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Laser Therapy Improves Healing of Bone Defects Submitted to Autologus Bone Graft

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ABSTRACT

Objective: **The aim of the present study was to assess histologically the effect of low-level laser thrapy (LLLT) (λ830 nm) on the healing of bone defects associated with autologous bone graft.** ***Background Data:*** **LLLT has been used on the modulation of bone healing because of the photo-physical and photochemical properties of some wavelengths. The use of correct and appropriate parameters has been shown to be effective in the promotion of a positive biomodulative effect on the healing bone.** ***Methods:*** **Sixty male Wistar rats were divided into four groups: G1 (control), G2 (LLLT on the surgical bed), G3 (LLLT on the graft), and G4 (LLLT on both the graft and the surgical bed). The dose per session was 10 J/cm^2, and it was applied to the surgical bed (G2/G4) and on the bone graft (G3/G4). LLLT was carried out every other day for 15 days (λ830 nm, ϕ = 0.5 cm^2, 50 Mw, 10 J/cm^2). The dose was fractioned in four points. The animals were sacrificed 15, 21, and 30 days after surgery; specimens were taken and routinely processed (wax, cut, and stain with H&E and Sirius red stains). Light microscopic analysis was performed by a pathologist.** ***Results:*** **In the groups in which the LLLT was used trans-operatively on the surgical bed (G2/G4), bone remodeling was both quantitatively and qualitatively more evident when compared to subjects of groups G1 and G3.** ***Conclusion:*** **The present study indicates that the use of LLLT trans-operatively resulted in a positive biomodulative effect on the healing of bone defects associated with autologous bone grafts.**

INTRODUCTION

Bone loss may occur due to trauma, pathologies, or some surgical procedures, and modern surgery has employed grafting procedures to replace these losses. 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.[1] Very often, autologous bone grafts are used, and they may be taken from several parts of the skeleton. Autologous bone grafts are characterized by biocompatibility and osteointegration, as well as significative osteogenic protential.[2]

The use of low-level laser therapy (LLLT) on the modulation of healing of different bones has resulted in several *in vivo* or *in vitro* studies to promote a more comfortable postoperative period and quicker healing.[3] Wound healing is a complex process that involves both local and systemic responses. Usually, bone healing is slower than that observed in soft tissue. Despite several reports found in the literature suggesting the benefits of LLLT in soft tissue, its effect in bone is still not completely understood, in part due to several conflicting results.[3,4]

METHODS

Sixty healthy male Wistar rats weighing an average of 336 g were kept at the Laboratório de Experimentação Animal of the Faculdade de Odontologia da Universidade Federal da Bahia; they were fed standard pelted laboratory diet and had water *ad*

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libitum during the experimental period. The animals were kept in groups of four in plastic cages lined with shredded paper for bedding, and they were maintained under natural daylight cycles and controlled temperature.

The animals were randomly distributed into four groups of 15 animals each: group 1 (Control, group 2 (LLLT on the surgical bed), group 3 (LLLT on the bone graft), and group 4 (LLLT on both surgical bed and bone graft). The animals of all groups were humanely sacrificed after 15, 21, and 30 days with an overdose of general anesthetics. Each group was divided into three subgroups ($n = 5$) according to sacrifice time. Under GA (Zoletil®, 20 mg/kg), the right femoral area was shaved and cleaned with 2% chlorohexidine. The femur was then surgically exposed, and a graft was then routinely raised from the lateral surface of the femur and was kept on saline solution. LLLT was then used according to the group in which the animal was placed (G2, G3, G4). Control subjects had no laser treatment. The autologous bone graft was then repositioned on the surgical bed, and the soft tissues were routinely sutured.

LLLT was performed with a diode laser. The dose used trans-operatively on the surgical bed was 10 J/cm^2 (λ830 nm, continuous wave [CW], ϕ = 0.5 cm^2, 50 mW, focal distance of 2 cm) in G2 and G4. LLLT was also applied at the graft surface before repositioning it on the defect on G3 and G4 with the same dose. The animals in experimental groups received laser treatment every other day for the following 15 days (λ830 nm, CW, ϕ; = 0.5 cm^2, 50 mW). Treatment consisted of a session dose of 10 J/cm^2 applied transcutaneously and punctually on four points around the surgical defect (4 $\times$ 2.5 J/cm^2), except on control specimens. The total treatment doses were 80 J/cm^2 for G2 and G3 and 90 J/cm^2 for G4.

The animals were humanely sacrificed 15, 21, and 30 days after surgery by means of a intraperitoneal lethal dose of 10% chloral hydrate. The specimens taken were macroscopically assessed and then kept on 10% formaline solution during 24 h at the Laboratório de Anatomia Patológica do Hospital Universitário Edgar Santos. The specimens were routinely processed (wax and stain with H&E and Sirius red stains). All slides were analyzed by light microscopy by two pathologists who were previously calibrated.[3,4,26] Each scoring was double checked in order to confirm the equivalence of the rating of each event. For the analysis, the following parameters were used: inflammatory infiltrate, bone reabsorption and neoformation, and collagen deposition (Table 1).

RESULTS

Macroscopic analysis showed a lack of chronic inflammatory reaction in all specimens. Many specimens showed formation of bone callous on the grafted region, except on the control specimen at the 15th day. This bone callous was seen callous on all subjects at day 30. A summary of the histological observations can be seen in Table 2.

Controls (group 1)

In non-irradiated subjects (G1), fragments of the bone graft were present in all specimens at day 15. In all the specimens, inflammatory reabsorption was present, and it was characterized by the presence of irregularities in the internal cortical bone surface of the graft and osteoblasts. An intense process of bone neoformation was obscerved at this time, which was characterized by the deposition of delicate trabeculae distributed among cancellous bone and the use of the graft as a support for bone deposition. This process was characterized by the presence of osteoblasts secreting mineralized matrix. Dispersed among the newly formed trabeculae, Sirius red staining highlighted the presence of a delicate mesh of collagen fibers, as well as the presence of a mild mononuclear inflammatory infiltrate (Fig. 1). On the 21st day, a decrease of reabsorption activity was observed. The presence of residual fragments of the grafted area persisted, and there was also a decrease of both inflammatory response and osteoblastic activity. The newly formed bone at this time exhibited a more lamellar aspect. On day 30, stabilization of the bone remodeling was observed, as was incorporation of the graft to the cortical bone.

Irradiation of the surgical bed (group 2)

Up to day 21, a more advanced reabsorption of the graft was observed in G2, as was significant bone neoformation different from that seen in controls. Larger amounts of newly formed bone tissue were observed, and its distribution was seen in the central portion of the medullar tissue. In controls, this was only seen at the vicinity of the graft (Fig. 2). Different from in controls, the presence of collagen fibers associated with newly formed bone tissue was not observed. However, the presence of reabsorption in the cortical plate was observed. The presence of a mild mononuclear inflammatory infiltrate and osteoclasts to the picture seen on controls at day 15 was also

Table 1. Scoring System of the Inflammatory Infiltrate, Bone Reabsorption and Neoformation, and Collagen Deposition[3,4,26]

	Inflammatory infiltrate	*Bone reabsorption*	*Bone neoformation*	*Collagen deposition*
None	0	0	0	0
Mild	+	+	+	+
Moderate	++	++	++	++
Severe/intense	+++	+++	+++	+++

TABLE 2. SUMMARY OF THE HISTOLOGICAL OBSERVATIONS DURING THE EXPERIMENTAL PERIOD

Subjects	*Observations*	*Group*	*Staining*	*15th day*	*21st day*	*30th day*
$n = 15$	$n = 30$	Control	H&E, Sirius red	Remnants of the graft Intense graft reabsorption Delicate mesh of collagen Delicate bone trabeculae Mild inflammation	Remnants of the graft Reduced graft reabsorption Bone maturation Lamellar bone Mild inflammation	Incorporation of the graft Bone remodeling
$n = 15$	$n = 30$	Irradiation of the surgical bed	H&E, Sirius red	Moderate graft reabsorption Exuberant bone deposition No collagen deposition Mild inflammation	Graft reabsorption Exuberant bone deposition	Bone remodeling Intense bone deposition
$n = 15$	$n = 30$	Irradiation of the autologus bone graft	H&E, Sirius red	Remnants of the graft Moderate graft reabsorption Bone Deposition Delicate bone trabeculae Moderate inflammation No collagen deposition	Increased bone deposition	Vestiges of the graft Intense bone deposition and remodeling
$n = 15$	$n = 30$	Irradiation of the surgical bed and autologus bone graft	H&E, Sirius red	Remnants of the graft Intense graft reabsorption Intense bone remodeling Bone deposition No collagen deposition	Increased bone deposition	Incorporation of the graft Bone maturation

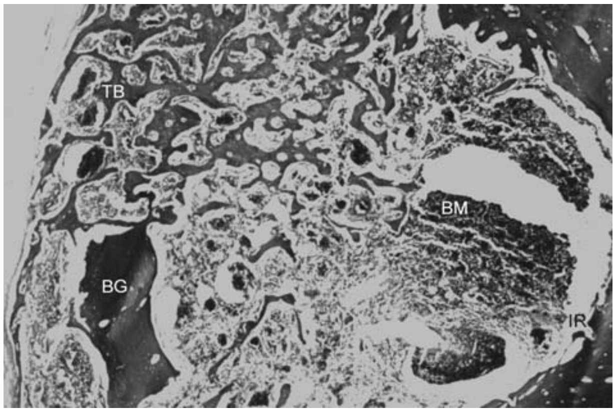

FIG. 1. Photomicrography of group 2A (laser on surgical defect, 15 days). The presence of bone graft (BG) fragment with great amount of neoformed trabecular bone (TB) toward bone marrow (BM), inflammatory reabsorption (IR) of internal cortex. H&E, approximately ×40.

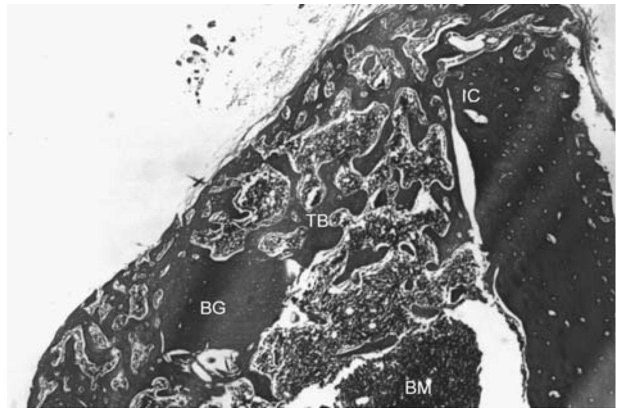

FIG. 3. Photomicrography of group 4A (laser on surgical defect and on bone graft, 15 days). The presence of bone graft (BG) fragment with inflammatory reabsorption and neoformed trabecular bone (TB) from the internal cortices (IC) of surgical defect remaining towards medullar tissue (BM). H&E, approximately ×40.

noticed. At the end of the experimental period, increased bone deposition was observed in all G2 specimens.

Irradiation of the autologus bone graft (group 3)

When bone graft was irradiated (in G3), the pattern of reabsorption of the bone graft and presence of osteoclasts at day 15 was considered more extensive when compared to all other groups. The grafted fragment was almost completely reabsorbed in all the specimens. In relation to the internal cortical surface, reabsorption was observed in three of five cases, and it was less severe than seen in G1 and G2 (Fig. 3). In 80% of G3 cases, the formation of delicate bone trabeculae was observed growing from the internal cortical plate to the center of the medullar space. The mononuclear inflammatory infiltrate seen on day 15 was moderate, and deposition of collagen matrix was not observed inside the cavity. By day 21, a larger increment of bone was formed from the internal surface of the cortical plate.

At day 30, it was verified that, in spite of vestiges of permanence of the bone graft within the cavity, this did not induce neoformation of bone, which stayed in large amounts predominantly starting from the internal surface of the cortical plate and showed better organization compared to previous times.

Irradiation of the surgical bed and autologus bone graft (group 4)

Irradiation of both the surgical bed and the bone graft resulted in intense remodeling of the bone at day 15. The fragment of grafted bone showed intense inflammatory reabsorption, while bone neoformation was seen, starting from the residual cortical bone as well as in the middle of the cancellous bone, which presented a normal aspect (Fig. 4). Sirius red staining showed bone neoformation, and deposition of collagen fibers was not verified. The maintenance of the integrity of

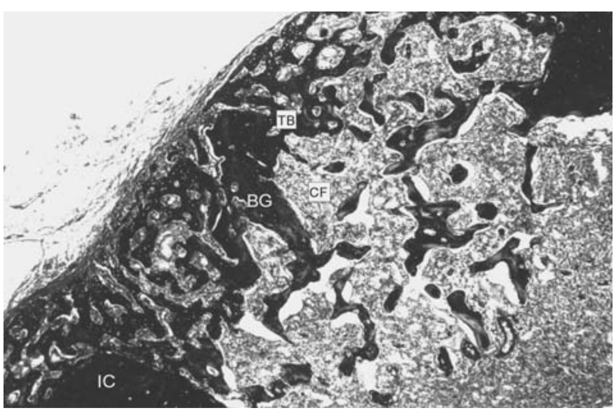

FIG. 2. Photomicrography of group 1A (control, 15 days). The presence of bone graft (BG) fragment is observed, with neoformed trabecular bone (TB), internal cortex (IC) of surgical defect and soft frame of collagen fibers (CF). Sirius red, approximately ×40.

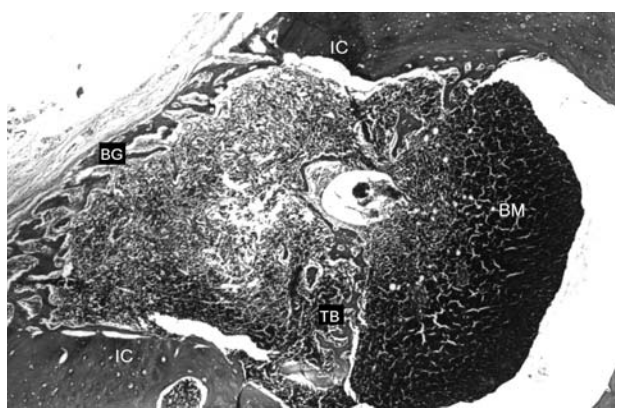

FIG. 4. Photomicrography of group 3A (laser on bone graft, 15 days). The presence of bone graft fragment (BG) with great reabsorption, internal cortex (IC) of surgical defect, trabeculae bone (TB) towards the center of medullar space (BM). H&E, approximately ×40.

the internal surface of the cortical plate was verified, unlike in G3, which showed larger reabsorption activity and the presence of osteoblasts. Mild chronic mononuclear inflammatory infiltrate was observed, as was also seen in the other experimental groups. At day 21 there was an increased deposition of bone, in which trabeculae were seen to present a more mature aspect. At the end of the experimental period of 30 days, the same histological pattern of bone neoformation was observed, starting from the remnants of cortical plates as observed at the previous times. The incorporation of the fragment of graft to the remnants of the cortical bone was also present.

DISCUSSION

Laser radiation possesses a wavelength-dependent capacity to alter cellular behavior in the absence of significant heating.[5,6] The dispersion of laser light in tissues is very complex, as tissue components influence the dispersion of light.[7] When this light passes from air to the interior of another substance, such as a biological tissue, its speed changes, but the frequency remains the same, and the nominal wavelength can be used to characterize the laser.[8]

Laser's biomodulation is one of the areas of controversy in the use of lasers,[9] and most applications of LLLT are in soft tissues. However, there is a growing interest in its application in mineralized tissue.[10]

Bone healing differs from the healing of soft tissues in that both the morphology and composition are slower than in soft tissue and consecutive phases are involved, which differ depending upon the type and the intensity of the trauma, and the extent of damage to the bone.[4] Bone trauma is immediately followed by a sequence of reparative processes in which the periostal osteogenic cells begin to proliferate and to differentiate into osteoblasts.[11]

In the present study, the effect of LLLT was evaluated after the use of autologous bone grafts. Bone neoformation in the rat has been detected as early as 6 days after surgical procedures.[12] Systemic effects may not be disregarded, as other studies failed in reveal significant effects when comparing contralateral wounds.[13–18] Infrared laser light has been used because of its deeper penetration of tissues, especially in subcutaneous tissues.[16,19] Several works have shown the effectiveness of LLLT in positive modulation of healing of both bone defects and fractures.[3,11,20–28] However, some authors did not find the same results.[14,16]

During our surgery, the dose was 10 J/cm^2 on the surgical bed and/or on the bone graft according to experimental group. In the postoperative applications, the dose was fractioned in four and applied around the grafted area. The use of such a protocol was due to the fact that a single spot application on a wounded site may not reach the borders of the surgical bed and stimulate the cells, and 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. So, better results are achieved when the irradiation is carried out on the borders of the wounded area.[3,4]

This protocol was considered effective in previous reports, where doses ranging from 1.8 to 5.4 J/cm^2 were utilized.[29] Some authors, however, reported positive responses to LLLT with much higher doses. A previous study[11] showed that bone neoformation was more evident when laser was used, with the effect 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 level of energy, but the recommended energy for a positive effect varies greatly.[30]

In groups in which the laser was applied to the surgical bed trans-operatively (G2/G4), bone reabsorption was more intense. Bone neoformation was also more significant, on both qualitative and quantitative grounds. Confirming the biomodulative effect of LLLT, previous works found similar results for the same observation periods. Previous reports from our group,[3,20] using both organic or inorganic bone grafts and reabsorbable membrane, found that the use of LLLT had a positive biomodulative effect on healing bone. Similarly, another study[31] showed positive biomodulative effect of LLLT on cultured rat fibroblasts—a report of great clinical interest for bone regeneration.

