Effects of low-level laser therapy on bone healing of critical-size defects treated with bovine bone graft

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1. Introduction

Bone loss in the maxillofacial region presents a challenging clinical issue, especially in the case of large defects, where their physiological regenerative capability is exceeded [1]. A variety of bone grafting or substitutes [2,3,4,5] have been suggested in order to regenerate these defects [6].

Among the materials used for bone regeneration, autogenous bone has been considered the ideal graft material [7,8,9] because of its osteoinductive, osteoconductive, and osteogenic characteristics [10]. However, its collection is associated with significant donor site morbidity, including damage to anatomic structures [11], infections [11,12], pain [13,14], hematoma formation [12,15], and unpredictable graft resorption [7,8,16]. Obtaining bone tissue from donor site sufficient to fill the defect also becomes a challenge in some complex clinical conditions that require bone regeneration in large quantity, such as bone defects resulting from trauma, infection, tumor resection, skeletal abnormalities, atrophic non-unions and osteoporosis conditions [17]. Moreover, grafts are often resorbed before osteogenesis is accomplished in large defects [15].

Consequently, a search for bone biomaterials that could replace the autologous bone, with the advantages of unlimited supply and no need for a donor site [16], has taken place [10]. Nonetheless, these are not always graced with the advantages of osteogenesis and osteoinduction inherent of the autologous grafts [10,18]. Xenogeneic bovine bone grafts (BBG) are the most commonly used material [19].
The literature reports its superior biocompatibility and osteoconductivity compared to other bone substitutes [19,22]. However, this material still lacks factors that promote osteogenesis and osteoinduction [10]. In turn, this increases healing time compared to autologous bone, which feature live cells and growth factors, fulfilling their osteogenic and osteoinductive potentials [18]. Such properties reflect positively on the time required for bone healing [24].

Low-level laser therapy (LLLT) has emerged as a strategy to accelerate the healing of bone defects treated with xenogeneic BBG [16,23] and others bone substitutes materials [25], since it can act as an osteoinductive factor [26,27]. The exact mechanism of action of LLLT on bone healing is not well understood [23], but it has been reported that it can promote angiogenesis [28] and increase local blood flow (enhancing the supply of circulating cells, nutrition, oxygen, and inorganic salts to the bone defect) [29], stimulate cell growth such as fibroblasts (which are related to collagen production) [30], increase osteoblast proliferation and differentiation [31] and promote mitochondrial respiration and ATP synthesis [32]. Specifically regarding xenogeneic BBG, there is a report that LLLT can improve bone formation process and accelerate particles resorption in the interior of bone defects [16], since it can increases osteoblastic [33] and osteoclastic activity [34]. This is a valuable finding when particles fail to resorb and remain like a motionless body surrounded by the host bone [35,36].

Few studies have addressed the action of LLLT on the interaction of implanted biomaterial and tissue during bone healing process [37,38]. It has been shown that LLLT promotes bone healing and bone mineralization [39]. In the search for the optimal biomaterials tissue interaction, the effect of LLLT on these cells is an important field of investigation [39]. Thus, the purpose of the present study was to analyze histomorphometrically the effect of LLLT on bone formation process in surgically created critical-size defects (CSDs) treated with BBG and its influence over particles resorption of BBG.

2. Materials and Methods

2.1. Animals and Experimental Groups

After careful planning of a double-blind interventional animal study and an ethical approval by the Ethics Committee on Animal Use (protocol # 003162/2007) of the School of Dentistry, Araçatuba Campus, São Paulo State University, sixty four 3-month-old male rats (Rattus norvegicus albinus, Wistar) weighing 250 to 300 g (UNESP, Dental School of Araçatuba, Animal Care Unit) were included in the study. This study conforms to ARRIVE (Animal Research: Reporting of In Vivo Experiments) [40]. The animals were kept in plastic cages with access to food and water ad libitum, in a room with a 12-h light-dark cycle and a temperature between 22 and 24 °C. Prior to surgical procedures, animals were allowed to acclimatize to the laboratory environment for a period of 7 days. Following a table generated by a computer program, the animals were distributed into 4 experimental groups (n = 16): the C group (control), only blood clot; the LLLT group, LLLT and blood clot; the BBG group, CSD filled with BBG; BBG/LLLT group, LLLT and CSD filled with BBG.

2.2. Creation of the CSD

For surgical procedures, the animals were anesthetized by intramuscular injection with ketamine (70 mg/kg) (Vetaset, Zoetis, Florham Park, NJ) and xylazine (6 mg/kg) (Coopazine, Coopers, São Paulo, São Paulo, Brazil). After aseptic preparation, a semilunar incision was made in the scalp in the anterior region of the calvarium, allowing reflection of a full thickness flap in a posterior direction. A 10-mm CSD was made with a trephine (3i Implant Innovations Inc., FL, USA) in a low-speed hand piece under continuous sterile saline irrigation. Extreme care was taken not to damage the dura mater during the creation of the CSD. The defect included a portion of the sagittal suture. The CSD of each animal was filled with particles of 250 to 1000 μm of BBG (Gen-Mix Baumer S.A., São Paulo, SP, Brazil) using a 6 mm3 measuring cup [6]. The soft tissues were then repositioned and sutured (4-0 Silk; Ethicon, São Paulo, SP, Brazil) to achieve primary closure. Each animal received post-surgical intramuscular injections of 24,000 IU of penicillin G benzathine (Fort Dodge, Saúde Animal Ltd., Campinas, SP, Brazil).

2.3. LLLT Protocol

In the LLLT and BBG/LLLT groups the LLLT was used after the displacement of the total retail and clothing of the surgical defect. The laser used in this study was gallium aluminum-arsenide (Bio Wave; Kondortech Equipment Ltd., São Carlos, São Paulo, Brazil), with a wavelength of 660 nm, power of 35 mW, and spot size of 0.07 cm2. LLLT was performed once in eight points around the CSD, in contact with the bone, and also in a central point of the CSD in the scanning procedure [41]. The treatment laser was emitted with power of 0.03 W during 72 s/point, irradiance of 0.42 W/cm2, and fluency of 30.85 J/cm2/point. The area received a total energy of 19.44 J.

2.4. Tissue Processing

Eight animals from each group were euthanized at 30 or 60 days post-operation. The area of the original surgical defect and the surrounding tissues were removed in block. The blocks were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 48 h, rinsed with water, and then demineralized in a solution of 10% EDTA. After decalcification, they were processed and embedded in paraffin. Serial 6 mm-thick sections were cut in a longitudinal direction. The sections were stained with hematoxylin and eosin (H&E) for analysis under light microscopy. Two sections from the central area were selected for histological and histomorphometric analyses.

2.5. Histomorphometric Analysis

Two histological sections, representing the center of the original surgical defect, were selected for histologic and histomorphometric analysis to increase the reliability of the data used in the statistical analysis. These analyses were performed by an examiner blinded to the treatment rendered (LRC). The images of the histologic sections were captured by a digital camera (Olympus DP 10, Olympus Optical Co. Ltd., Tokyo, Japan) coupled to a light microscope (Olympus BX 50 F4, Olympus Optical Co. Ltd., Tokyo, Japan) with an original magnification of 32 ×. The digital images were saved on a computer. A composite digital image was then created by combining three smaller images, because it was not possible to capture the entire defect in one image at the level of magnification used. The composite image was created based on anatomic reference structures (such as blood vessels and bone trabeculae) within each of the histologic sections. The ImageLab 2000 software (Diracon Bio Informática Ltd., Vargem Grande do Sul, São Paulo, Brazil) was used for the histomorphometric analysis. The following criteria [42]...
were used to standardize the histomorphometric analysis of the digital images:

(a) The total area to be analyzed corresponded to the entire area of the original surgical defect. This area was determined by first identifying the external and internal surfaces of the original calvarium at the right and left margins of the surgical defect, and then connecting them with lines drawn following their respective curvatures. The newly formed bone area (NFBA) was delineated within the confines of the total area.

(b) The total area was measured in square millimeters and was considered to represent 100% of the area to be analyzed. The NFBA and remaining particles areas (RPA) of BBG was also measured in square millimeters and calculated as a percentage of the total area.

2.6. Statistical Analysis

The values of NFBA for each animal were represented by the mean percentage of the two histologic sections. The data were subjected to the Shapiro-Wilk test to confirm a normal distribution, and the data were analyzed by a two-way ANOVA (p ≤ 0.05) with Tukey’s post hoc test for individual comparisons (p ≤ 0.05). All analyses were performed using BioStat 3.0 software (Bioestat Windows 1995 Sonopress; Manaus, Amazonas, Brazil).

3. Results

3.1. Qualitative Histologic Analysis

At 30 days post-operation, almost all specimens of the C group exhibited absence of new bone formation at the edges of surgical defect while some specimens exhibited discrete new bone formation (Fig. 1: A(a)/(c)). The connective tissue presented thin and with rare fibroblasts (Fig. 1: A(b)). In the LLLT group, we observed discrete new bone formation at the edges of surgical defect (Fig. 1: B(a)/(b)). Inside it, the collagen fibers of connective tissue presented more organized than the C group and with small number of fibroblasts. Areas of osteoid matrix with a large number of osteoblasts also were observed (Fig. 1: B(b)). The BBG and BBG/LLLT groups exhibited extensive areas occupied by implanted material (Fig. 1: C, D) encircled by a range of well-vascularized fibrous and osteoid matrix with a large number of osteoblasts (Fig. 1: C(b), D(b)). In the BBG group also was observed discrete new bone formation at the edges of surgical defect (Fig. 1: C(a)/(c)) and the presence of small spurs of new bone formation adjacent to implanted material in the interior of defect (Fig. 1: C(b)). In the BBG/LLLT group was observed discrete new bone formation at the edges of surgical defect (Fig. 1: D(a)/(c)), however, higher than BBB and the LLLT groups. The interior of defect presented connective tissue with moderate number of fibroblasts and osteoid matrix with a large number of osteoblasts adjacent to implanted material (Fig. 1: D(b)).

At 60 days post-operation, the histological characteristics were similar to those previously described. All specimens of C group presented similar characteristics in relation to connective tissue and arrangement of collagen fibers, which exhibited a moderate number of fibroblasts dispersed throughout the defect (Fig. 2: A(b)). In the LLLT group was observed areas of new bone formation at the edges (Fig. 2: B(a)/(b)) and inside the surgical defect (Fig. 2: B(b)). In BBG and BBG/LLLT groups, the interior of defect was occupied by granules of implanted material (Fig. 2: C, D), without an extensive inflammatory response (Fig. 2: C(b), D(b)). With respect to bone formation, the histological characteristics of the C, LLLT groups were very similar to 30 days post-operation. The specimens of the BBG/LLLT group presented moderate new bone formation at the edges of surgical defect (Fig. 2: D(a)/(c)) and more quantity of new bone formation spurs adjacent to implanted material (Fig. 2: D(c)) compared to C, LLLT and the BBG groups. The BBG and BBG/LLLT groups presented specimens with granules of BBG encircled by new bone formation and extensive areas of osteoid matrix formation with large number of osteoblasts (Fig. 2: C(b), D(b)/(c)). No CSD in any of the groups and time points studied were completely regenerated with bone. At the end of the experimental period, the defect in the BBG and BBG/LLLT groups was still filled by BBG and the area was densely filled by collagen fibers.

3.2. Histometric Analyses

3.2.1. NFBA

The LLLT (5.82 ± 2.05; 7.34 ± 1.01) group presented significantly greater NFBA when compared to the C group (1.61 ± 0.30; 5.59 ± 0.94) at 30 and 60 days post-operation (p < 0.05). The BBG/LLLT group (7.39 ± 1.45; 9.44 ± 2.36) presented significantly greater NFBA when compared to the BBG group (3.85 ± 1.56; 8.02 ± 0.63) at 30 and 60 days post-operation (p < 0.05). Means and standard deviations of NFBA for each group are presented in Table 1.

3.2.2. RPA

Comparison between periods within the same group showed that solely BBG Group showed lower (p ≤ 0.05) mean RPA percentage for the implanted material following 60 post-operation days (22.72 ± 10.23) when compared to 30 post-operation days (27.78 ± 11.22). BBG/LLLT Group did not present any statistically meaningful difference in mean RPA percentage of implanted material in 30 (21.98 ± 4.10) and 60 (27.20 ± 6.39) post-operation days. There was no statistically meaningful difference at the mean RPA percentage of implanted material between groups at the same period. Mean and standard deviations of RPA, with inter and intra-groups comparisons, are presented at Table 2.

4. Discussion

This study used the classical calvarial defect model [43] to evaluate, from a histological and histometric point of view, the effect of LLLT on bone healing processes of surgically created CSDs treated with BBG. Considering previous findings about the action of LLLT on osteoblastic [33] and osteoclastic activity [34], our hypotheses were: (1) LLLT could improve bone formation process; (2) LLLT could accelerate the particles resorption of BBG in the interior of the bone defects.

In the present study, in both experimental periods, the LLLT group presented greater new bone formation when compared to C (control) group, and the BBG/LLLT group presented greater new bone formation when compared to BBG group, suggesting that LLLT can increase the bone formation and accelerate the bone healing process in CSDs treated or not with BBG. These results are in agreement with previous studies that support the positive effect of laser on bone formation both when used alone or associated with bone graft [16,23]. The literature reports that bone healing process triggered by injury results in a local inflammatory immune reaction whose development is thought to highly influence the outcome of such process [44,45,46]. The inflammatory events contribute to the local production/release of growth factors classically associated with bone neoformation, as well as the promotion of chemotaxis of cells associated with the repair process [46,47,48,49,50,51]. It

Fig. 2. Panoramic views of the surgical defects and detailed histological appearance of the edges and center of the surgical defect at 60 postoperative days. Photomicrographs showing the NFB close to the edges of the surgical defect; remnants of granules of BBG and formation areas of osteoid matrix in (A) C; (B) LLLT; (C) BBG and (D) BBG/LLLT. NFB restricted to areas close to the edges of the surgical defect in C; LLLT; BBG and BBG/LLLT – A(a)/(c), B(a)/(c), C(a)/(c) and D(a)/(c). Range of well-vascularized fibrous CT in C – A(b). NFB adjacent to area of osteoid matrix with osteoblasts in LLLT – B(b). Granules of BBG, distant from edges of the surgical defect, encircled by spurs of NFB and areas of osteoid matrix with a large number of osteoblasts (asterisk) in BBG and BBG/LLLT – C(b) and D(b). (Hematoxylin and eosin staining; original magnification × 50 in A, B, C and D; original magnification × 100 in A(a)/(b)/(c), B(a)/(b)/(c), C(a)/(b)/(c) and D(a)/(b)/(c). Abbreviations: NFB, newly formed bone; BBG, bovine bone graft; CT, connective tissue.
have evaluated the effect of infrared laser light on bone healing [59,61,62,63,64] because of its deeper penetration. However, recent studies of Garcia et al. [38,41] have demonstrated that visible laser light (660 nm wavelength) also presents biomodulatory effect on bone healing, when used transoperatively at the borders of surgical defects, in contact with bone tissue, and also in a central point of the CSD [38,41]. On the contrary, Jakse et al. [65] did not confirm the positive LLTL effect on bone healing in cancellous sinus graft, because of its inadequate irradiation power (< 4 J/cm²) and absorption of most irradiated light by thick sinus cortical bone and deep sinus of the sheep. In both studies of Garcia et al. [38,41], the CSD was irradiated only during the surgery procedure. As in the mentioned studies, in the present, the CSD also was irradiated with visible laser light (660 nm of wavelength, 30.85 J/cm² of dose), only during the surgical procedure at the borders of wound, in contact with bone tissue, and also in a central point of the surgical defect.

Although the use of LLTL on the biostimulation of bone repair has been growing steadily, and several studies have demonstrated positive results on the healing of bone tissue, there are few previous reports on the association of LLTL and bone grafts or biomaterials [55,66]. Some preclinical studies have demonstrated the effects of LLTL on implanted biomaterials in order to improve bone healing process in different models of study with bone augmentation procedures. Among the models used are: calvarial CSDs [16,23,38,62], surgical defects created in femur [59,67], tibia fractures [68], mandibular trauma [69] and titanium implants associated to biomaterial on tibia of rats [60,68].

The calvarial bone defect model is appropriate for the examination of maxillary bone regeneration because its several similarities to the maxillofacial area [70,71]. Anatomically, the calvarium consists of two cortical sheets separated by reticulated bone, like the mandible; physiologically, the healing pattern is also similar to that of the maxilla [72]. In terms of morphology and embryology, the calvarium develops from a precursor membrane, like the bones of the face, including the maxilla and the mandible [73,74]. This process is called intramembranous bone formation because it occurs in the interiors of the membranes of loose connective tissue and not on a cartilaginous mold, which characterizes endochondral ossification [75]. Once the graft is placed within the calvarial surgical defect, the particles of material are engulfed by a blood clot. This supplies the necessary proteins/growth factors needed to begin the cell adhesion process and ultimately the reconstruction and repairing of bone [6].

The qualitative histological analysis demonstrated that, excepting for some specimens of control group at 30 days post-operation, new bone formation was observed close to the borders of the surgical defect. In the groups treated with BBG, it was possible to observe particles encircled by immature bone trabeculae or collagen fibers with no signs of an extensive inflammatory response, suggesting that the grafting material placed in the defects was biocompatible and, at the same time, osteoconductive. However, either in the BBG or BBG/LLLT group, the particles remained within the defect. This observation is important because of the reports that LLTL can accelerate the resorption of these particles in the interior of bone defects [16]. In the present study, the quantitative histological analysis showed that while in the BBG group there was a decrease in the mean percentage of implanted material particles over the time, in the BBG/LLLT group it didn’t occurred. It was also observed that there was no significant difference in the mean percentage of particles between these two groups in both experimental periods studied. These results suggest that, on the contrary reported by Cunha et al. [16] LLTL is not able to accelerate particles resorption of BBG. Despite these findings, these materials have been used successfully in numerous clinical and preclinical studies, including guided bone regeneration [76,77], sinus augmentation [78,79] and socket augmentation [80,81]. Considering that little is known about the effects of LLTL on xenografts BBG and the high rate in medical and dental clinics of these material in bone reconstruction procedures, we believe that the present study has important implications in the clinical setting. Within the limits of the present study, it can be concluded that LLTL can improve bone formation process in CSD filled or not with BBG in rat calvaria, but it is not able to accelerate particles resorption of this material in the interior of bone defect.

### Table 1

Mean percentage (%) and standard deviations (M ± SD) of RPA within the surgically created defects with comparison among groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Periods</th>
<th>n</th>
<th>30 days</th>
<th>60 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (control)</td>
<td>16</td>
<td>16</td>
<td>1.61 ± 0.39</td>
<td>5.59 ± 0.94</td>
</tr>
<tr>
<td>LLTL</td>
<td>16</td>
<td>16</td>
<td>5.82 ± 2.05*</td>
<td>7.34 ± 1.01*</td>
</tr>
<tr>
<td>BBG</td>
<td>16</td>
<td>16</td>
<td>3.85 ± 1.56*</td>
<td>8.02 ± 0.63*</td>
</tr>
<tr>
<td>BBG/LLTL</td>
<td>16</td>
<td>16</td>
<td>7.39 ± 1.45,#</td>
<td>9.44 ± 2.36,#</td>
</tr>
</tbody>
</table>

Comparison inter-groups.

* Significant difference with C group (ANOVA, Tukey’s test) (p < 0.05).

# Significant difference with BBG group (ANOVA, Tukey’s test) (p < 0.05).

### Table 2

Mean percentage (%) and standard deviations (M ± SD) of RPA within the surgically created defects with comparison among groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Periods</th>
<th>n</th>
<th>30 days</th>
<th>60 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBG</td>
<td>16</td>
<td>16</td>
<td>27.78 ± 1.12</td>
<td>22.72 ± 1.02*</td>
</tr>
<tr>
<td>BBG/LLTL</td>
<td>16</td>
<td>16</td>
<td>21.98 ± 4.10</td>
<td>27.20 ± 6.39</td>
</tr>
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</table>

n = 32

* Significant difference with BBG group in the same period (ANOVA, Tukey’s test) (p ≤ 0.05).
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References


