Effectiveness and Acceleration of Bone Repair in Critical-Sized Rat Calvarial Defects Using Low-Level Laser Therapy

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Background and Objective: Tissue regeneration remains a challenge for orthopedic and craniomaxillofacial surgery to treat bone loss. The use of low-level laser therapy suggests a promise on this road with positive results for narrow defects. However, temporal and quantitative evaluations are required to understand the healing process of large injuries. The aim of this study was to investigate the repair of critical-size bone defects in rat calvaria using a GaAlAs laser.

Study Design/Materials and Methods: Bone defects (9 mm in diameter) were created on the skull of 30 Wistar rats separated in control or irradiated group. GaAlAs laser (λ = 830 nm, energy density = 2.5 J/cm² and output power = 50 mW) was applied after surgery and six times more at 48 hours intervals. The animals were euthanized after 2, 4, and 8 weeks. Digital radiographs, descriptive histological and histomorphometric analyses were carried out.

Results: Radiographic analysis showed greater bone formation in the irradiated group than control at 8 weeks, covering 45% and 28% of the defect, respectively (P < 0.05). Histological analysis showed that the irradiated groups had a higher amount of bone neoformation and greater maturity at 4 and 8 weeks. Histomorphometric analysis showed that the volume density of bone tissue at 4 weeks in the irradiated group was two times higher than the control (P < 0.01).

Conclusion: The photobiomodulation of low-level laser therapy using 830 nm wavelength light was effective in promoting bone healing in critical defects despite the unfavorable prognosis as well as it accelerated the maturation of bone tissue. Lasers Surg. Med. 46:61–67, 2014.

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Key words: low-level laser therapy; bone repair; critical-size defects

INTRODUCTION

According to the World Health Organization there are more of 150 diseases and syndromes related to skeletal and joint problems. Despite the great progress in development of surgical approaches and bone substitutes with their osteopromotive, osteoconductive or osteoinductive properties, there still is not established “gold standard” clinical technique to mimic the performance of autogenous cells. Tissue regeneration remains a challenge for orthopedic and craniomaxillofacial surgery to treat severe bone loss and to improve the quality of life for these patients [1].

In response to this demand, photobiomodulation or low-level laser therapy (LLLT) needs to be considered for promoting the bone repair process without thermal damage or tissue injury [2–6]. It is believed that the main structures responsible for absorption of light are proteins and their photobiomodulation involve reactions in cell membrane (by regulation of signal-transduction pathways) and organelles (e.g., mitochondria), which would generate acceleration of cellular metabolism. A short-term evaluation

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demonstrated acceleration of ATP synthesis (glycolysis and oxidative phosphorylation) and a long-term period had increased DNA synthesis and cell proliferation [7].

Beyond the common effects of LLLT as improving tissue repair in skin and mucosa, anti-inflammatory and analgesic behavior and increasing local microcirculation, due to its greater penetrating power is also used in inflammatory diseases of the joints, to treat muscle injuries, to repair peripheral nerves and to improve bone repair [2–6,8–11].

The isolated use of LLLT has been described as a method that can promote bone repair [2–5]. GaAlAs laser in different animal models has been increasingly studied in vivo with promising results in various experimental sites [3–5,8,9,12–15]. Histomorphometric study in rats indicated an orthodontic movement in molars faster in irradiated group ($\lambda = 830$ nm, energy density $= 18$ J/cm$^2$), with increased bone deposition (overexpression of labeled cells by PCNA) on the traction zone and a larger number of osteoclasts in the compression zone [15]. GaAlAs laser ($\lambda = 904$ nm) on rat mandibular bone promoted an increased transport of calcium from bone callus with output power $= 4$ mW/cm$^2$ and energy density $= 0.72$ J/cm$^2$ at 2 and 4 weeks, while a higher incidence (output power $= 22.4$ mW/cm$^2$, energy density $= 4.32$ J/cm$^2$) did not cause any effect under biochemical analysis [9]. Bilateral defects on rat calvaria (2.7 mm) using laser (energy density $= 3$ J/cm$^2$ daily for 7 days afterwards surgery) presented a greater amount of calcium, phosphorous and proteins, as well as increased revascularization and connective tissue at 14 and 28 days indicating the positive effect of biostimulation of GaAlAs laser [4]. Femoral bone defects (1 mm) in Wistar rats submitted to laser application ($\lambda = 660$ nm, energy density $= 10$ J/cm$^2$ every 2 days) had higher cellular activity surrounding them, increased osteoblastic activity in 5 days, higher mineral apposition rate in 15 days and increased osteoclastic activity in 25 days, however, without changes in the architecture of normal bone structure [3]. Femoral defects (2 mm) after use of laser ($\lambda = 830$ nm, energy density $= 4.8$ J/cm$^2$) showed difference at 7-days follow-up, with higher amount of neoformed trabecular bone in irradiated group, suggesting that the laser have favored the process of bone repair in the early phase of healing [10]. Rabbit tibial fractures received 4 daily applications of laser ($\lambda = 830$ nm) in the region of the fracture (cranial, caudal, medial and lateral, energy density $= 40$ J/cm$^2$ per session) and displayed at 3 and 4 weeks using X-ray microtomographic evaluation greater bone remodeling in irradiated group and under histological analysis a lesser amount of fibrocartilage surrounding the fracture and increased bone volume by stimulating both from peristomeum and endosteum [14]. Osseointegrated implants (5.5 mm × 10 mm) placed in iliac crest of baboons after 5 days of exposure to laser ($\lambda = 690$ nm, energy density $= 6.5$ J/cm$^2$) already had a larger number of osteocytes and vital bone around themselves than control group, without influence on peri-implant bone resorption in this short period of time [12].

The association of LLLT with biomaterials displays a potential synergistic effect for bone repair. Laser ($\lambda = 830$ nm, energy density $= 16.5$ J/cm$^2$ per session, every 48 h during 15 days) associated to bovine organic bone [2,6] or synthetic hydroxyapatite [6,8] and demineralized freeze-dried membrane, in the treatment of femoral bone defects of Wistar rats, promoted greater bone formation and deposition of collagen fibers in 15 days and greater density and organization of cancellous bone in 30 days [2,6,8]. Under the previous experimental conditions, the same positive effect occurred with the use of laser and its combination to bone xenograft, membrane and growth factors (BMP) [5]. In the same murine model, trans-operative use of laser (energy density $= 10$ J/cm$^2$) on autologous bone graft and its daily supplementation for 15 days also promoted earlier maturation and more advanced level of tissue repair [11].

However critical-size defects (CSD) treated with LLLT has not yet been described in the literature. Extensive bone loss or critical bone defects are those that do not repair spontaneously during the lifetime of the animal or human. The creation of CSD in animal models aids the development of better assessment techniques and materials that positively influence bone healing [16–24]. The objective of this study was to investigate the response of bone tissue in CSD on rat calvaria after GaAlAs laser irradiation.

**MATERIALS AND METHODS**

**Experimental Groups and Surgical Procedure**

For this study, 30 Wistar rats (*Rattus norvegicus albinus*), adult males, $w = 250–300$ g, 50 days old, were chosen. Rats were provided by the Center of Laboratory Animals (NAL-UFF). They were monitored daily to verify their general condition and maintained throughout the experiment in appropriate boxes, respecting light and dark cycles and receiving a balanced diet and water ad libitum. All handling and experimentation procedures followed the norms of the Brazilian College of Animal Experimentation (COBEA). This project was approved by the Ethics Committee on Animal Research, Fluminense Federal University (CEPA-UFF, registration protocol #002/07).

All surgical procedures were conducted under general anesthesia by intramuscular injection in the rectus femoris muscle with a solution (ratio 3:1) of 60 mg/kg ketamine (Clortamina$^\circledR$ 50 mg/ml, Pharmaceutical Biochemistry Institute Ltda, RJ, Brazil) and 15 mg/kg xylazine (Dopas$^\circledR$ 20 mg/ml, Animal Health Hertape Calier S/A, MG, Brazil), with a dose of 0.2 ml/100 g weight of the mixture. When necessary, an incremental dose of $\frac{1}{3}$ of the original was established to deep the anesthesia [10,19].

After trichotomy and antisepsis, a half-moon shaped skin incision and full-thickness flap were made on each rat calvaria. Using a trephine drill with a 9 mm-external diameter (National Implant System-SIN, SP, Brazil), a bicortical craniotomy was performed in the interparietal region, exposing the dura matter (Fig. 1A). The osteotomized fragment was gently removed to avoid any injury to the dura matter or brain. The flap was repositioned and sutured with nylon 5.0 (Ethicon$^\circledR$, Johnson & Johnson, SP, Brazil) and the rat skin was marked with a dermographic pen to facilitate the application of laser (Fig. 1B).
The animals were randomly divided into two experimental groups: control (GI) and irradiated (GII). Three experimental periods (2, 4, and 8 weeks) were defined with each condition containing five animals. A GaAlAs diode laser (Inova®, Laserline, SP, Brazil) was superimposed on the rat skin, in a continuous mode of application ($\lambda = 830$ nm, output power $= 50$ mW, energy density $= 2.5$ J/cm$^2$, diameter of fiber-optic output $= 9$ mm, $t = 45$ s) (Fig. 1C). The irradiation protocol began afterwards the surgery and followed six times more at 48 hours intervals. The 48 hour-interval application was adapted from other studies [2,5,8,11]. The animals were euthanized with an overdose of anesthesia.

