodulatory effect on the healing of bone defects, and that this effect was more evident when LPBM was performed on the surgical bed intraoperatively, prior to the placement of the autologous bone graft.

Introduction

WOUND HEALING is a complex process that involves both local and systemic responses. Usually, bone healing is slower than that observed in soft tissue. Bone tissue has an enormous regenerating capacity, and most of the time it is able to restore its usual architecture and mechanical properties. However, there are limits for this capacity, and complete recovery may not occur if there is deficient blood supply, mechanical instability, or competition with highly proliferating tissues. The loss of bone fragments or the removal of necrotic or pathologic bone, and even some surgical procedures may create bone defects. These defects may be too large for spontaneous and physiologic repair. Bone loss may occur due to trauma, pathology, or a surgical procedure, and modern techniques have employed grafting to replace these losses.[1]

Bone is the most common type of graft used in oral implantology, in prosthetic surgery, in the treatment of congenital defects, and in reconstructive procedures of the jaws.[2,3] Often autologous bone grafts are used, and they may be taken from several parts of the skeleton.[1] Autologous bone grafts are characterized by biocompatibility and osteointegration, as well as substantial osteogenic potential.[1,4–6] The use of bone morphogenetic proteins (BMPs, proteins that belong to the transforming growth factor-β superfamily of proteins) is not new,[7,8] and they have been widely used in reconstruction of the alveolar ridge,[9] for the recovery of bone losses, and on several types of bone defects.[10–17]

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Surgeons are challenged daily by the need to recover bone losses due to several etiologic factors.[1] Several autologus and xenografts have been used to provide a framework or stimulate new bone formation, and many times these grafts respond positively to the use of some wavelengths of EM energy.[1] The use of laser light for the modulation of healing of different bones has resulted in several *in vivo* or *in vitro* studies to find ways to promote a more comfortable postoperative period and quicker healing.[1]

Despite several reports in the literature suggesting the benefits of laser photobiomodulation (LPBM) on soft tissue, its effects on bone are still not completely understood, in part due to conflicting results,[1] and there have been few studies assessing the association of laser energy with the use of biomaterials.[1,13,14,15,17]

A pioneering work by our team histologically assessed the effect of LPBM on the healing of bone defects associated with autologous bone grafting. In that study, laser therapy was applied to the surgical bed, to the graft, and to both the graft and the surgical bed. The dose per session was 10 J/cm^2. LPBM was carried out every other day for 15 days (830 nm, Ø ~ 0.5 cm^2, 50 mW, and 10 J/cm^2). In the groups in which LPBM was used intraoperatively on the surgical bed, bone remodeling was both quantitatively and qualitatively improved compared to the subjects in the other groups, indicating that the use of laser therapy intraoperatively resulted in a positive biomodulating effect on the healing of bone defects associated with autologous bone grafting.[18]

In another study, we histologically assessed the effect of LPBM on the repair of surgical defects treated or not treated with BMPs, organic bovine bone grafts, and guided bone regeneration (GBR) in a rat model. GBR is a procedure in which a barrier (membrane) is placed over a healing bone to avoid migration of soft tissues into the defect. The irradiated groups received seven irradiations every 48 h, with the first immediately after the surgical procedure. LPBM (830 nm, 40 mW, CW, Ø ~ 0.6 mm) consisted of 16 J/cm^2 per session divided over four points (4 J/cm^2) around the defect. The results showed histological evidence of increased deposition of collagen fibers (at 15 and 21 d), as well as an increased amount of well-organized bone trabeculi at the end of the experimental period (30 d) in the irradiated animals compared to the non-irradiated controls. We concluded that the association of laser therapy with BMPs, organic bovine bone grafts, and GBR increases the positive biomodulating effects of laser light.[7]

A previous report investigated the influence of 650-nm laser light on the action of BMPs in bone defects produced in rat femurs. In this study the authors used a mechanically-induced bone defect that was filled or not with a bovine bone compound (an aggregation of organic bone matrix, inorganic bovine bone, BMPs, hydroxyapatite, and collagen), used with or without laser therapy. Those researchers found that the association of low-power laser application and Gen-Tech bone-inducing substance achieved a better result than laser application or BMP use alone.[19]

Our previous studies[1,7,18] have pointed out the need to assess the possible benefits to the bone healing response to autologous bone grafting associated with BMPs and LPBM. This would provide us data to help determine both the most effective parameters for LPBM, and the best use of biomaterials for this new treatment modality.