In this study, all experimental groups presented higher bone remodeling as early as day 15, showing a tendency to stabilize beginning at day 21. This may indicate that the effect of LLLT is more effective when the treatment is carried out at early stages, that is, when high cellular proliferation occurs.[3,20,25,26,32] These results are corroborated by a previous study by our group.[26] Another group,[24] using computerized morphometry, also showed a positive effect of LLLT on bone remodeling in rats.

Some other works also mentioned a great concentration of collagen fibers within irradiated bone cavity, unlike in the control group, which did not receive LLLT.[3,20] In the present study, however, histological examination failed to reveal deposition of collagen matrix associated with newly formed bone, except in control subjects (G1), where it was observed at day 15 after the experiment, as evidenced by Sirius red staining. The implications of this fact remain unclear.

The true mechanism that leads to a positive effect of laser light on different tissues is not fully understood, and this aspects makes the comparative analysis of the present results difficult because of the diversity of techniques, methods, and experimental models, as well as treatment protocols reported.[33] Several hypotheses have been suggested for this mechanism. One report[11] suggested that laser energy stimulates porphyrins and cytochromes to increase cellular activity, thus increasing the concentration of ATP and AlP, and the release of Ca.

One study[34] has suggested that the magnitude of the biomodulative effect depends on the physiologic status of the cell at the time of irradiation. This may explain why the biomodulative effect is not always detectable. Another study[32] showed that the stimulant effect of laser light occurs during the initial phase of proliferation and initial differentiation of undifferentiated cells; this does not occur during more advanced stages.

In the present study, such a mechanism may explain the differences between the subjects in which the surgical bed was irradiated trans-operatively and the ones in which only the bone graft was irradiated. In individuals in which the bone marrow that contained immature cells was directly irradiated (G2/G4), the biomodulative effect was more evident when compared to the group in which laser light was applied during the same period but the tissue irradiated was the bone graft, which possessed fewer or no immature or undifferentiated cells (G3).

One report[25] pointed out that, although the use of laser light during early stages of healing was more effective on the process of bone healing, treatment with laser light during later periods might have an important role on the maintenance of the bone regeneration. Considering this aspect, the protocol of radiation used on the present study, besides the trans-operative irradiation, on experimental groups, included the irradiation on the postoperative period and was repeated at every 48 hours during 15 days, using the same protocol to all groups but not to controls.

Also investigating the possible mechanisms of action of the Laser on tissue, a previous report[33] assessed the effect of Laser light on rat bone marrow cells in culture and observed an increase of the mitotic activity on bone marrow cell on experimental groups.

Vascular responses to LLLT were also suggested as one of the possible mechanism responsible for the positive clinical results observed following LLLT. Therefore vascularization is an important and decisive factor for the healing of wounds and for the relief of the pain. The improvement of the vascularization following LLLT is one of the possible mechanisms for the clinical effectiveness of this treatment that has been used on the control of the pain or to improve wound healing.[35]

Mitochondrial changes have also been suggested as responsible for the positive results of LLLT. A report[36] demonstrated that the Laser light might induce several changes on the respiratory cycle of the mitochondria. This paper suggested that the Laser light of λ352nm and λ458nm might damage the mitochondrial membrane while λ514,5mm Laser light may slightly increase the synthesis of ATP.

The photo biological response has been suggested as a response of the absorption of a specific wavelength by some unknown molecular photoreceptor[37]. Such molecular photoreceptor usually participates on metabolic reactions on the cell, which cannot necessarily be directly linked to the responses to the Laser light itself. After the absorption of a specific wavelength and the resulting excitation primary molecular processes, which occurs on molecular receptors, may lead to the photo biological response.

The treatment protocol used on this investigation is in agreement to our group experience as no existing parameters are universally accepted. Besides, many authors who used similar protocols have reported conflicting results. A unique parameter able to produce itself a photo biological response doesn't exist, but the conjugation of different parameters and its variations are in agreement with the experimental model.[3,4] It still remains uncertain if bone stimulation by Laser light is a general effect or if the isolate stimulation of osteoblasts is possible.[31]

It is concluded that LLLT, carried out with the parameters of the present investigation, resulted in a positive biomodulatory effect on the healing of bone defects on the femur of the rat submitted to autologous bone graft being this effect more evident when Laser irradiation is performed on the surgical bed trans-operatively prior the placement of the autologous bone graft and post-operatively.

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