X-ray Analysis

The necropsies were fixed in 4% buffered formalin at pH 7.2 for 48 hours. Then radiographs of the rat calvarias were computed at the Nuclear Instrumentation Laboratory, Federal University of Rio de Janeiro (LIN-COPPE-UFRJ). A microfocus equipment (FXS-100.10®, FeinFocus, CT) and a desk scanner (CR-50P®, GE IT System, WI) were used following the parameters described: 40 kV (tension), 125 $\mu$A (electric current), 600 seconds (exposition time), 2 m (source-detector distance), 40 cm (source-object distance), $\times 5$ (magnification factor), 50 $\mu$m (pixel size) and 10 $\mu$m (focal spot size of X-ray).

The images were analyzed with Image Pro-Plus® 6.0 software (MediaCybernetics, CA) to determine the area of bone repair in the rats. The total radiopaque area was accounted by manual marking of the defect, characterization of black and white in the radiograph on a gray scale and accounting for white areas. The effect of time and treatment on bone healing between groups was evaluated using InStat 3.01® and Prism 5.0® softwares (GraphPad), previously calibrated in micrometers/pixel and with a superimposed 100 points grade. This procedure was performed by two calibrated independent examiners, which means the maximum variation was less than 0.01% (paired t-test, InStat 3.01®, GraphPad).

Statistical Analysis

The means and standard deviations obtained were submitted to variance analysis (ANOVA) using InStat 3.01® and Prism 5.0® softwares (GraphPad), defining significant differences if $P < 0.05$ (Tukey test). In cases where the standard deviations of groups were significantly different the nonparametric Kruskal–Wallis test was applied.

RESULTS

X-ray Analysis

The radiographic images obtained showed a pattern of bone growth from the edges of the CSD, irregular in their distribution. There was a strong change in optical density represented by shades of gray in the same group of animals tested, as well as the presence of islets with higher optical density suggesting new bone. The variation in the area of

Fig. 1. Critical-size defect and GaAlAs laser. (A) Superior view of skull of the animal with the osteotomized area (9 mm) and (B) markings on the rat skin and lateral delimitation sutures. (C) Application of laser and positioning of the laser pen on the rat skin.
newly formed bone was regular in GI in opposition to GII, which had a significant increase in the bone area when compared 2 weeks to 4 weeks (2.5 times; \(P < 0.01\)) and 8 weeks (\(P < 0.05\)). In relation to the effect of treatment between the groups, GI and GII had significant difference only at 8 weeks (\(P < 0.05\)), with 28.4 ± 6.5% and 44.8 ± 15.6% of the closure of the CSD, respectively (Fig. 2).

**Descriptive Histological Analysis**

At 2 weeks, the inflammatory reaction was mild in GI, with the trabecular bone formation from the edge toward the middle of CSD and also growing in its thickness. There was a slight increase of new bone formation in GII with characteristics of more mature bone than GI. The presence of congested vessels of large and small caliber and reversal lines was noted in both groups (Fig. 3A and B).

At 4 weeks, GI showed trabecular bone formation with a high number of osteocytes and cortical bone formation in margins of the CSD, while in GII there was a larger amount of bone formation with a greater presence of compact bone in the periphery and few trabecular spaces. In this group the bone growth was more pronounced and obvious toward the center of CSD with the formation of islets of bone tissue than bone growth toward the endosteum and periosteum, with characteristics of mature (cortical) bone (Fig. 4A and B).

At 8 weeks, presence of newly formed bone with few marrow spaces, and a moderate amount of reversal lines in GI (Fig. 5A) with few osteoblasts and occasional presence of osteoclasts. In GII a greater amount of newly formed bone was noted with characteristics of compact bone (toward the center of the lesion), and islets of new bone. This group had the lowest osteoblastic paving, and the presence of larger amount of mature bone becoming less clear the margin of the CSD, with moderate amount of reversal lines (Fig. 5B and C) and osteoclasts.

**Histomorphometric Analysis**

The volume density of connective tissue decreased significantly in GI when compared 2 and 8 weeks (20.6 ± 6.3% and 15.8 ± 5.2%; \(P < 0.05\)) in opposition to GII wherein there was no significant intra- or inter-group variance. Similarly, there was a significant decrease in the density of newly formed vessels in GI when compared 2 and 8 weeks (1.1 ± 0.9% and 0.8 ± 0.9%; \(P < 0.05\)) in opposition to GII wherein there was no significant intra- or inter-group variance (data not shown).

