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Infrared Laser Light Further Improves Bone Healing When Associated with Bone Morphogenetic Proteins and Guided Bone Regeneration: An *in Vivo* Study in a Rodent Model

ANTONIO LUIZ B. PINHEIRO, Ph.D.,[1] MARLENY E. MARTINEZ GERBI, Ph.D.,[2]
ELIZABETH ARRUDA CARNEIRO PONZI, M.S.,[2] LUCIANA MARIA PEDREIRA RAMALHO, Ph.D.,[3]
APARECIDA M.C. MARQUES, Ph.D.,[1] CAROLINA MONTAGN CARVALHO, D.D.S.,[1]
RAFAEL DE CARNEIRO SANTOS, D.D.S.,[1] PRISCILA CHAGAS OLIVEIRA, D.D.S.,[1]
and MANUELA NÓIA, D.D.S.[1]

ABSTRACT

Objective: **This study assessed histologically the effect of laser photobiomodulation on the repair of surgical defects created in the femurs of Wistar rats treated or not treated with bone morphogenetic proteins (BMPs) and organic bovine bone graft.** ***Background Data:*** **This paper is part of an ongoing series of works in which biomaterials and/or guided bone regeneration (GBR) are used in association with laser photobiomodulation. Several previous reports from our group have shown that the use of laser photobiomodulation improves the treatment of bone defects.** ***Materials and Methods:*** **Forty-eight adult male Wistar rats were divided into four randomized groups: group 1 (controls, n = 12); group 2 (laser photobiomodulation, n = 12); group 3 (BMPs + organic bovine bone graft + GBR, n = 12); and group 4 (BMPs + organic bovine bone graft + GBR + laser photobiomodulation, n = 12). The irradiated groups received seven irradiations every 48 h, the first immediately after the surgical procedure. Laser photobiomodulation (830 nm, 40 mW, CW, ϕ ~0.6 mm) consisted of a total of 16 J/cm^2 per session at four points (4 J/cm^2 each) equally spaced around the periphery of the defect. The animals were sacrificed after 15, 21, and 30 d, and the specimens were routinely embedded in wax and stained with hematoxylin and eosin and Sirius red stains and analyzed under light microscopy.** ***Results:*** **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 irradiated animals compared to non-irradiated controls.** ***Conclusion:*** **We concluded that the use of laser photobiomodulation in association with BMPs, organic bovine bone grafts, and GBR increases the positive biomodulating effects of laser energy.**

INTRODUCTION

BONE LOSS is a major problem in many medical and dental specialties, and may occur due to several physiologic and pathologic conditions. Physiologic bone loss occurs primarily due to aging. Bone tissue has enormous regenerating capacity, and is generally able to restore its usual architectural and mechanical properties. However, there are limits to this capacity, and complete recovery may not occur if there is insufficient blood supply, mechanical instability, or competition with highly proliferating tissues. The loss of bone fragments, the removal of necrotic or pathologic bone, or even some surgical procedures may also create bone defects. These defects may be too large for spontaneous and physiologic repair. There are several methods that can be used to ameliorate bone repair, including the use of grafts, and recently the use of laser photobiomodulation (LPBM).[1]

[1]Laser Center, School of Dentistry, Department of Propedeutica and Clínica Integrada, Universidade Federal da Bahia, Salvador, and Instituto de Pequisa e Desenvolvimento (IPD), Vale do Paraíba University, São José dos Campos, [2]School of Dentistry, Federal University of Pernambuco, Recife-PE, and [3]School of Dentistry, Department of Propedeutica and Clínica Integrada, University Federal of Bahia, Salvador, Brazil.

A major problem in modern dentistry is the repair of bone defects caused by trauma, surgical procedures, or pathology. Several types of biomaterials have been used to improve the repair of these defects. Several autologous grafts and xenografts have been used to provide a framework or to stimulate new bone formation, and many times these grafts are associated with guided bone regeneration (GBR) techniques.[2–4]

Healing of bone differs from the healing of soft tissues, due to both the morphology and composition of bone, and healing occurs more slowly in bone than in soft tissues. It occurs in several phases, which differ depending upon the type and the intensity of the trauma, as well as the extent of the damage to the bone. The bone trauma is immediately followed by a sequence of reparative processes in which periostal osteogenic cells begin to proliferate and to differentiate in osteoblasts.[1]

The use of bone morphogenetic proteins (BMPs) is not new.[5–7] BMPs have been widely used for the reconstruction of the alveolar ridge,[8] in the recovery of bone loss, for repair of several types of bone defects, and for GBR procedures.[1–6,9–13]

The effects of LPBM on bone are still controversial, as previous reports have differing or conflicting results. It is possible that the effect of LPBM on bone regeneration depends not only on the total dose of irradiation, but also on the irradiation time and the irradiation mode. Most importantly, a recent study suggested that the threshold energy density and intensity are biologically independent of one another. This independence accounts for both the success and the failure of LPBM at low energy density levels. The possibility of ameliorating the repair of bone is an important step toward the application of photoengineering on living tissues.[1]

Despite the growing successful application of LPBM in the biomodulation of bone repair, there are few studies assessing the use of laser energy in association with biomaterials.[2–5,11]

The use of LPBM for the biomodulation of bone repair has been growing steadily, and several studies have demonstrated positive results on the healing of bone tissue. LPBM has been successfully used for improving bone healing in several conditions, such as in alveoli after tooth extraction, in bone fractures, during orthodontic treatments, and after dental implant operations.[1]

The results of our studies indicate that LPBM is more effective when the treatment is carried out at the early stages of healing, when rates of cellular proliferation are high. The mechanism of the positive effect of laser energy on different tissues remains unclear, but possibilities include stimulation by laser energy of porphyrins and cytochromes to increase cellular activity and increase the concentration of adenosine triphosphate 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, and the stimulant effect of laser energy appears to be maximal during the initial phase of proliferation and differentiation of undifferentiated cells, and is less during more advanced stages of healing.[1]

These issues indicate a need for further study to determine the most effective parameters for LPBM, and its interaction with different biomaterials for this new modality of treatment.

MATERIALS AND METHODS

This study was approved by the Animal Ethics Committee of the School of Dentistry of the Federal University of Bahia. Forty-eight healthy male and female young adult Wistar rats weighting 270–320 grams were kept at the Laboratório de Experimentação Animal of the School of Dentistry of the Federal University of Bahia during the experimental period. The animals were fed with a pelleted laboratory diet and had water *ad libitum*. The animals were kept in plastic cages bedded with sterilized wood chips and were kept in a day/night light cycle and controlled temperature during the experimental period.

Under intraperitoneal general anesthesia (10% chloral hydrate, 0.4 mL/100 g) the animals had their left legs shaved and the femurs exposed. A standardized 2 mm^2 cavity was created with a drill on the superior third of the lateral side of the bone of each animal. The animals were then randomly distributed into four groups: group 1 (control, bone cavity only); group 2 (bone cavity + LPBM); group 3 (bone cavity + biomaterial + GBR); and group 4 (bone cavity + biomaterial + GBR + LPBM). Each group was then divided into three subgroups (Table 1).

In group 1 (untreated controls), the periosteum was repositioned and suturing was performed with catgut and the skin closed with nylon. In the animals in groups 2 and 4, the wound margins were tattooed with nankin ink at four points. These were used to guide the application of laser treatment. The laser light was delivered on the medial side of each mark, to avoid any possible interference by the dye with the absorption of the laser energy. In groups 3 and 4 the cavity was completely filled with a biomaterial (organic lyophilized decalcified bovine bone, GenOx® + collagen gel, Gencol® + BMP pool, Genpro®) (Baumer S.A., Mogi Mirim, SP, Brazil), using the technique suggested by the manufacturer, and then sutured closed. Groups 3 and 4 also underwent GBR (bone resorbable decalcified cortical bone membrane, Genderm®; Baumer, S.A., Mogi Mirim, SP, Brazil).

Laser photobiomodulation (830 nm, 40 mW, ϕ ~0.60 mm, CW; DMC Equipamentos, São Carlos, SP, Brazil) was given

Table 1. Distribution of the Animals in the Study Groups

Group	*Subgroup*	*n*	*Treatment*
1	C15**/C21***C30****	12	Control-bone cavity only
2	CL15/CL21/CL30	12	Bone cavity + LPBM
3	PM15/PM21/PM30	12	Bone cavity + BMPs + organic bovine bone graft + GBR
4	PML15/PML21/PML30	12	Bone cavity + BMPs + organic bovine bone graft + GBR + LPBM

to groups 2 and 4, and was begun immediately after suturing, and consisted of transcutaneous application of laser energy at four points around the surgical site, and was repeated every other day for 15 d. The dose per point was 4 J/cm^2, the total dose per session was 16 J/cm^2, and the total treatment dose was 112 J/cm^2.

The animals were humanely sacrificed 15, 21, and 30 d postsurgery with an intraperitoneal overdose of 10% chloral hydrate. Specimens were routinely taken, kept in 4% formalin solution for 5 d, and were then cut, embedded in wax, and routinely stained with hematoxylin and eosin (H&E) and Sirius red at the Oral Pathology Department of the School of Dentistry of the Federal University of Bahia. All slides were analyzed using light microscopy by a single experienced pathologist. The descriptive and semi-quantitative histological analyses were performed based on the following parameters: reabsorption of the cortical plate and of the biomaterial inserted into the bone defect; the presence of medullary tissue and/or granulation tissue; the inflammatory reaction; the presence of giant cells, collagen fibers, or haversian systems; and the amount and quality of the newly formed bone.

RESULTS

Macroscopic observations showed that among the animals in groups 3 and 4, only one animal from group 3 showed remnants of the Genderm membrane at day 15, and at the end of the experimental period no remnants could be seen in either group. There was no cortical repair in most control animals on day 21, but repair was seen in 50% of the specimens from the control group at the end of the 30-day experimental period. In group 2 at day 15, 75% of the specimens showed complete cortical repair, and all specimens from group 2 showed complete cortical repair on day 21. Twenty-five percent of the specimens from group 3 showed complete cortical repair at day 15, and

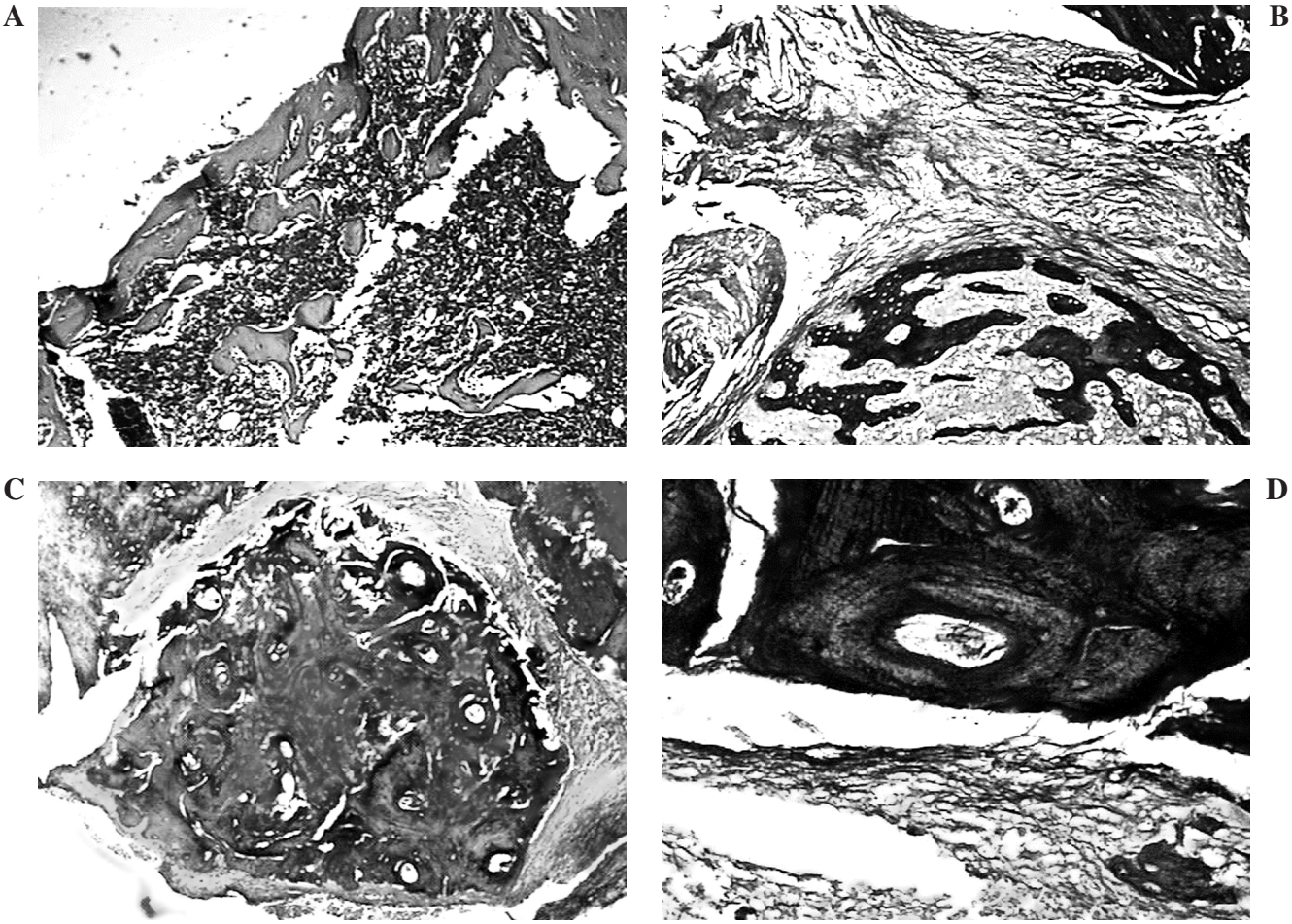

FIG. 1. (**A**) Photomicrograph of control specimen at day 15. A small amount of bone formation is seen at the cortical level. The defect is largely filled with medullary tissue and a few bony trabeculi can be seen (H&E, approximately ×40). (**B**) Photomicrograph of a specimen from an animal that had LPBM at day 15, showing newly formed trabeculi and many collagen fibers within the cavity (Sirius red, approximately ×100). (**C**) Photomicrograph of a specimen from an animal that had grafting and GBR at day 15. Note the incomplete cortical repair (Sirius red, approximately ×40). (**D**) Photomicrograph of a specimen from an animal that had grafting, GBR, and LPBM at day 15, showing haversian systems in the remodeling phase encircled by collagen fibers, indicating formation of new bone (Sirius red, approximately ×200).

this percentage increased to 50% at the end of the 30-day experimental period. In group 4 at day 15 75% of the subjects showed complete cortical repair, and by day 30 complete repair could be seen in all specimens.

Light microscopy

Controls. At day 15, the defect was filled by medullary tissue and osteoblastic activity was present in most specimens, and was seen as a small amount of bone matrix within the medullary tissue. No cortical repair was seen in most specimens. Osteoclastic activity was detected as lacunae in the cortical area. At this time medullary tissue and a few bony trabeculi can be seen within the defect (Fig. 1A). At day 21, most specimens showed early signs of cortical repair, and the defect was mostly filled by medullary tissue (Fig. 2A). At the end of the 30-day experimental period, the defect remained mainly filled by medullary tissue and most specimens showed complete cortical repair (Fig. 3A).

Laser photobiomodulation

At day 15, the defect was filled by medullary tissue and large amounts of newly formed bone were seen in most specimens. Delicate trabeculi and sparse collagen fibers were seen within the defect. In most specimens cortical repair was complete at this time. Osteoclastic activity was seen as several lacunae and giant cells were seen within the defect. Many collagen fibers could be seen (Fig. 1B). At day 21, a small amount of osteoblastic activity was observed. Small lacunae were present and most of the defect was filled by medullary tissue, with no evidence of spongy bony or collagen fibers. Cortical repair was complete at this time (Fig. 2B). At the end of the 30-day experimental period, the picture was similar to that seen on day

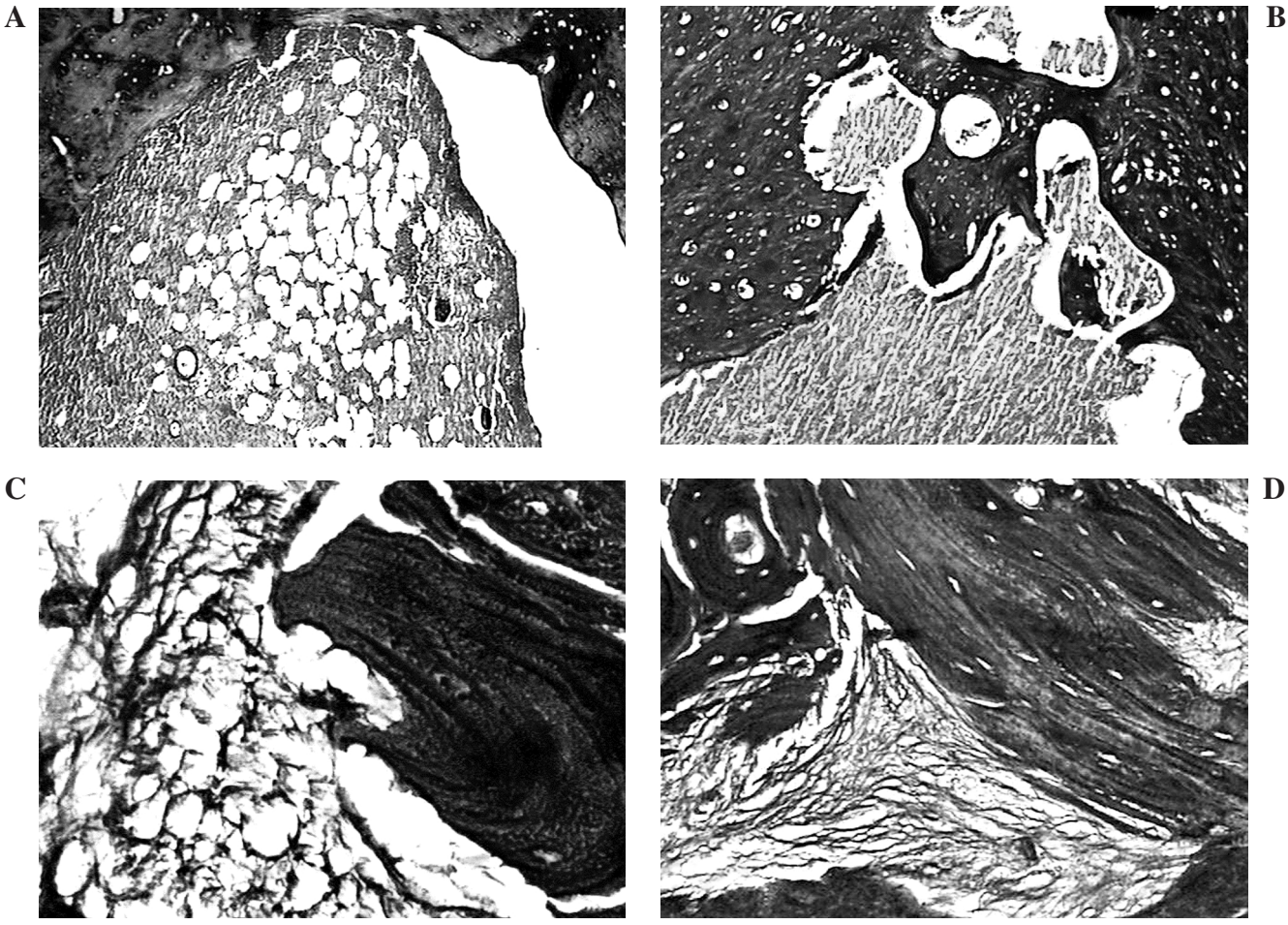

FIG. 2. (**A**) Photomicrograph of control specimen at day 21, showing a large amount of medullary tissue filling the defect, along with a large number of adipocytes (Sirius red, approximately ×40). (**B**) Photomicrograph of a specimen from an animal that had LPBM at day 21, showing the formation of bony trabeculi from the cortical plate, along with some medullary tissue within the defect (Sirius red, approximately ×100). (**C**) Photomicrograph of a specimen from an animal that had grafting and GBR at day 21. Note the presence of collagen fibers encircling the remnants of the graft (Sirius red, approximately ×200). (**D**) Photomicrograph of a specimen from an animal that had grafting, GBR, and LPBM at day 21. Note the presence of large amounts of collagen fibers and the deposition of osteoid matrix around the haversian system, indicating remodeling and new bone formation (Sirius red, approximately ×200).

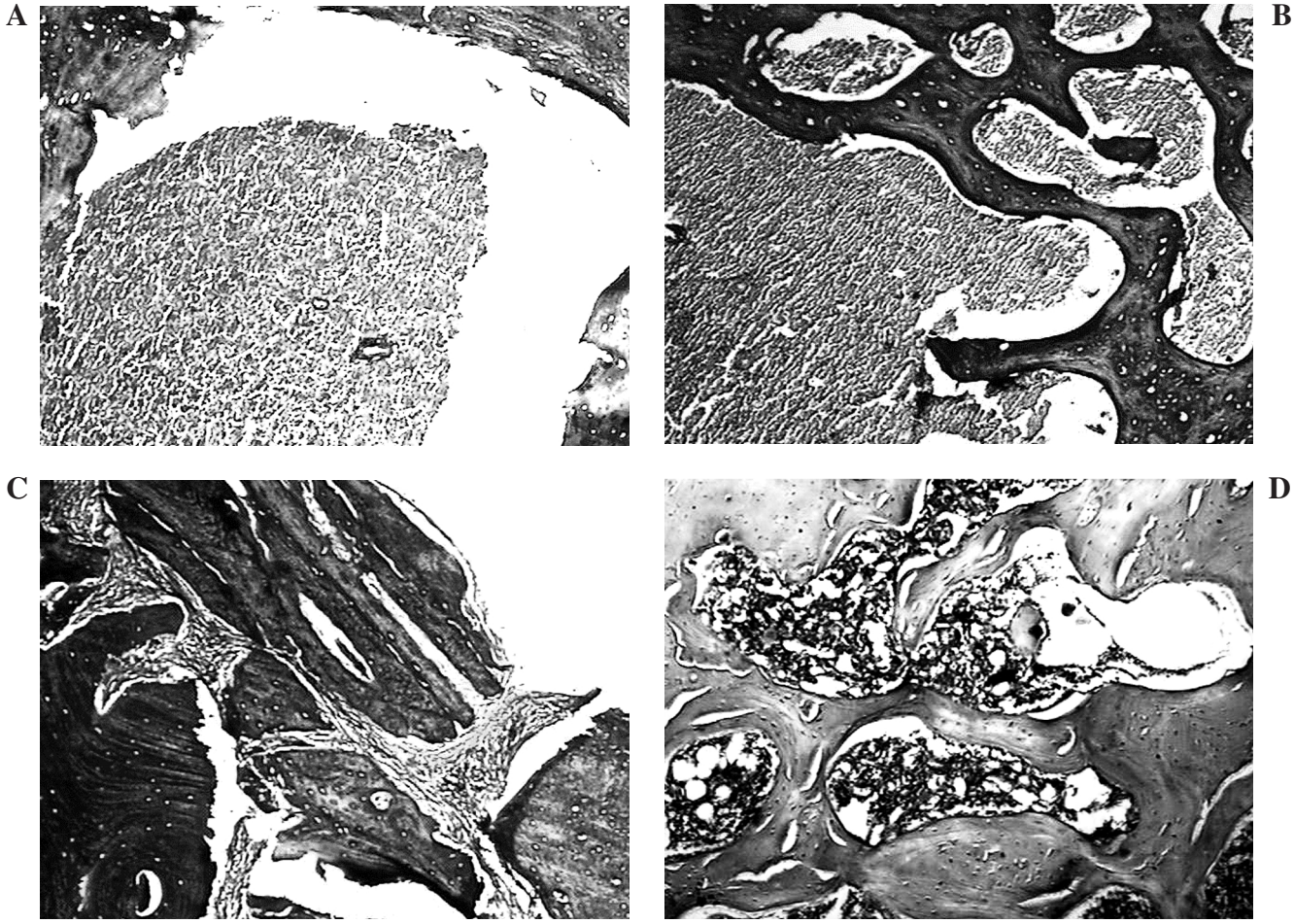

FIG. 3. (**A**) Photomicrograph of control specimen at day 30. The appearance here is similar to that seen on days 15 and 21. The defect was mostly filled by medullary tissue (Sirius red, approximately ×40). (**B**) Photomicrograph of a specimen from an animal that had LPBM at day 30, showing newly formed bone trabeculi originating from the cortical area at a more advanced stage of maturation (Sirius red, approximately ×100). (**C**) Photomicrograph of a specimen from an animal that had grafting and GBR at day 30, showing partial cortical repair and remnants of the graft within the defect (Sirius red, approximately ×40). (**D**) Photomicrograph of a specimen from an animal that had grafting, GBR, and LPBM at day 30. Note that the defect is completely filled by mature newly formed bone and medullary tissue with no visible remnant of the graft (Sirius red, approximately ×40).

21, and cortical repair was complete in all specimens. No necrosis could be seen (Fig. 3B).

Biomaterial + GBR

At day 15, most specimens showed incomplete cortical repair. Some bone formation and a moderate amount of osteoid tissue deposition were seen within the defect, and most specimens displayed replacement of the implant by some newly formed bone. A moderate amount of chronic inflammation was present around the implanted material (Fig. 1C). At day 21, cortical repair was seen in half of the specimens, and collagen deposition around the remnants of the implant was evident (Fig. 2C). At the end of the 30-day experimental period complete cortical repair was seen in half the specimens. Collagen fibers were seen encircling the remnants of the implanted material, and mineralization and some bone trabeculi were seen at this stage. No necrosis was seen (Fig. 3C).

Biomaterial + LPBM + GBR

At day 15, complete cortical repair was seen in most the specimens. The newly formed bone was similar to that seen in untreated areas. Within the defect, intense bone deposition replacing the implanted material was evident. Remodeling haversian systems were also seen at this stage. Remnants of the implanted material were encircled by collagen fibers and there was some lymphocytic inflammatory infiltrate seen (Fig. 1D). At day 21, there was an evident increase in the amount of newly formed bone. The newly formed bone showed remodeling haversian systems and characteristics of mature bone. Bone trabeculi were seen at the center of the defect, and were also spreading from the cortical area toward the center of the defect. Large amounts of collagen fibers and osteoid tissue were seen around the remnants of the implanted material (Fig. 2D). At the end of the 30-day experimental period there was an evident increase in the amount of bone trabeculi seen within the defect.

In half of the specimens no remnants of the implanted material were seen, and large amounts of collagen fibers and osteoid tissue were seen. Complete cortical repair was seen in most the specimens, and this newly formed bone was similar to that seen in untreated areas. No inflammation or necrosis was seen (Fig. 3D).

DISCUSSION

LPBM is an exciting new modality that has been a real boon to medical science. However, the protocols previously reported elsewhere in the literature have so far failed to provide a comprehensive set of parameters for the achievement of optimal results. The large number of possible combinations of laser parameters makes the establishment of an acceptable protocol even more complex.[1]

There is still controversy in the literature with regard to the biological effects of LPBM. Several researchers have reported positive results of *in vivo* studies.[2–6,9–13] Previous researchers have suggested that there are several aspects that may have influenced the results of studies that failed to find positive results with LPBM, including a genuine lack of biological response to the protocol implemented, inappropriate dosimetry, and the use of inappropriate models.[1,14]

In this study we used a dose of 16 J/cm^2 per session, and a total of 112 J/cm^2 for the entire treatment regimen, as recommended previously by our team. We have found that the previously recommended protocol induced an intense cellular and humoral response at the early stages of healing.[1]

The results of the present investigation showed that all of the experimental groups showed improved qualitative and quantitative formation of new bone compared to controls up to day 30. In control subjects, on day 30 we found only medullary and granulation tissue, with only partial cortical repair. On the other hand, when LPBM was used the bone quality and the amount of newly formed bone was much more evident and confirmed results of our previous reports.[2–6,9–13]

One interesting result of this paper that is in conflict with the literature is the early appearance of haversian systems, that usually are not seen until 4–6 wk after the use of BMPs.[5–7] It is important to understand that the use of either LPBM or biomaterial alone did not show results similar to those seen when both treatments were used together. This is evidence that the use of laser energy actually improves healing. This improved process could also be seen at the end of the experimental period, a fact in agreement with findings of previous reports in the literature.[2–6,9–13]

Another important finding of this study concerns neovascularization. We found early signs of newly formed blood vessels at day 15 in all laser-treated subjects, as well as in animals grafted with BMP, but not in control animals. We have previously commented that these results may be related to the effect of laser energy on cells at an earlier stage of healing. At this early stage, laser energy stimulates cells to both differentiate and proliferate, and later on it promotes cell maturation. This is why we chose to begin irradiation immediately post-surgery, and to continue it for 15 d, with treatment sessions every other day.[1,5] The effects of laser energy on pain, edema, inflammation, and wound healing have already been documented in the literature.[1]

Cortical repair was also different between the control and experimental groups. Most of the experimental subjects showed complete cortical repair at day 21, and it was apparent that the defect was nearly filled by newly formed, compact, well-vascularized bone, with no trace of the graft in the animals from group 4. It is important to note that bone repair was seen at this stage in all irradiated animals, and that this growth was less vigorous in animals that were neither grafted with BMPs nor those having GBR. The growth was much more apparent when the treatment modalities were combined.[1–5,11] The effects of laser energy on bone growth are due to increased deposition of collagen fibers, and more advanced stages of maturation were seen in irradiated subjects versus controls. This is important because it makes the newly formed bone more compact and of sound quality.[1–6,9–13]

The use of BMPs results in several changes in the healing of bone. Initially there is an increased release of several growth factors such PDGF, TGF-β, FGF, and IGFs, which are important for the migration of endothelial cells and for the formation and proliferation of capillaries. PDGF stimulates cell division and promotes neoangiogenesis in the vascular complex within the graft. BMPs belong to the TGF-β family, and initially they stimulate mitosis of osteoblasts and their precursors. Later they stimulate the precursors to differentiate into osteoblasts, that will later mature and become functional. TGF-β also stimulates both osteoblasts and fibroblasts to secrete collagen and bone matrix, respectively.

The literature shows that despite the fact that LPBM and BMPs both have positive effects on the healing of bone, they do so differently. However, the trigger for the cascade of events leading to bone healing appears to be similar for both, and includes chemotaxis, cell differentiation, stimulation of collagen synthesis and deposition, differentiation and maturation of both osteoblasts and chondroblasts, increased deposition of osteoid matrix, and mineralization of bone tissue. Both therapies accelerate bone healing and their combination results in a synergistic effect that greatly promotes bone healing. Also, it is important to note that neither adverse effects nor foreign body reactions were seen in any of the animals included in this study.[1–6,9–13]

The results of the present study may be useful for patients who have undergone radiotherapy (RT), as it has been suggested that osseous healing may be impaired by radiation-induced decreases in BMP2/4 expression in combination with increases in TGF-β_1 expression, and that BMPs play a major role in the synthesis of bone matrix proteins. Reduced osteocalcin expression in areas of osseous consolidation post-RT, as well as depletion of other cellular elements may result.[15] Also, the absence of osteogenic cells in areas of healing bone may also impair bone repair. Furthermore, mesenchymal stem cells are destroyed by RT, and differentiation of the osteoblasts and chondrocytes required for bone regeneration is impaired. BMP2/4 is expressed differently in irradiated and non-irradiated bony structures. It was demonstrated that BMP2 expression correlates with the differentiation of mesenchymal stem cells into osteoblasts and chondrocytes.[16,17] Thus previously irradiated tissue expresses reduced levels of BMP2/4. Significantly increased TGF-β_1 expression was seen in previously irradiated graft beds compared with non-irradiated tissues, along with increased fibrosis and collagen I expression.[18] Compared with a non-irradiated graft

