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Original Research

Histomorphometric Assessment of the Influence of Low-Level Laser Therapy on Peri-Implant Tissue Healing in the Rabbit Mandible

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Abstract

Objective: The purpose of this study was to demonstrate the effect of low-level laser therapy (LLLT) on the peri-implant bone healing process in the rabbit mandible. ***Background data:*** LLLT has been shown to accelerate tissue repair and osseointegration of implants placed into the rabbit tibia. However, the beneficial effects of LLLT have never been tested in the rabbit mandible, which would more closely mimic the human situation. ***Materials and methods:*** Twenty-four male New Zealand rabbits were randomly divided into four groups of six animals each. All animals had their left mandibular incisors extracted, followed by immediate insertion of a titanium dental implant in the fresh socket. Three groups received LLLT [aluminum-gallium-arsenide (AlGaAs), $\lambda = 830$nm, 50 mW, continuous wave (CW)] at three different energy densities per treatment session (E-5, 5 J/cm^2; E-10, 10 J/cm^2; and E-20, 20 J/cm^2). Irradiation was performed every 48 h for 13 days, totaling seven sessions. One group received sham treatment (controls). Histological sections were obtained from each of the 24 mandibles dissected, without first decalcifying the specimens, and were stained with hematoxylin and eosin and Picrosirius red for histomorphometric evaluation. Bone-to-implant contact (BIC), bone formation area, and collagen fiber area were assessed by light microscopy. ***Results:*** Significant differences were found between group E-20 and all other groups ($p < 0.05$). Histomorphometric evaluation showed significantly higher BIC and significantly more collagen fibers in group E-20. ***Conclusions:*** Photobiostimulation with LLLT at an energy density of 20 J/cm^2 per session had a significant positive effect on new bone formation around dental implants inserted in the rabbit mandible.

Introduction

Low-level laser therapy (LLLT) is a treatment modality widely used in dentistry to accelerate the healing process, and has proven to be effective in various dental procedures even with the use of lasers of different wavelengths.[1–3] The biomodulatory effects of LLLT on the healing process are associated with increased collagen synthesis, increased epithelial and fibroblast proliferation, reduced inflammation, and attenuation of postoperative pain.[4–7] