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Effect of Photobiomodulation Therapy Associated With Biphasic Phosphate Calcium on Bone Repair: A Histomorphometric Study in Rats

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Abstract

Introduction: This study aimed to investigate the effects of photobiomodulation (PBM) therapy associated with biphasic calcium phosphate on calvaria critical defects in rats.

Methods: Forty-eight (90 days old) adult male rats (*Rattus norvegicus*, Albinus variation, Wistar) received critical defects of 5 mm in diameter, which were made on their skull, and they were randomly assigned into the following groups: C-blood clot, B-biphasic calcium phosphate, L-photobiomodulation therapy, and B + L-biphasic calcium phosphate + photobiomodulation therapy. A low-level a gallium aluminum arsenide (GaAlAs) laser was applied in a single dose during surgery, in a wavelength of 660 nm and total energy density of 45 J/cm^2. On 30th and 60th days, the animals from each group were euthanized. Histological and histomorphometric analyses were performed.

Results: In 30 days, almost all specimens (C, L, B and B + L) showed bone neoformation areas in regions near the borders of the surgical defect. In 60 days, in many specimens (C, L, B, B + L), it was possible to see a narrow neoformed bone structure along almost the whole extension of the surgical defect, though it was thinner than the original calvary bone. Data were recorded as mean ± standard deviation, and after normality was tested, a suitable statistical test was applied (α = 5%). On day 60, there was a statistically significant difference when comparing the proportion of neoformation area between group L (0.52% ± 0.13) and group B + L (0.20% ± 0.08). Group L showed a difference compared with all the groups when we compared the remaining distance between the edges of neoformed bone (C × L, $P = 0.0431$; B × L, $P = 0.0386$; L × B + L, $P = 0.0352$), demonstrating a great defect closure.

Conclusion: Our findings suggest that although biphasic calcium phosphate exerts some osteogenic activity during bone repair, PBM therapy is not able to modulate this process.

Keywords: Bone regeneration; Bone substitutes; Biocompatible materials; Laser therapy; Rats.

Introduction

In the context of current dentistry and oral rehabilitation, the need for bone repair and the use of bone grafts are very frequent. Autogenous bone is considered the gold standard for bone graft materials. It comprises osteogenic cells and molecular signs that can trigger cell differentiation.[1,2] In addition, it exhibits osteoconductive properties, which, along with its osteoinductive and osteogenic effects, can promote regenerative potential.[3] However, autogenous graft removal involves a second surgical donor site and increases patient morbidity.[4,5] In addition, complications such as damage to the alveolar and mental nerves, devitalization of teeth near the surgical site, compromised facial esthetics, and increased risk of mandibular fractures[6,7] may occur once an intraoral site is selected as the donor.

A variety of bone replacement materials have been demonstrated to achieve acceptable results.[3,8,9] Ideally, bone replacement materials should exhibit good bone tissue integration, osteoinduction, and long-term

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stability.[7] However, no such material has been found in its pure form.[7] Biphasic calcium phosphate (BCP) is a new type of bone graft material that is purely synthetic and consists of a mixture of 60% hydroxyapatite and 40% beta-tricalcium phosphate (β-TCP). The use of BCP has produced favorable results in preclinical studies and animal models by promoting bone formation and successfully repairing critical-size cranial defects.[10,11] Additional benefits were found during the use of enhanced calcium phosphate-coated poly (propylene fumarate) scaffolds for the treatment of bone defects,[11] particularly with respect to the percentage of bone volume, which was significantly higher in the treated defect.[12] In a comparative evaluation of BCP materials with different configurations in a guided bone regeneration setting with regard to new bone formation, BCP showed higher amounts of newly formed bone compared with the other groups despite a higher remaining graft volume.[13]

Owing to its degradability, biocompatibility, and porous structure, BCP is efficiently incorporated into bone tissue and facilitates the intergrowth of newly formed bone. However, the osteoinductive properties of synthetic ceramics have been shown to be insufficient for healing extensive bone defects.[14] The osteoconductivity of calcium phosphate is derived from its ability to accommodate material on its surface, enabling the formation of carbonate hydroxyapatite, which represents 65% of the total bone mass.[15] Hence, in the search for alternatives to minimize failures, several associations of therapies have been used for bone tissue engineering, such as growth factors,[16] fibrin Biopolymer, and photobiomodulation (PBM) therapy.[17]

Evidence demonstrates that PBM therapy has a positive effect on bone metabolism and may enhance bone healing.[18,19] PBM has the potential to stimulate the proliferation of osteoblast precursor cells and cell differentiation, thereby increasing the number of osteoblasts, which promote new bone formation.[20] PBM can accelerate the bone healing process in rats and increase bone density.[21] However, little attention has been paid to the possible positive effect of the association between bone replacement graft materials and PBM. Oliveira et al[22] stated that PBM could not modulate the osteogenic activity of Biosilicate®. Conversely, Fangel et al[23] demonstrated positive results when PBM at 60 J/cm^2 was combined with Biosilicate® for bone-fracture consolidation in osteoporotic rats, evidencing more bone regeneration and better biomechanical properties. The use of autogenous bone or bovine bone grafts has been suggested as an effective therapy for critical-size defects, and its combination with PBM induces accelerated bone healing and resorption of the graft particles.[24]

Although positive effects have been observed when different bone graft materials were associated with PBM to enhance bone healing, further studies are needed to determine the best combination of bone graft material and PBM parameters. In this context, the hypothesis of this study was that the use of PBM combined with BCP could result in better outcomes, so the aim was to investigate the effects of BCP and PBM applied via a single application protocol on the bone repair process in rats, using histomorphometric analyses.

Materials and Methods

This study was conducted according to the Ethical Principles for Animal Experimentation adopted by the National Council of Animal Experimentation (CONCEA), and it was approved by the Institutional Review Board of the Institute of Science and Technology/UNESP (08/2014-PA/CEP).

Study Design

The sample size calculation was made based on a previous study[25] where it was possible to obtain the variability of the data around 0.5 mm^2 for the area of bone neoformation. Thus, the test of the study power was carried out using the Minitab program (version 17.1, 2013) and the statistical program PIFACE Russ Lenth (Softpedia, Bucharest, Romania) Russel V. Lenth (version 1.76, 2011). It was found that with n = 6 for each subgroup, it was possible to detect an average difference of the "Treatment" effect equal to 0.8 mm^2 with a power test up to 80%.

Forty-eight 90-day-old adult male rats (*Rattus norvegicus,* Albinus variation, Wistar) weighing approximately 300 g each were used. The rats were housed in suitable cages and fed with water and food *ad libitum*. The animals were randomly divided through a draw into four groups: C (blood clot), B (BoneCeramic™), L (PBM), and B + L (BoneCeramic™ + PBM). The four groups were subdivided according to observation periods of 30 and 60 days, and each subgroup contained six rats.

Surgical Procedure

For the surgical procedure, the animals were anesthetized with a solution of 13 mg/kg of 2-(2,6-xylidine)-5-6-dihydro-4*H*-1,3-thiazin-2-amine (Xylazine, Rompun - Bayer, Brazil) and 33 mg/kg of base ketamine (Dopalen, Agribands, Brazil). After sensitivity tests, a trichotomy of the upper region of each animal's head was performed, with posterior asepsis using iodized alcohol. Subsequently, a longitudinal 1-cm incision was made on the skin along the sagittal suture, thus exposing the calvaria bone. A critical bone defect with a diameter of 5 mm was introduced laterally to the sagittal suture and central calvaria using an electric motor with the speed set at 800 rpm (Driller, Carapicuiba, SP, Brazil) and a trephine drill (Neodent®; Curitiba, PR, Brazil) under constant irrigation with sterile saline solution.[26,27] Then, the biomaterial (BoneCeramic™, Straumann®; Switzerland) was placed, completely filling the inner diameter of the bone defect,

and PBM was performed. In the control group, the bone defect did not receive any kind of treatment, so after the defect was made, the next step was suturing. The suturing was done using 4.0 silk sutures, and dipyrone monohydrate (150 mg/kg) via subcutaneous injection was administered every 12 hours for 2 days. The day of surgery was set as day 0.

Photobiomodulation Therapy

The PBM protocol was performed using a gallium aluminum arsenide (GaAlAs) Laser DUO (MMOptics Ltda., Sao Carlos, SP, Brazil) with a wavelength of 660 nm, power of 30 mW, and tip area of 0.04 cm^2, with continuous laser beam emission (CW) using a laser pen nib[28,29] in contact with the bone defect. PBM was applied at five points so that the entire surgical wound received the treatment evenly. Four application points were distributed along the edges of the surgical wound at 3, 6, 9, and 12 o'clock respectively, and the fifth was located in the central region of the surgical wound. Each point was irradiated for 12 seconds with a total energy density of 45 J/cm^2, which was achieved during the surgical procedure in a single application directly on the exposed bone[25,28,29] (Table 1). After 30 and 60 days, the six rats in each group were euthanized and their calvarias were removed for analysis.

Histological and Histomorphometric Analyses

For histological and histomorphometric analyses, specimens were immersed in 10% formaldehyde for a maximum period of 48 hours, and each specimen was cataloged. Then, the decalcification process was initiated with 10% EDTA at pH 7.8 and room temperature. Once decalcification was complete, each specimen was divided longitudinally into two blocks along the central line of the surgical defect, producing two blocks that were embedded in paraffin and cut from their central portions. Serial 5-μm-thick sections were made longitudinally from the center of the surgical defect. The sections were stained with hematoxylin–eosin and examined under an Axiophot 2 light microscope (Carl Zeiss, Oberköchen, Germany) coupled with an AxioCam MRc 5 digital camera (Carl Zeiss).

Table 1. Laser's Parameters

Type of laser	Low level gallium aluminum arsenide lasers (GaAlAs)
Wavelength	660 nm
Power	30 mW
Energy distribution	45 J
Spot diameter at the focus	4 mm^2
Focus spot area	12.56 mm^2
Emission mode	Continuous laser beam emission (CW)
Time of irradiation	12 s at each application point
Total time of irradiation	60 s
Delivery system	Single trans-surgical application by focusing handpiece

Three equidistant histological sections were selected for histological and histomorphometric analyses to increase the reliability of the data.[25,30] A blinded examiner was calibrated by evaluating the measurements of twelve total areas of the defect on two separate occasions. The values obtained were analyzed using the Pearson correlation coefficient, and if the similarity was at the 90% level, the calibration was accepted.

The AxioVision Release 4.7.2 software was used for the histomorphometric analysis of the images. The first step to assess the proportion of the area of bone neoformation within the surgically created defects was to determine the total area of the defect. Thus, initially, the right and left edges of the created defect were delimited, and these edges were identified as the limit between the native bone and the newly formed bone. Afterward, these edges were connected horizontally, delimiting the area that would correspond to the cortical if there was no defect, which determined the area comprised our 100%" for "this area was determined as 100%.[30]

Subsequently, the neoformation area was measured trough limiting the new bone identified in the defect area; in this step, only the newly formed bone tissue was selected. Therefore, the remnants of biomaterial and connective tissue were excluded. After obtaining the area, the proportion of bone neoformation to the total defect area was calculated; therefore, the total neoformation area (NA) was the proportion of the bone neoformation area within the defect.[28]

After the measurements related to the area, three linear measurements that represent the remaining distance between the edges of the newly formed bone were made: the remaining distance between the edges of the neoformed bone was denoted as DBE-NB. The remaining distance between corticals formed from the neoformed bone on the edges in the upper and lower regions was denoted as DBSC-NB and DBIC-NB respectively.[28,31] These measures provided us with complementary data regarding the dimension of defect closure.

Statistical Analysis

Quantitative data were recorded as means ± standard deviations (SD), and to verify normality, we used the Shapiro–Wilk test. Normality was only observed on DBE-NB parameter, so an analysis of variance (two-way ANOVA) followed by Tukey's test was performed to evaluate the differences within and between groups ($\alpha = 5\%$). For non-parametric data on the NA, DBSC-NB, and DBIC-NB parameters, the Kruskal–Wallis test and Dunn post hoc test for multiple comparisons ($\alpha = 5\%$) were performed. The data were statistically analyzed using GraphPad Prism (version 6, 2014) and SigmaPlot

12.0 (for Windows, version 12) software.

Results

Qualitative Histological Analysis

After 30 days, almost all the specimens from the four groups (C, L, B, and B + L) showed bone neoformation areas in regions near the borders of the surgical defect (Figure 1A, 1B, 1C, 1D). After 60 days, most specimens presented similar bone neoformation when compared with 30-day specimens, whereas some showed increased bone formation. After 60 days, many specimens of the four groups (C, L, B, B + L) contained a narrow neoformed bone structure along almost the entire extension of the surgical defect, although it was thinner than the original calvaria bone (Figure 1E, 1F, 1G, 1H).

It was also possible to observe dense connective and infiltrated chronic inflammatory tissue along the entire surgical defect during all the experimental periods. Specimens belonging to groups B and B + L contained gaps that were filled with residual biomaterial particles after 30 and 60 days (Figure 1B, 1D, 1F, 1H, Figure 2).

Histomorphometric and Statistical Analyses

On day 30, there was no difference between the treatment groups in the total NA. Only after 60 days, there was an intergroup difference between groups L and B + L ($L \times B + L$, $P < 0.05$) with respect to the NA parameter. Between periods, there was a difference between groups C30 and L60 ($C30 \times L60$, $P < 0.05$) and groups B30 and L60 ($B30 \times L60$, $P < 0.05$) (Figure 3A, Table 2, Supplementary file 1).

Within linear measurements, the distance between the edges of the neoformed bone (DBE-NB) parameter showed an intragroup difference for group L ($L30 \times L60$, $P = 0.0165$) (Table 2, Supplementary file 1). After 30 days, there was a difference between groups B and B + L ($B \times B + L$, $P = 0.0241$), and after 60 days, group L showed a difference compared with all the groups ($C \times L$, $P = 0.0431$; $B \times L$, $P = 0.0386$; $L \times B + L$, $P = 0.0352$) (Figure 3B). With respect to the distance between the upper corticals formed in the newly formed bone (DBSC-NB), there was only an intragroup difference in group L ($L30 \times L60$, $P < 0.05$), and there was no intergroup difference (Figure 3C). With respect to the parameter of the distance between the lower corticals formed in the newly formed bone (DBIC-NB), there were no intragroup or intergroup differences after 30 and 60 days (Figure 3D, Table 2, Supplementary file 1).

Discussion

Few studies have investigated the effect of the combination of PBM and BCP on bone repair. Hence, the aim of this study was to investigate the effects of PBM (660 nm, 45 J/cm^2) via a single application protocol in association with BCP on critical bone defects in rats.

The highest percentage of the bone neoformation area was observed in group L; this difference in the neoformation area was statistically significant in group B + L after 60 days. These findings are in agreement with those of previous studies that demonstrated that the effect of PBM increases and accelerates the bone repair process in critical-size defects,[29] spinal cord injury,[32] and within titanium scaffolds in both healthy and ovariectomized rats.[33] Although there was no statistical difference in 30 days between groups B and B + L, the PBM application seemed to accelerate bone formation in the defect, as observed by Freitas et al.[34]

On the other hand, Fangel et al[23] demonstrated that the group exposed to a treatment combining PBM (60 J/cm^2) with Biosilicate had the largest amount of bone neoformation. Garcia et al[25] demonstrated that

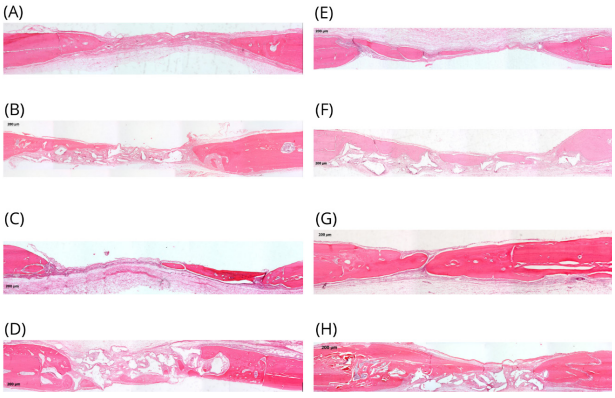

Figure 1. Photomicrographs of the Panoramic View of Defects on 30th and 60th days. (A) Group C-30 days, (B) Group B-30 days, (C) Group L-30 days, (D) Group B + L-30 days, (E) Group C-60days, (F) Group B-60days, (G) Group L-60 days, (H) Group B + L-60days. HE staining, original magnification X5

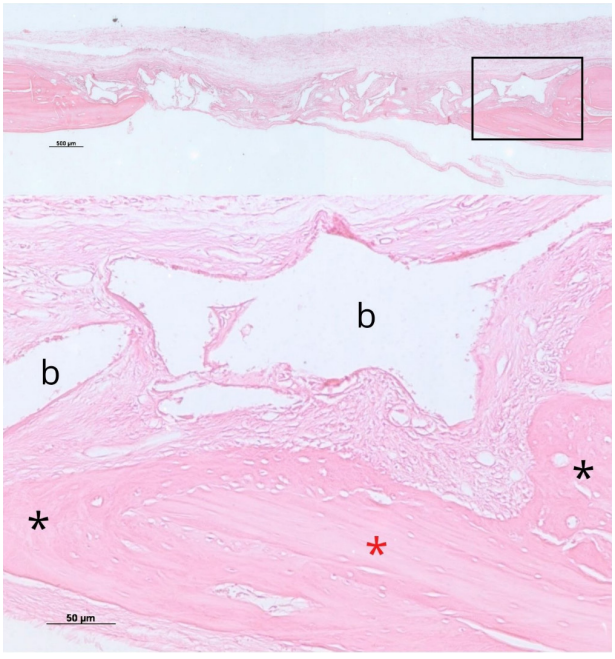

Figure 2. Photomicrograph at higher magnification of specimen in group B + L-30d showing the area of bone neoformation (*) close to the edge of the surgical defect (red *) and spaces that would be occupied by biomaterial particles (b) (HE staining, original magnification X10)

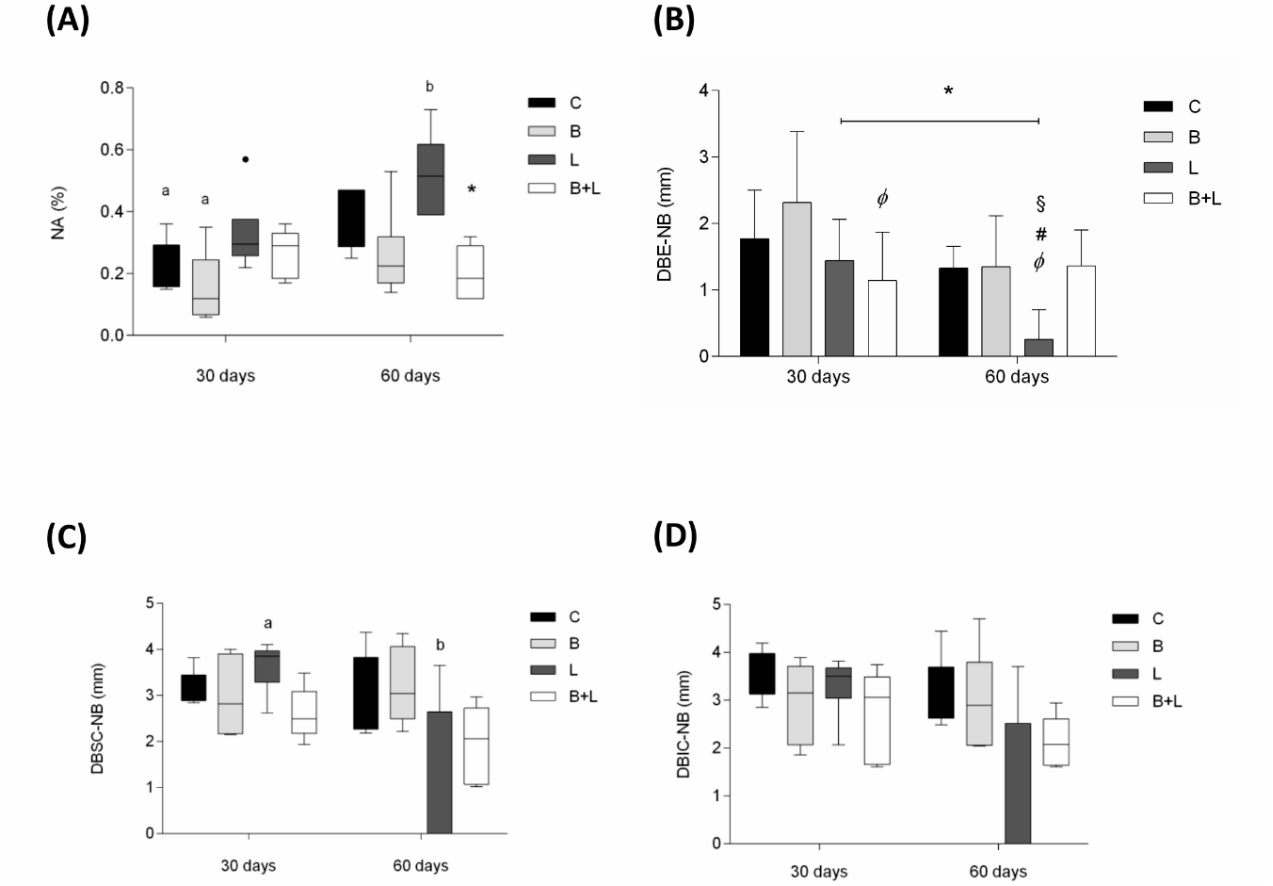

Figure 3. Graphics of Parameters NA, DBE-NB, DBSC-NB and BBIC-NB. (A) Graphic of NA parameter: Intragroup statistically significant differences for L (*). Different letters represent different intergroups. Kruskal-Wallis, Dunn Test, $P<0.05$; (B) Graphic of DBE-NB parameter: Statistical differences intra and intergroups for L (*), C (#), B (ϕ) and B+L (§). ANOVA two-way, Tukey test, $P<0.05$; (C) Graphic of DBSC-NB parameter: Statistical differences intergroups for L group (*). Kruskal-Wallis, Dunn test, $P<0.05$; (D) Graphic of DBIC-NB parameter: Comparisons inter and intragroups of the remaining distance between the corticals already formed from the newly formed bone at the edges in the lower region

Table 2. Mean and Standard Deviation Values of Measures Regarding Time and Treatment Modality

Parameters	Time	C	B	L	B+L
NA (%)	30 days	0.22±0.08	0.16±0.11	0.33±0.12	0.26±0.08
	60 days	0.37±0.10	0.26±0.14	0.52±0.13	0.20±0.08
DBE-NB (%)	30 days	1.77±0.73	2.32±1.07	1.45±0.62	1.15±0.72
	60 days	1.33±0.33	1.35±0.77	0.26±0.45	1.36±0.54
DBSC-NB (mm)	30 days	3.21±0.36	2.97±0.84	3.64±0.54	2.60±0.56
	60 days	3.17±0.83	3.20±0.81	0.99±1.59	1.94±0.84
DBIC-NB (mm)	30 days	3.48±0.49	2.97±0.85	3.32±0.63	2.67±0.96
	60 days	3.31±0.69	3.01±1.06	0.97±1.58	2.14±0.57

Data represented are mean and standard deviation.
NA: proportion of the bone neoformation area within the defect; DBE-NB: central distance between the edges of newly formed bone; DBSC-NB: the distance between the upper corticals formed in the newly formed bone; DBIC-NB: the distance between the lower corticals formed in the newly formed bone.

the association of autogenous bone grafting with PBM stimulates bone neoformation in critical defects of calvarias in immunosuppressed rats with an amazing degree of accuracy, and they concluded that PBM, either in association with the treatment using bisphosphonates or not, was effective in stimulating bone formation in critical calvarial defects in ovariectomized rats.

In a study that assessed the effect of PBM on critical calvarial defects in rats treated with autogenous or bovine autologous bone grafts, Cunha et al[24] demonstrated that the groups that had been irradiated with PBM had a larger bone neoformation area than the ones that had not. Therefore, it was concluded that PBM accelerates bone defect repair and the reabsorption of particles from the material used for grafting.

Over both periods of observation, most of the newly formed bone in the groups that received the bone allograft was in the form of lamellar bone close to the

edges of the surgical defect, as reported by Bosco et al.[35] In general, in group L, the newly formed bone was evenly distributed between the lamellar and intramembranous formation; this could be explained by tissue stimulation promoted by PBM irradiation, which was justified by the presence of some important biomarkers in the central region of the defect after PBM, such as osteocalcin and osteopontin—proteins secreted during the process of osteoblast differentiation and mineralization—and vascular endothelial growth factor—a signal protein that stimulates vasculogenesis and angiogenesis.[36]

Ghahroudi et al[37] demonstrated greater effectiveness of PBM in repairing bone in the early stages, i.e. at 4 weeks post-operation. Marques et al[36] demonstrated that the greatest bone neoformation occurred within a period of 15 days and concluded that PBM is more effective in the early stages of bone repair, thus reporting efficacy in the stages of cell proliferation, mineralization, and maturation of the bone matrix. The results of Garcia et al[25] indicated that the group that had received autologous bone grafting associated with PBM demonstrated significant bone neoformation in 30 days; therefore, the animal model and methodology used for the experiment have been espoused in the literature.[25,27,36,38] The filling of defects with particulate biomaterials is an effective strategy. The difficulty in placing and retaining the biomaterial particles in defects can be overcome by associating the bone graft with a membrane or fibrin, as demonstrated by Della Coletta et al,[17] and PBM had a great impact on guided bone regeneration with fibrin membrane.[17] However, in the absence of membrane use, this difficulty can be minimized by inducing the formation of a cohesive mass of the biomaterial and clot; thus, the particles remain in place. Fangel et al[23] have reported similar phenomena.

Garcia et al[25] demonstrated that the combination of autogenous bone grafts with PBM produced good results; this type of graft is regarded as a major touchstone for guided bone regeneration. In the present study, such a combination (BCP + PBM) did not show statistically significant results regarding the percentage of the bone neoformation area, unlike those of Garcia et al[25] and Fangel et al,[23] who evaluated the combination of bioactive glass-ceramic and PBM. A possible explanation for this is that a synthetic biomaterial composed of hydroxyapatite and β-TCP was used, and it had a slow absorption rate.[39]

In a clinical study by Schmitt et al,[7] the group that received BCP exhibited a moderate amount of bone neoformation when compared with the group that received an autogenous bone graft. Clinically, there is no evidence that this situation can be regarded as advantageous or disadvantageous. The use of bone replacement can be considered an advantage because it represents a type of protection against bone resorption, thus guaranteeing long-term stability for guided bone regeneration surgeries.

Since BCP is a completely synthetic material, it exhibits integration and bone formation after 5 months of repair, which guarantees long-term stability for implant placement because, as the quantity of new bone formation increases, the amount of bone replacement material decreases.[7] The 30- and 60-day periods in this study can be considered very early stages to evaluate the percentage of the bone neoformation area when using BCP. Barbeck et al[40] investigated the influence of the particle size of BCP on the induction of mononuclear giant cells and bed vascularization, and their results demonstrated that the size of the granules had an impact on the initial vascularization of the bed and on the induction of mononuclear cells in later phases of tissue reaction. This impact is an important factor as it can slow down the bone repair process.

In the present study, group L presented the best parameters at the end of the experiment, compared with other treatments, with regard to the bone defect closure represented by the DBE-NB. However, even with no statistically significant difference, all the groups presented greater closure of the surgical defect after 60 days. The positive action of PBM with respect to this parameter has been previously reported.[28,29,31] PBM is able to create environmental conditions that accelerate bone healing by stimulating the proliferation and differentiation of osteoblasts *in vivo* and *in vitro*, which would consequently lead to an increase in bone formation.[41]

Therefore, PBM can be considered a non-invasive biostimulation method based on this study, and it is promising for stimulating osteogenesis.[18,19] However, it is still challenging to compare the effect of PBM on bone tissue and implanted biomaterials owing to variations in experimental models, materials used, duration of treatments, wavelength, energy, exposure time, power, and the biological state of the cell when receiving PBM.

Analysis of the results of the present study showed that PBM irradiation resulted in the highest percentage of the bone neoformation area. Despite the association of PBM with biomaterials, it was not effective in improving the parameters related to the area of bone neoformation and wound closure; however, it must be taken into account that biomaterials help maintain bone volume and architecture. In this context, more studies that assess other types of laser treatments, different doses, and parameters are necessary to evaluate the clinical application of PBM combined with BCP so that it can be efficiently established.

The present study has some limitations that may hinder the transposition of the data obtained, such as the absence of a group that could show the initial effects of the application of PBM on bone regeneration. However, a previous study by the research group did not show statistically significant histomorphometric results[28] to justify the increase in the number of animals

in the present study that sought to follow the animal reduction policy. Also, there are studies with longer initial periods for PBM analysis in the literature.[31,42] Moreover, the presence of complementary analyses, such as immunohistochemistry, could open discussions about the action of PBM at the cellular level, which is not possible to be done only with histomorphometry, and the addition of micro-computed microtomography (micro-CT) would bring us the dimension of closing the defect in an assertive and concrete way. Therefore, more pre-clinical studies are necessary to determine specific protocols of the association between BCP and PBM.

Conclusion

Based on this study, it was possible to conclude that the application of PBM at 45 J/cm² resulted in the highest percentage of the bone neoformation area after 60 days. The combination of PBM at 660 nm and 45 J/cm² with BCP did not induce a significant increase in the bone neoformation area in critical calvarial defects of rats when compared with other study groups. Our findings suggest that although BCP exerts some osteogenic activity during bone repair, PBM therapy is not able to modulate this process.

Acknowledgments

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Conflict of Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics Considerations

This study was submitted and approved by the Ethics Committee of Research in Animals of the Institute of Science and Technology of Sao Jose dos Campos/UNESP (08/2014-PA/CEP -CEUA/ICT-UNESP).

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Supplementary Materials

Supplementary file 1 contains Tables S1-S4.

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