The density of new bone tissue showed a significant increase in GII between 2 and 4 weeks (3.5 times; \(P < 0.02\)), but the difference was not significant in GI (\(P > 0.05\)). When comparing GII and GI, the density of bone volume was about two times higher in GII than GI within 4 weeks. There were no significant differences at 2 and 8 weeks (Fig. 6).

**DISCUSSION**

Standardized protocols for research and universalization of the LLLT were not done yet and for this reason, it could be complicated the interpretation of heterogeneous outcomes with different devices and, consequently, it becomes a challenge to the reproducibility and transferability of LLLT to other experiments. [4,5,9,12–15].

The positive correlation between the application of LLLT and the acceleration of bone repair is widely described in literature [4–8,10,14,15]. However, bone defects with small diameter are not reliable to demonstrate the quantitative contribution of lasers as biomodulatory therapy for bone repair. Thus, there is still controversy regarding the actual effectiveness of laser therapy in bone repair [3–5,8,11–13].

In this study, we demonstrate that LLLT intensified and accelerated bone healing, especially at 4 weeks wherein there was a marked reduction of bone marrow spaces and at 8 weeks with a greater amount of newly formed compact bone and reversal lines indicating maturation of bone tissue. It is important to note that unlike other studies [2,3,5,8–10,14], the use of CSD is a more difficult model for bone repair than competitor tissues, since the loose connective tissue that fills the defect is rich in fibroblasts which release cytokines decreasing the osteogenesis [25].

The radiographic analysis suggested bone growth from the edge to the center of the defect, as previously reported in other studies [16,19,20,22], confirming at 8 weeks a significant area of bone tissue formation versus control. Interestingly, there was greater bone formation for both groups in the posterior side of CSD due to already expected greater neovascularization by middle meningeal artery that presents larger caliber in this rat anatomical region. Histomorphometric analysis showed the volume density of bone tissue was about 6% or two times higher in irradiated group at 4 weeks. Considering that the normal bone repair

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Fig. 2. Digital radiographs of experimental groups. Note the greater degree of bone formation in the GaAlAs laser group (GII) than control (GI).
of CSD on rat calvaria varies from 2% in 4 weeks [21] to 4–7% in 8 weeks [18,21], our study confirms the importance of GaAlAs laser in bone biomodulation. We credited the heterogeneous results between radiographic and histomorphometric analyses to their different anatomical planes. For radiographic analysis, assessment was carried out throughout the area of the defect, while for histomorphometry were performed cuts in the central portion of CSD with a laterolateral perspective. Anyway, similar intergroup variances were found in both methods highlighting the effect of GaAlAs laser.

The time of laser application, which was performed until the second postoperative week in our study, may lead to a greater stimulus of tissue repair in the initial periods by generating a higher cell proliferation, as previously described [5,8–10,14]. It is possible to hypothesize that the maintenance of laser applications for a long-term period of the lifetime of the animal could promote more intense bone repair [5].

For our study, we created a pen applicator with 9 mm in diameter to encompass the entire CSD, allowing the equivalent stimulus across the border of the defect. This

Fig. 3. Histological analysis. Photomicrographs of the (A) control group/GI and (B) GaAlAs laser group/GII. At 2 weeks note a greater presence of blood vessels (V) and a small degree of bone neoformation (ON). The arrows indicate the borders of the bone defect. LR, reversal lines and AC, cutaneous appendages.

Fig. 4. Histological analysis. Photomicrographs of the (A) control group/GI and (B) GaAlAs laser group/GII. At 4 weeks, note a significantly greater quantity of neoformed bone (ON) in the GaAlAs laser group. Formation of cortical bone (OC) in the bone defect margin.
strategy is newness into literature since studies published have only reported point applications in specific areas and wherein it is difficult to determine if the entire area of bone damage was stimulated by laser [3–5,8,9,12–15]. Furthermore, the available literature does not determine the values of scattering in different biological tissues, since there are no reports of such measurements in living tissue. On this way, other studies should be performed to understand the cellular and molecular effects of GaAlAs laser on the bone repair, including irradiation protocols, long-term evaluations and quantitative analyses.

CONCLUSION

The use of low-level laser therapy is safe and does not cause tissue pathologic changes. LLLT using 830 nm wavelength promotes the acceleration and increase of bone repair and the development of more mature bone tissue than the control group.

REFERENCES