bed, fewer functional mesenchymal cells, which are required for primary callus formation, are seen in previously irradiated tissues, and there is a complete loss of lacunar cells in irradiated bone.[19] The "reseeding" of pre-osteoblasts from the surrounding tissue via blood circulation may be related to the regenerating capability, and eventually to the vasculature, seen in irradiated tissues. In addition to radiation-induced stem cell depletion, competitive inhibition of BMP2/4 via TGF-β_1 has to be considered, because the receptors for TGF-β_1 and BMP2/4 display a close homology, which is differentiated via three amino acids, and they also share SMAD proteins as signal transducers. The absence of BMP expression causes insufficient signal transduction and less synthesis of bone matrix proteins. Radiation-induced TGF-β_1 activation leads to increased synthesis of extracellular matrix via activation of transcription factors such as phospho-SMAD4.[18]

Biological activity in the osseous healing area is characterized by the presence of active, proliferating progenitor cells that stimulate bone regeneration. It may be that the limiting factor for bone healing after RT is not the remaining level of osteogenetic potency and the vascularity of the new bone, but rather is the absence of biological activity of the healing bone.[20]

New therapies must be directed toward stimulation of improved osteogenetic potency of osteoblasts in order to overcome radiation-induced apoptosis of mesenchymal cells, as well as the suppression of BMP2/4 and BMP2/4-associated signal transduction[21–24] achieved via osteoinduction in non-irradiated and previously irradiated bone through recombinant BMP2. Adenoviral and liposomal BMP-coded vector systems could be one approach for inducing bone matrix protein synthesis in irradiated bone tissue, or perhaps osteogenic cells could be grafted into healing bone.[25–27]

It may be possible to develop new therapeutic approaches for improving bone repair in patients subjected to RT by using the protocol explored here. It is well known that LPBM can induce differentiation and proliferation of osteoblasts.[1–6,9–13]

The treatment of bone defects in irradiated bone is likely to involve the use of biological substances or their recombinants, such as growth factors and cytokines. Bone formation and regeneration are complex processes involving interactions of numerous cells with local and systemic regulatory factors. These regulators include cellular mediators, cytokines, hormones, and components of the extracellular matrix. Clearly, bone formation can be stimulated by BMPs and certain cytokines as well as by LPBM. Bone resorption also can be modulated, stimulated, or repressed by numerous other cytokines, as well as LPBM. In addition, neovascularization can be enhanced by some LPBM protocols.[1–6,9–13]

During the past decade, there has been an explosive increase in the number of growth factors and other cytokines that have been identified and characterized. At least three of these groups are potential candidates for use as therapeutic modalities for treatment of osteonecrosis: cytokines (including interleukins, tumor necrosis factors, and signaling molecules such as FGFs, PDGF, IGFs, TGF-βs); BMPs; and angiogenic factors.[28]

BMPs are a family of proteins with proven osteogenic potential. This group of proteins has the capacity to promote osteogenesis at non-osseous sites,[29] and may be useful adjuncts to the treatments of osteonecrosis due to their pleiotropic effects, such as stimulation of new bone formation, neovascularization, and promotion of articular cartilage repair.

A characteristic feature of osteonecrosis is that the bone lesion is avascular. One possible approach to the treatment of this disease might involve stimulating angiogenesis. Angiogenesis, also called neovascularization, involves several steps including degradation of the existing basement membrane and recruitment of endothelial cells. These steps are likely to be controlled by soluble angiogenic factors. Such factors may include angiogenin, prostaglandin E_2, FGF-α and FGF-β, angiogenic lipid fraction, IL-1, and TNF-α. Fibroblast growth factors have been shown to promote the growth of mesodermal cells such as vascular endothelial cells and fibroblasts. They also have been shown to stimulate angiogenesis when given in small amounts.[30] It is well described in our previous report that LPBM is capable of stimulating angiogenesis.[1–6,9–13]

CONCLUSION

We conclude that the use of infrared laser energy was effective in hastening the healing process in bone defects, and is even more effective when combined with bone morphogenetic proteins, organic bovine bone grafting, and guided bone regeneration, as shown by our findings of increased collagen deposition, faster cortical repair, and earlier development of haversian systems.

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Address reprint requests to:
Prof. Antonio Luiz Barbosa Pinheiro, Ph.D.
Laser Center
Faculdade de Odontologia
Universidade Federal da Bahia
Av. Araújo Pinho, 62, Canela
Salvador, BA, CEP 40140-110, Brazil

E-mail: albp@ufba.br