The aim of the present investigation was to histologically assess the effects of laser photobiomodulation on the repair of autologous bone grafts in a rodent model.

Materials and Methods

Following the approval of the Animal Experimentation Ethics Committee of the School of Dentistry of the Federal University of Bahia, 24 healthy young adult Wistar rats weighting 270–320 g were obtained from the Centro de Criação de animais da Faculdade de Medicina Veterinária da Universidade Federal da Bahia, and were kept at the Animal Experimentation Laboratory of the School of Dentistry of the Federal University of Bahia. The animals were kept in individual plastic cages bedded with wood chips and maintained at 22°C in a day/night light cycle. The animals were fed a standard pelleted laboratory diet and had water *ad libitum*. After regular quarantine the animals were randomly distributed into four groups: group I (bone graft; n = 6); group II (bone graft + LPBM of the bed; n = 6); group III (BMPs on the bed + bone graft; n = 6); and group IV (BMPs on the bed + bone graft + LPBM of the bed; n = 6).

Table 1. Semi-Quantitative Criteria Used for the Light Microscopy Analysis

Score criterion	*Slight*	*Moderate*	*Intense*
Bone reabsorption	Presence of <25% of the reabsorption of the graft remnants and/or the surgical bed	Presence of 25–50% of the reabsorption of the graf remnants and/or the surgical bed	Presence of >50% of the reabsorption of the graft remnants and/or the surgical bed
Bone neoformation	Presence of <25% of the formed bone similar to adjacent untreated bone tissue	Presence of 25–50% of newly formed bone similar to adjacent untreated bone tissue	Presence of >50% of newly formed bone similar to adjacent untreated bone tissue
Inflammatory infiltrate	Presence of <25% of neutrophils in the area	Presence of 25–50% of neutrophils in the area	Presence of >50% of neutrophils in the area
Collage deposition	Presence of <25% of collagen deposition in the area	Presence of 25–50% of collagen deposition in the area	Presence of >50% of collagen deposition in the area

TABLE 2. SUMMARY OF THE LIGHT MICROSCOPIC ANALYSIS

Criterion	*Bone reabsorption*	*Bone neoformation*	*Inflammatory infiltrate*	*Collagen deposition*
Group I	33.3% slight 16.6% moderate	83.3% slight 16.6% moderate	Absent	66.6% slight 33.3% moderate
Group II	33.3% slight	66.6% slight 33.3% moderate	Absent	16.6% slight 66.6% moderate 16.6% intense
Group III	Absent	16.6% slight 66.6% moderate 16.6% intense	Absent	33.3% slight 66.6% moderate 16.6% intense
Group IV	Absent	33.3% moderate 66.6% intense	Absent	66.6% intense 33.3% moderate

Under general anesthesia (Zoletil®, 20 mg/kg), the right femoral area was shaved and cleaned with 2% chlorhexidine. The femur was then surgically exposed, and a cortical graft measuring 0.5 × 0.5 cm was then routinely raised from the lateral surface of the femur using a low-speed drill under constant saline solution irrigation. The graft was kept in saline solution. In all cases the operative time was 9 min. BMPs and/or LPBM were then used according to the treatment group to which the animal belonged. Control subjects had no treatment. The autologous bone graft was then repositioned on the surgical bed, and the soft tissues were routinely sutured.[18]

LPBM was performed with a diode laser. The dose used intraoperatively on the surgical bed was 10 J/cm^2 (790 nm, continuous wave [CW], $\phi \sim 2$ mm, 50 mW in groups II and IV). Laser therapy was also applied at the same dose to the graft surface before repositioning it on the defect in groups II and IV. The animals in the experimental groups received laser treatment every other day for the following 15 d. Treatment consisted of a total session dose of 10 J/cm^2 applied transcutaneously and punctually on four points around the surgical defect (4 × 2.5 J/cm^2), except for the control animals. The total treatment doses were 80 J/cm^2 for group II and 90 J/cm^2 for group IV.

The animals were humanely sacrificed 40 d after surgery by means of an intraperitoneal lethal dose of 10% chloral hydrate. The specimens taken were macroscopically assessed and then kept on 10% formalin solution for 24 h at the Oral Pathology Laboratory of the School of Dentistry of the Federal University of Bahia. The specimens were decalcified (with formic acid and formalin solution), routinely wax embedded, and stained with hematoxylin and eosin and Sirius red. All slides were analyzed by light microscopy by one pathologist who was previously calibrated. Each score was double checked to confirm the consistency of the rating for each specimen. For the analysis, the following parameters were used: bone reabsorption and neoformation, and collagen deposition (Table 1). The results were analyzed by Fischer's exact test (95%; $p = 0.05$).

Results

Macroscopic examination showed that while the incorporation of the graft into the surgical bed was sometimes irregular, mostly it was uniform. Control specimens showed the most irregularities on the graft-bed interface, which had a crater-like aspect. When LBPM was applied to the graft, a complete union of the graft to the bed could be seen, and

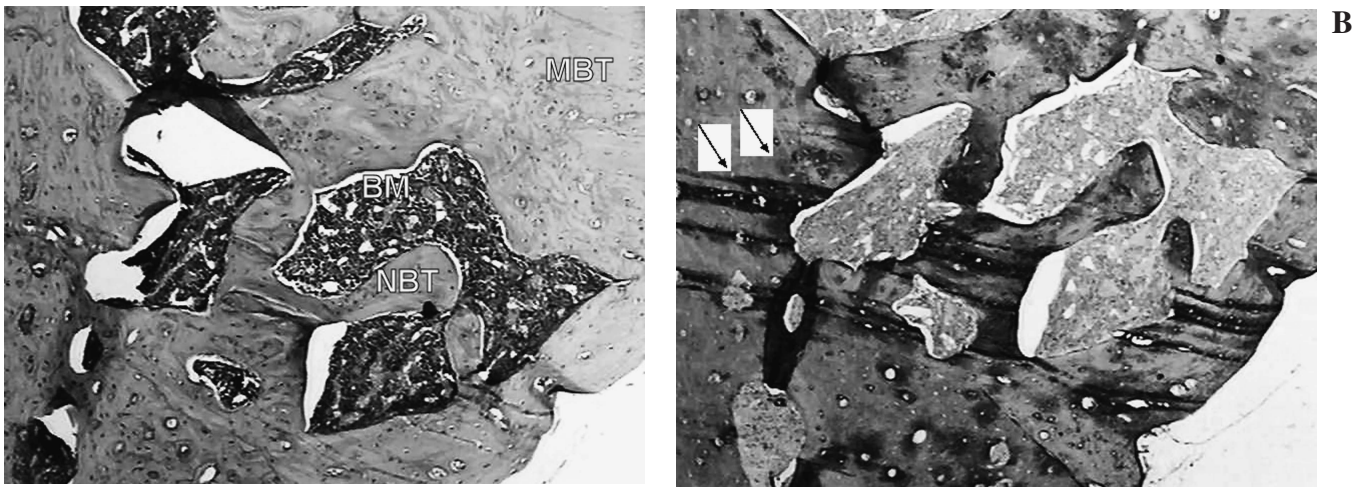

FIG. 1. (**A**) Photomicrograph of a control specimen showing newly-formed bone tissue (NBT) and medullary tissue (BM). Note the integration of the new bone and the surgical bed (approximately 40×; hematoxylin and eosin). (**B**). Sirius red stain showed interconnected mature bone trabeculi with varying amounts of collagen (arrows) (approximately 40×).

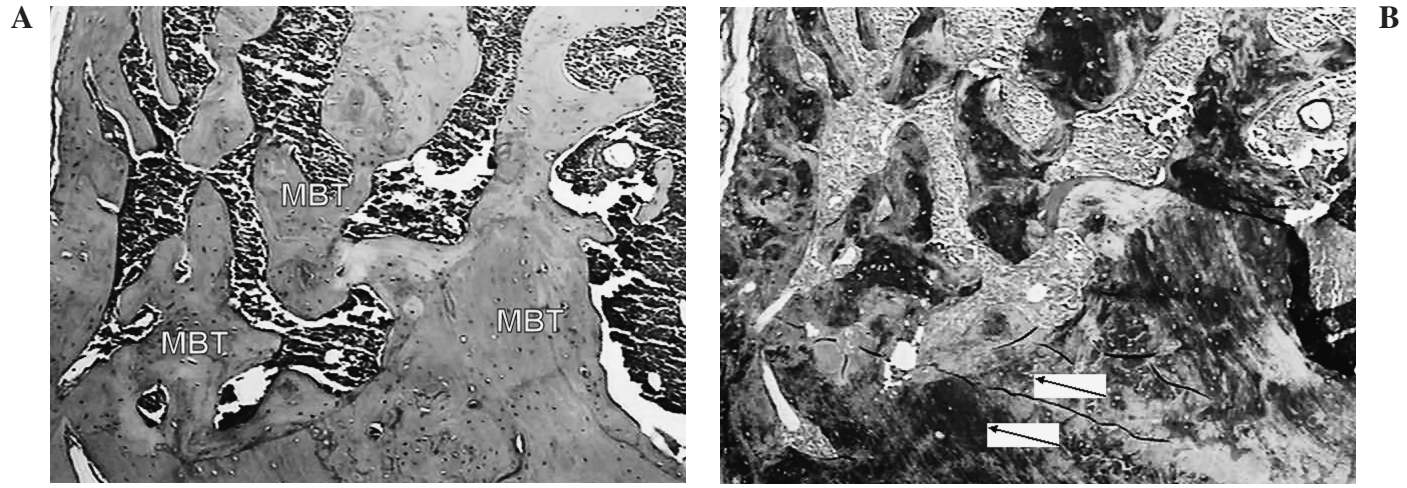

FIG. 2. (**A**) Photomicrograph of a specimen that received LPBM of the bone graft (group II), showing interconnected mature bone trabeculi and medullary tissue. Note the integration between the newly-formed bone and the surgical bed (MBT) (approximately 40×; hematoxylin and eosin). (**B**) The interconnected mature bone trabeculi showed varying levels of mineralization of the collagen matrix(arrows) (approximately 40×; Sirius red).

discrete areas of irregularity of the graft-bed interface could be seen in some specimens. When BMPs were used, complete union of the graft with the bed could be seen, and no irregularities were seen on the graft-bed interface. Using LPBM along with BMPs resulted in a well-defined union between the graft and the surgical bed.

Our results showed that in most cases, the specimens showed the presence of mature connected or unconnected bone trabeculi filling the defect. A basophilic line representing deposition/reabsorption was seen within the newly-formed medullary tissue, and the level of collagenization of the trabeculi varied.

A summary of the results can be seen in Table 2. The results of the light microscopy analysis showed that at the end of the experimental period all the specimens showed mature bone trabeculi that were connected or unconnected, filling the defect. A basophilic line of deposition/reabsorption was also seen among the medullary bone. The bone trabeculi showed a variable level of collagen. Control specimens had bone trabeculi with osteoblasts at their surface, and some remnants of cartilage were also seen. Bone remodeling and the incorporation of the autologous bone graft into the bed were adequate (Fig. 1A and B). When LPBM was used (group II), an increased amount of well-organized bone deposition from the internal cortical plate was seen (Fig. 2A and B). When the BMPs were used (group III) on the surgical bed, remnants of the graft were seen at the center of the bed. No signs of bone reabsorption were seen. Areas of intense fibroblastic activity and bone neoformation from the remnant of the cortical plate were seen at the end of the experimen-

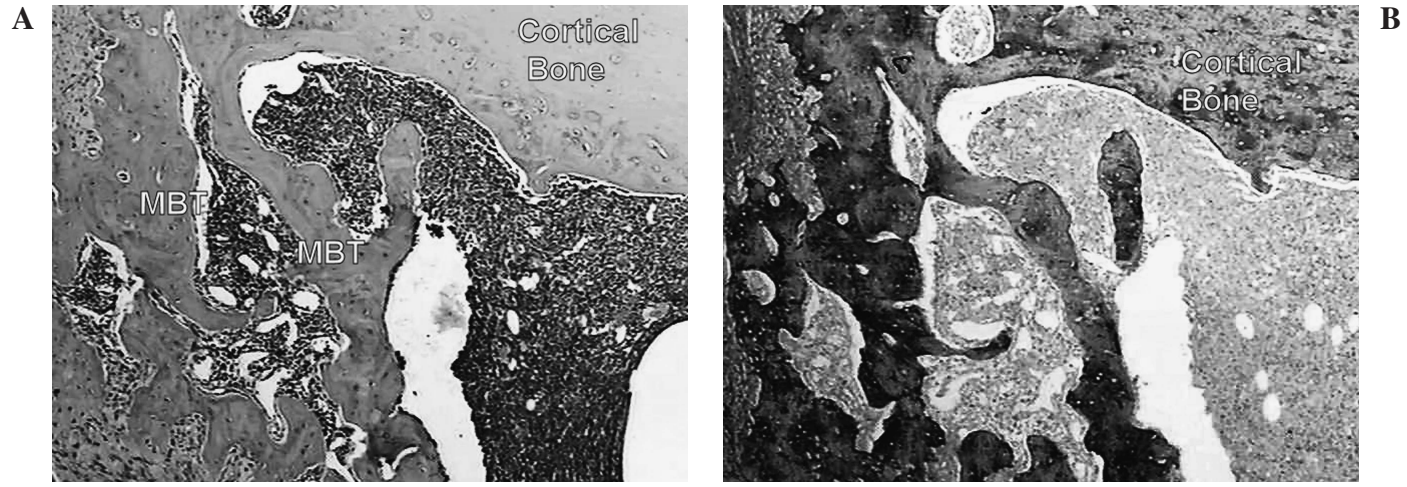

FIG. 3. (**A**) Photomicrograph of a specimen in which BMPs were used between the graft and the bed (group III), showing the presence of osteocytes on the newly-formed bone (MBT) (approximately 40×; hematoxylin and eosin). (**B**) Interconnected bone trabeculi are seen originating from the cortical surface and medullary tissue (approximately 40×; Sirius red).

