Effect of Low-Level Laser Therapy on the Healing of Sites Grafted With Coagulum, Deproteinized Bovine Bone, and Biphasic Ceramic Made of Hydroxyapatite and ß-Tricalcium Phosphate. In Vivo Study in Rats

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Objective: The aim of this study was to evaluate the effect of low-level laser therapy (LLLT) on the healing of biomaterial graft areas (i.e., coagulum, deproteinized bovine bone, and biphasic ceramics comprising hydroxyapatite and ß-tricalcium phosphate).

Material and Methods: Ninety rats were divided into two groups according to laser irradiation use (λ 808 nm, 100 mW, ϕ ~ 600 μm, seven sessions with 28 J of irradiation dose in total): a laser group and a control group. Each of these groups was divided into three subgroups of 15 animals each according to the type of biomaterial used: Coagulum (COA), deproteinized bovine bone (DBB), and hydroxyapatite/ß-tricalcium phosphate (HA/ßTCP). Biomaterials were inserted into Teflon domes, and these domes were grafted to the lateral aspect of the mandibular branch of the rats. The animals were sacrificed after 30, 60, and 90 days. Scarring patterns were evaluated by microtomography and histometry. The expression levels of BMP2, osteocalcin (OCN), and alkaline phosphatase (ALP) were evaluated by immunohistochemistry. The mRNA expression levels of ALP, BMP2, Jagged1, Osterix, Runx2, and TGFβ1 were determined by RT-qPCR.

Results: The animals treated with LLLT exhibited increased mineralized tissues and bone, particularly after 90 days. These increases were associated with increased BMP2, OCN, and ALP protein expression and ALP, BMP2, and Jagged1 mRNA expression.


Key words: bone repair; bone substitutes; low-level laser therapy; pre-clinical study

INTRODUCTION

Damaged bone tissues have high regenerative potential due to high plasticity induced by remodeling mechanisms, which depend on the vascular supply and mechanical stability of wounds [1]. However, in some situations, including trauma-induced defects and resective surgical procedures, the extent of a defect to be repaired may limit their complete healing [2,3]. Because the formation of critical defects can influence the quality of life of patients, the use of biomaterials as bone substitutes may be required to stimulate repair [4].

Among the biomaterials proposed for the treatment of bone defects, the use of autogenous grafts has been considered the gold standard because of the combination of osteoinduction, osteoconduction, and osteogenesis properties [5]. However, due to technical limitations and induced morbidity at the host donor site, uses of other types of biomaterials have been considered [4,5]. Biomaterials of xenogeneic (bovine) and alloplastic origin have been extensively used and have shown good results in repairing bone defects in surgically resected areas [6,7], in maxillary sinus floor augmentation [8], and in bone reconstruction before placement of dental implants [9,10]. Although osteoconductive biomaterials have produced favourable clinical outcomes, their biological capacity for bone formation solely via osteoconduction decreases the formation of vital bone tissue compared with the use of autogenous bone grafts [11]. For this reason, these biomaterials have been combined with autogenous bone [12] and growth factors [13] to improve the characteristics of bone tissue repair.

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However, limitations in the use of autografts [5,14] and the high cost-to-benefit ratio of the use of growth factors [15] limit the clinical applicability of these associations.

The use of low-level laser therapy (LLLT) with diode lasers has been shown to improve wound repair [16], management of muscle lesions [17], and management of nervous tissue lesions [18]. Moreover, LLLT stimulates the formation of blood vessels [19], decreases the levels of pro-inflammatory cytokines [20], and reduces bacterial load during treatment of infectious diseases when associated with a photosensitizer [20]. In bone tissue, LLLT stimulates several physiological processes, including fracture repair [21], osseointegration of implants [22,23], repair of periodontal tissue [24], and repair of non-critical defects filled [25,26] or not filled with biomaterials [27,28].

Although preclinical studies have evaluated the effects of LLLT on the healing of grafted areas, many previous models involved non-critical defects, which heal spontaneously without the use of biomaterials [26,27,29]. However, this type of healing hardly occurs in daily clinical practice. Additionally, little is known about molecular events specific to bone metabolism associated with bone repair induced by LLLT in areas grafted with osteoconductive biomaterials. Therefore, this study aimed to evaluate the effects of LLLT on the healing of areas grafted with biomaterials of xenogeneic and alloplastic origin and coagulum and to further evaluate the effects of LLLT on biological markers of osteoblast activity and differentiation.

MATERIALS AND METHODS

Distribution of Animals and Groups

Ninety adult male rats (Rattus norvegicus albinus, Holtzman) with approximately 3-months age, with body weights between 200 and 250 g, were reared in the animal facility of the School of Dentistry of Araquara (Faculdade de Odontologia de Araquara—FOAr) of the State University of Sao Paulo (Universidade Estadual Paulista—UNESP). The animals were fed with solid chow and water ad libitum before and during the experimental period under controlled conditions of light and temperature. The rats were maintained in propylene cages with five animals each. The study was approved by the Animal Research Ethics Committee (Comissão de Ética no Uso de Animais—CEUA) of FOAr-UNESP under CEUA No. 08/2014 and was conducted according the ARRIVE guidelines.

The animals were randomly divided into two groups according to the use of laser irradiation: a control group and a laser group. Each of these groups was subdivided into three groups (n = 15) according to the type of biomaterial used: coagulum (COA), deproteinized bovine bone (DBB; Bio-Oss®, Geistlich AG, Wolhusen, Switzerland), and biphasic ceramic comprising hydroxyapatite and β-tricalcium phosphate (HA/βTCP; Straumann® Bone Ceramic, Straumann AG, Basel, Switzerland). The animals were euthanized 30, 60, and 90 days after surgery (n = 5 animals per group per period; Fig. 1).

Surgical Procedure

After 1 week of acclimation to the rearing conditions, the animals were anesthetized with a combination of ketamine-xylazine at 0.08 ml/100 g of body weight of ketamine hydrochloride (Francotar, Virbac do Brasil Ind., Com., Ltda.) and xylazine hydrochloride at 0.04 ml/100 g of body weight (Virbaxyl at 2%, Virbac Brazil Ltd., São Paulo, Brazil). Subsequently, the mandibular branch of each animal was subjected to bilateral trichotomy.

Horizontal incisions were performed on the outer part in the lower region of the mandibular branch, and the muscle tissue and periosteum were detached to expose the lateral aspect of the ascending mandibular branch of each animal. Four perforations of 0.5 mm in diameter were made using a round bur exceeding the bone plate. These perforations were parallel to the base of the jaw and were made 6 mm from each other to form the edges of a square. The region between the perforations was scarified with the same bur to promote bleeding in the region. A dome-shaped Teflon capsule with an outer diameter of 5 mm, a height of 2.5 mm, and a peripheral ring of 1 mm was placed on the lateral aspect of the jaw so that the ring superimposed the perforations (two capsules per animal, one on each side). A 0.032 mm³ volume of the biomaterial was inserted in the internal region of the dome. The capsules were fixed in the jaw using 4.0 silk sutures (Ethicon, Johnson Products, São Paulo, SP, Brazil), and the sutures pierced the capsule and perforations made on the mandibular branch (Fig. 2) [30]. Each animal received the same type of treatment at random on both sides of the jaw. The soft tissues were repositioned on the capsule and sutured with Vicryl 4.0 (Ethicon, Johnson Products). After surgery, the animals received an intramuscular dose of veterinary pentabiotic at 0.3 ml/kg (Zoetis Dodge, São Paulo, SP, Brazil, subcutaneous) and an intramuscular dose of ketoprofen at 0.3 ml/kg (Ketoflex, Mundo Animal, São Paulo, SP, Brazil). After 30, 60, and 90 days of surgery, the animals were sacrificed by anaesthetic overdose. All the surgical procedures were performed by a single operator (G.J.O.).
Low-Level Laser Therapy

A gallium aluminium arsenide laser (Therapy XT, λ 808 nm, 100 mW, beam divergence 0.37 rad, φ ~600 μm, CW, DMC Equipment, São Carlos, SP, Brazil) was used during the radiation therapy. The treated area was defined with a marker pen after suturing the surgical site. Four points 3 mm equidistant were marked to delimit the entire area of the dome, and these points served as guides for laser irradiation. Laser irradiation was conducted transcutaneously with the laser tip in contact with the skin for 10 seconds at each point, totalling 40 seconds of irradiation per session. Seven sessions were conducted, and these sessions were repeated every 48 hours for 13 days after surgery [26,31]. The first session was applied immediately after surgery after the tissues were sutured (Fig. 1). The energy of irradiation at each point was 1 J, which amounted to 4 J of energy of irradiation per session and 28 J of energy of irradiation during treatment on each side. The energy fluence at each point of irradiation was approximately 354 J/cm².

Samples

After euthanizing the animals, samples of the mandibular branch together with the domes were collected on both sides. One side was immediately placed in liquid nitrogen and maintained at −80°C for reverse transcription polymerase chain reaction (RT-qPCR) analysis. The other side was placed in a 4% buffered paraformaldehyde solution for 48 hours and then in a 70% alcohol solution until microtomographic analysis. The side used for each analysis was selected at random.

Microtomography

The mandibular branches were scanned using a SkyScan device (SkyScan, Kontich, Belgium) with the following parameters: Camera Pixel: 12.45; x-ray tube potential: 65 kVP, X-ray intensity: 385 μA, integration time: 300 ms, filter: Al-1 mm, and voxel size: 18 μm. The generated images were later rebuilt, spatially reoriented, and analysed using a specific software (NRecon/DataViewer/CTan, SkyScan, Aartselaar, Belgium). The volume of the mineralized tissues was calculated within a region of interest (ROI), which encompassed the entire tissue between the dome and the lateral aspect of the mandibular branch (Fig. 3A). A gray scale threshold range was set at 65–255 to determine the volume of mineralized tissue within the ROI. This analysis was conducted by a trained examiner (M.A.T.) who was blinded to the experimental groups.

Histomorphometry

After microtomographic analysis, the samples were decalcified in 7% ethylenediamine tetraacetic acid (EDTA) and processed for paraffin embedding. The samples were cut in the central region and embedded in the transverse plane. Serial sections were made with thicknesses of 5 μm, and five slides with three specimens each were stained with haematoxylin-eosin (HE). Three equidistant sections (at a distance of 20 μm from each other) were selected, and the first section was selected at random. Histological images were acquired using a DIASTAR optical microscope (Leica Reichert and Jung Products, Wetzlar, Germany) at a magnification of 25× and were subsequently measured using ImageJ image analyser software (San Rafael, CA). The percentages of bone and biomaterial in the space between the dome and the lateral aspect of the mandibular branch were calculated (Fig. 3B). This analysis was conducted by an experienced examiner (G.J.O.) who was trained and blinded to the experimental groups.

Immunohistochemistry

Histological sections with thicknesses of 5 μm were mounted on silanized slides (Fisher Scientific Superfrost...
Plus, Fisher, New York, NY). After deparaffinization and rehydration of the sections, antigen retrieval was achieved by dipping the slides in a microwave-heated buffer solution of potassium citrate and cooling at room temperature. Endogenous peroxidase was inactivated by immersing the tissue sections in hydrogen peroxide solution at 1% for 30 min, and unspecific epitopes were blocked by incubating the slides in a 3% bovine serum albumin (BSA) solution for 1 hour. Subsequently, the sections were immersed in primary antibodies against bone morphogenetic protein 2 (BMP-2) at 1:400, osteocalcin (OCN) at 1:400, and alkaline phosphatase (ALP) at 1:800 (Abcam, Cambridge, UK) for 16 hours. The sections were then treated with the avidin-biotin-peroxidase complex (ABC, DAKO, Glostrup, Denmark) using the ABC Staining System kit (DAKO) according to the manufacturer instructions. Sections immersed in 1% PBS served as negative controls. The sections were stained with HE for nuclei visualization. Quantitative analysis was conducted in two histological sections per animal to calculate the number of cells that expressed proteins. Three areas were selected at random, two areas in the lower region of the dome covering part of the bone on the lateral aspect of the mandibular branch and one area in the upper region (Fig. 3C). These analyses were conducted under a DIASTAR optical microscope (Leica Reichert-Jung and Products, Wetzlar, Germany) at a magnification of 400×. The arithmetic average of the three values was considered the value of each sample. These experiments were conducted by an experienced examiner (G.J.O.) who was trained and blinded to the experimental groups.

RT-qPCR

Total RNA was extracted from the tissues of the grafted area (RNAqueous-4PCR kit, Ambion, Inc., Austin), quantified, analysed for purity in a spectrophotometer, and converted to cDNA using the High-Capacity cDNA Synthesis kit (Applied Biosystems, Foster City). The RT-qPCR reaction contained 1 μl of cDNA, 8 μl of nuclease-free water, 10 μl of TaqMan gene expression master mix (Applied Biosystems) and 1 μl of TaqMan gene expression assays (Applied Biosystems) for the mouse target genes, and the reaction was performed in the Step One Real-Time PCR System (Applied Biosystems). The relative expression levels of genes runt-related transcription factor 2 (Runx2), Jagged1, ALP, Osterix, BMP-2, and transforming growth factor beta 1 (TGFβ1) were calculated using the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as a normalizer.

Statistical Analysis

Data obtained in this study were analyzed using the Kolmogorov–Smirnov normality test. Data from the microtomographic, histometric, and immunohistochemical analyses showed normal distributions, whereas data from the RT-qPCR analysis had a non-normal distribution. Unpaired t-tests were used for the analysis of the normal data, and the Mann–Whitney test was used for the analysis of the non-normal data. Comparisons were made between the same subgroups from each study group (laser and control). GraphPad Prism software version 5.0 (San Diego, CA) was used for the statistical tests. All tests were applied with a confidence level of 95%.

RESULTS

All animals recovered well from the operation, and there was nothing remarkable regarding the post-operative healing period. Thus, all of the animals were included in all the analysis performed. A previous study that compared the bone repair in grafted areas with bioglass and DBB in an experimental model similar to that applied in this study showed that the minimum significant difference in the averages of

Fig. 3. Flowchart of the microtomographic, histometric, and immunohistochemical analyses. (A) Microtomography: The volume of mineralized tissue was assessed within a region of interest (ROI), which encompassed the tissue present between the lower surface of the dome and the lateral aspect of the mandibular branch (highlighted in red); (B) Histometry: The amount of bone tissue and biomaterial (black arrows) was calculated as the percentage of the total area of the dome (delimited area); (C) Immunohistochemistry: a quantitative analysis was conducted to calculate the number of stained cells present in three randomly selected areas, two on the base of the dome, covering part of the bone tissue in the lateral aspect of the mandibular branch, and one area in the upper portion (delimited by squares with black margins).
percentage of bone formed evaluated by the histometric analysis found was 10.5% associated with the mean standard deviation of 0.45% [32]. Thus, the use of five animals per group/period provided an α-type error of 0.05 and β-power higher than 0.90.

**Microtomography**

The amounts of mineralized tissue in the animals treated with LLLT were greater than those in the control group at 90 days in all the subgroups. Furthermore, the laser group presented higher volume of mineralized tissues than the control group at 60 days in the COA subgroup. (Fig. 4).

**Histometry**

The laser-irradiated group presented higher amounts of bone in the grafted area than did the control group for all subgroups and study periods, except for the HA/βTCP subgroup at 30 days. Furthermore, the grafted areas in subgroups DBB and HA/βTCP of the laser-irradiated group had fewer amount of biomaterial than those in the control group at 60 days (Fig. 5).

**Immunohistochemistry**

The expression levels of OCN, ALP, and BMP2 proteins in the laser-irradiated group were higher than those in the control group at 30 days. The higher expression of ALP in the laser-irradiated group persisted until 90 days in subgroups DBB and HA/βTCP. However, the expression levels of the other proteins become higher in the control group. The expression of OCN was higher in the HA/βTCP subgroup at 60 days. The expression levels of OCN and BMP2 were higher in the COA and DBB subgroups at 90 days. The graphs of the immunohistochemistry analyses are shown in Figure 6, and the representative images are shown in Figure 7.

**RT-qPCR Analysis**

At 30 days, the mRNA expression levels of Jagged1 and Runx2 in the laser group were higher in the tissues grafted with DBB and HA/βTCP. In this same period, the laser group presented higher mRNA expression levels of BMP2 (COA subgroup) and ALP (COA and DBB subgroups) than the control group, whereas the control group had a higher mRNA expression of TGFβ1 (HA/βTCP subgroup) than the laser group. At 60 days, the control group had higher mRNA expression levels of Jagged1 (COA subgroup), Runx2 (COA and DBB subgroups), and TGFβ1 (all subgroups) than the laser group. At 90 days, the laser group had higher mRNA expression levels of ALP (HA/βTCP subgroup), Jagged1 (HA/βTCP subgroup), and BMP2 (COA and HA/βTCP subgroups) than the control group, whereas the control group had higher mRNA expression levels of Runx2 (DBB subgroup) and TGFβ1 (COA and DBB subgroups) (Fig. 8).

**DISCUSSION**

Improvements in clinical results with the use of osteoconductive bone substitute biomaterials aim to produce outcomes similar to those obtained with autogenous bone grafts with respect to the period necessary to achieve the proper maturation of the repaired tissue. LLLT improved bone healing associated with the use of DBB and HA/βTCP. The result presented herein indicate that LLLT is a promising therapy for the stimulation of bone tissue repair and the improvement of the quality of the grafted area.

The model used to evaluate the bone repair associated to the use of biomaterials was previously used successfully in

![Fig. 4. Microtomography images and graphs. A greater amount of mineralized tissue in the laser group in the later period of evaluation can be observed.](image-url)
Fig. 5. Histological images and graphs of the histometry analysis. Note that the repaired tissue had a greater amount of bone tissue in the animal treated with LLLT. Despite the increase in the resorption of biomaterials induced by LLLT at 60 days, this difference was not maintained at 90 days (original magnification at 25× with haematoxylin-eosin).

Fig. 6. Immunohistochemistry graphs. The expression levels of BMP2, ALP, and OCN proteins were higher in the laser group at 30 days, whereas the expression levels of BMP2 and OCN were higher in the control group at 90 days.
other studies and would more efficiently to mimic the use guided bone regeneration technique in critical defects [30,32,33]. The use of Teflon has been shown to cause no adverse effects in pre-clinical studies [30,32]. In addition, this material has been used successfully in clinical studies of guided bone regeneration associated with the use of non-resorbable membranes [34,35].

The microtomographic analysis revealed an increase in the mineralized tissue in the animals subjected to LLLT, particularly in the longer study periods. The finding that the amount of mineralized tissue was greater in the COA subgroup from the laser group and the results of histometric analysis demonstrate that the increase in mineralized tissue was associated with increased bone formation in the sites subjected to LLLT. These results are consistent with those of previous studies, which demonstrated that LLLT stimulated bone repair in grafted areas [3,4,25,26,36], osseointegration [22,23,37], and fracture repair [21].

The histometric analysis indicated that the degradation of particles from both evaluated biomaterials increased in the laser group compared with the control group at 60 days.

![Fig. 7. Immunohistochemical images after 30 days of surgery. The staining of BMP2 and ALP proteins was higher in the periosteum and regions near blood vessels and biomaterials. OCN was expressed in all three evaluated areas, osteocytes, and areas of the mineralized matrix. The expression levels of all proteins were higher in the laser group after 30 days of surgery. The red arrows indicate the regions with higher protein expression (original magnification at 400×).](image)

![Fig. 8. Graphs representing mRNA expression of ALP, BMP2, Jagged1, Osterix, Runx2, and TGFβ1. The expression of the genes that encode proteins involved in osteoblastic activity (ALP and BMP2) was higher in the laser group than in the control group throughout the study period, whereas the expression of the genes that encode proteins that induce osteoblastic differentiation (Jagged1 and Runx2) was higher in the laser group in the early evaluation periods. These trends tended to reverse, with greater amounts of these genes in the control group in later evaluation periods. The expression of the TGFβ1 gene was higher in the control group than in the laser group. Furthermore, no significant differences in the expression of genes that encode Osterix were observed.](image)
The use of osteoconductive biomaterials is known to induce less formation of bone tissue in the grafted areas than in ungrafted areas. When biomaterials are not resorbed, they occupy the space of the new bone [38]. This result was observed in our study, wherein the COA subgroups had nearly twice the amount of bone tissue (approximately 60%) than the subgroups that received bone grafts at 90 days (20–30%). However, the microtomographic analysis demonstrated that the grafted areas presented higher amounts of mineralized tissue, which may indicate that the use of osteoconductive biomaterials is clinically more important for the achievement or maintenance of the morphology and proper function of bone than for the formation of bone tissue [39]. In fact, despite the accelerated degradation of the biomaterials, at the 90-day period of analysis, the amount of biomaterial in the grafted sites was not significantly different between the laser and control groups. This indicated that LLLT increased the osteoconductive potential of the biomaterials and maintained the volume of the biomaterial in the final period of the analysis. These findings have been reported in other preclinical studies [1,4,25].

The expression levels of BMP2, ALP, and OCN proteins were higher in the group subjected to LLLT, which may be the reason for the occurrence of greater bone formation in the laser group compared with the control group. Moreover, increases in the expression of genes associated with differentiation (Runx2 and Jagged1) [40,41] and osteoblastic activity (ALP and BMP2) [42,43] were observed at 30 days. However, there was a reversal in the pattern of gene expression for Runx2 and Jagged1; this was associated with increased expression of OCN and BMP2 proteins in the control group at 90 days. Furthermore, the gene expression of TGF\(\beta\)1 increased in the control group compared with the laser group in all evaluation periods. These findings may be due to the initial stimulation by LLLT of mesenchymal cells involved in bone repair, which differentiated faster and initiated bone synthesis and maturation in the early study periods. This phenomenon occurred later in the control group but was not sufficient for the formation of bone tissue in the study periods evaluated. The higher gene expression of TGF\(\beta\)1 in the control group may explain the lower rate of bone formation in this group compared with the laser group because, despite the involvement of this protein in bone formation [44], TGF\(\beta\)1 also participates in the formation of fibrous connective tissue [45]. The model used in this study could have hindered the recruitment of mesenchymal cells from the bone marrow and promoted the formation of fibroblasts instead of osteoblasts in the presence of TGF\(\beta\)1.

LLLT has been considered for application in the early stages of bone repair. At this stage, the healing tissues are formed by many differentiating cells. The later reduction in the numbers of these cells decreases the potential of LLLT to maintain its osteostimulatory activity in the later stages of healing [31,46]. Therefore, this study adopted the protocol for frequency and duration of LLLT used in other studies to stimulate bone healing [26,31]. In addition to the application duration of LLLT, the irradiation wavelength should also be considered in the stimulation of bone repair [46]. The use of lasers in the infrared wavelength range is necessary for the adequate penetration of light energy because lasers that radiate in this range have higher penetration ability than lasers that radiate in the red wavelength range [46,47]. Therefore, infrared laser had the most suitable wavelength range to induce bone repair.

Despite the limitations of extrapolating data from a preclinical study for clinical practice, the results of this study demonstrate the possibility of stimulating the healing process using biomaterials of xenogeneic and alloplastic origin. This may indicate that the grafted tissue will reach maturity faster than non-irradiated grafted areas. Furthermore, these materials may shorten the period of implantation and prosthetic loading of dental implants and therefore decrease the treatment period. Patients with diseases (e.g., diabetes), with lifestyles (e.g., smoking), and who use medications (e.g., corticosteroids) that impair bone metabolism may benefit from LLLT more than healthy patients because the repair of grafted areas is impaired in these situations [48]. Indeed, LLLT has been shown to accelerate the integration of autogenous bone grafts in immunosuppressed [49] or nicotine treated animals [50]. However, future studies are required to confirm if the LLLT can improved the bone repair in the defects grafted with osteoconductive biomaterials.

This study has some limitations. Despite the extended evaluation period of this animal study, the higher expression of biomarkers of bone formation at 90 days in the control group may indicate an equivalence of treatments in later periods. However, this hypothesis could not be confirmed in this study. The ability of the repaired tissue to withstand loads was not directly assessed, and only indirect assumptions could be made about the mechanical properties of this tissue, which limits the evaluation of the capacity of the repaired tissue to receive an osseointegrated implant. Despite the clear effects demonstrated by the LLLT on bone repair it was not determinate if this protocol of LLLT is an ideal.

We conclude that the use of LLLT stimulated bone healing with the use of osteoconductive biomaterials and the formation of bone tissue in ungrafted areas. This effect was associated with higher expression of proteins and genes related to osteoblastic activity and differentiation, particularly in the early periods of wound healing.

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