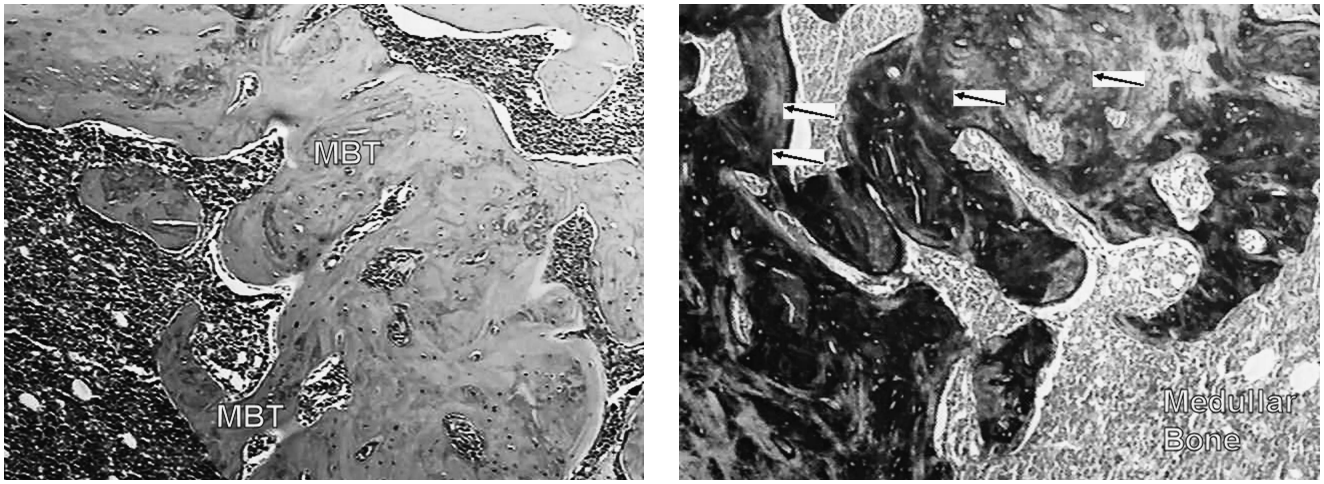

FIG. 4. (**A**) Photomicrograph of a specimen in which LBPM was applied to the surgical bed (BMP) and to the bone graft (group IV), showing the defect filled by newly-formed bone (MBT) and medullary tissue. Note that neoformation progressed from the cortical bone outward (approximately 40×; hematoxylin and eosin). (**B**) The newly-formed bone showed varying levels of collagen deposition on the site (arrows) (approximately 40×; Sirius red).

tal period (Fig. 3A and B). The use of both BMPs and LPBM applied to the surgical bed and the autologous bone graft (group IV) resulted in a cavity that was completely filled by cortical bone. The defect was filled with well-organized and mature bone trabeculi extending from one cortical side the other among some medullary tissue (Fig. 4A and B).

Statistical analysis showed significant differences ($p = 0.019$) with regard to bone neoformation between group I (bone graft only) and group IV (BMPs on the bed + bone graft + LPBM of the bed).

Discussion

Although bone tissue shows good regeneration, the capacity for repair may be impaired by poor blood supply, mechanical instability, and the presence of other tissues with higher proliferative activity. Large bone losses result in large defects that are too big for routine bone repair. As a means of improving the recovery of large bone defects, the use of several techniques, including photobioengineering, have been extensively studied.[1]

Autologous bone is considered the best type of graft for the treatment of bone defects. The success of this technique is strongly related to the blood supply to the surgical bed. Despite all the advantages of using autologous bone grafts, there are limitations on their use, including the size of the defect, the lack of a suitable donor area, and the need for two surgical procedures.[20]

Previous reports from our group,[7,8,13–15,17] using either organic or inorganic bone grafts or resorbable membrane, found that the use of LPBM had a positive biomodulating effect on healing bone. Similarly, another study[21,22] showed a positive biomodulating effect of LPBM on cultured rat fibroblasts, a report of great clinical interest for those studying bone regeneration.

Several previous reports have shown that the use of BMPs is effective in the improvement of bone repair *in vivo*[1,17,23] and *in vitro*, as well as with their association with GBR. However, the association of biomaterials, GBR, and LPBM is not as well documented in the literature.[1,7,8,13–15,17] A previous report[19] also found improvement in bone repair of defects grafted with biomaterials using visible red light, even if we take into account that our model is different from their model, in which a bone defect was filled with biomaterial, while in ours we used an autologous bone graft; however, the results were equivalent. It is important to mention that the results found in that report[19] corroborate those of our previous reports on the association of laser light and biomaterials.[1]

The results of our studies and those of others indicate that bone irradiated with infrared wavelengths shows increased osteoblastic proliferation, collagen deposition, and bone neoformation, compared to non-irradiated bone.[1]

It is well known that the stimulatory effect of laser light on bone occurs during the initial phase of proliferation of both fibroblasts and osteoblasts, as well as during the initial differentiation of mesenchymal cells. Fibroblastic proliferation and increased activity have been detected previously in irradiated subjects and cell cultures, and these are responsible for the great concentration of collagen fibers seen within irradiated bone.[1,7,8,13–15,17]

The results of the present study showed that in all treatment groups, bone neoformation was greater and qualitatively better in irradiated than in non-irradiated subjects. Control specimens showed less advanced repair after 40 d, and this was characterized by the presence of medullary tissue, a small amount of bone trabeculi, and cortical repair in 50% of cases. These results are in accordance with those of previous reports from our group, in which we used different protocols that resulted in dense, compact, and well-organized bone trabeculi in the irradiated subjects.[1,7,8,13–15,17]

In the present study, the effects of LPBM were evaluated after the use of autologous bone grafts and/or BMPs. Bone neoformation in the rat has been detected as early as 6 d after surgical procedures.[23] Systemic effects should also not be dismissed, as other studies failed to reveal significant effects when comparing contralateral wounds.[23–28]

Infrared laser light has been used because of its deeper penetration of tissues, especially in subcutaneous tis-

sues.[26–29] Several studies have shown the effectiveness of LPBM in improving healing of both bone defects and fractures.[7,14,32–35] However, some authors did not find these results.[30–35]

During our study, the dose was 10 J/cm^2 on the surgical bed and/or on the bone graft depending on the experimental group. In the postoperative applications, the dose was divided into four portions that were spread around the grafted area. We used this protocol was because a single, spot application to a wounded site may not reach the borders of the surgical bed and stimulate the cells there. Also, our group had already assessed several techniques for the irradiation of wounded sites, and we found that the presence of dental implants or membranes at a wounded site may make penetration difficult or increase the scattering of light. Thus, better results are achieved when the irradiation is carried out on the borders of the wounded area.[1]

This protocol was considered effective in previous reports, in which doses ranging from 1.8–5.4 J/cm^2 were utilized.[1,7,8,13–15,17] Some authors, however, reported positive responses to LPBM with much higher doses. A previous study[32,33] showed that bone neoformation was more evident when the laser was used, and that the effect was more evident at the highest dose, and when treatment was initiated 24 h after surgery. Many authors have emphasized the importance of choosing an appropriate energy level, but the proper energy level needed to attain the optimal positive result varies greatly.[1]

The treatment protocols used in our various studies worked well in our experience, but no current parameters are universally accepted. A unique parameter that alone is able to produce a photobiological response does not exist, and we used a combination of different parameters in our experimental model. It remains uncertain if bone stimulation by laser light is a generalized effect, or if the isolated stimulation of osteoblasts is possible. It is possible that laser therapy's effect on bone regeneration depends not only on the total dose of irradiation, but also on the duration and mode of irradiation. Most importantly, recent studies have suggested that the threshold parameter energy density and intensity are biologically independent of one another. This independence accounts for both the success and failure of laser therapy applied at low-energy density levels.[36]

One report[34] pointed out that although the use of laser light during early stages of healing was more effective in improving bone healing, treatment with laser light during later periods might have an important role in the maintenance of bone regeneration. This is why we chose the protocol used here, which included both intraoperative and postoperative irradiation that was repeated every other day for 15 d.

The results of our studies indicate that LPBM is more effective if the treatment is carried out at the early stages of healing, when cellular proliferation levels are high. The mechanism behind the positive effects of laser light on different tissues remains unclear, as there are many possibilities to be considered, such as stimulation by the laser energy of porphyrins and cytochromes to increase cellular activity, thus increasing the concentration of ATP and alkaline phosphatase and the release of calcium. Our experience also indicates that the magnitude of the biomodulative effect depends on the physiologic status of the cell at the time of irradiation,[1] or the stimulant effects of laser energy may occur during the initial phases of proliferation and differentiation of undifferentiated cells. However, this effect is not seen during more advanced stages of cellular development.

Conclusion

We conclude that LPBM, when carried out with the parameters used here, resulted in a statistically significant positive biomodulatory effect on the healing of bone defects in rat femurs subjected to autologous bone grafting and grafting with BMPs, and that this effect was more pronounced when laser irradiation was performed on the surgical bed intraoperatively, prior to the placement of the autologous bone graft, as well as postoperatively.

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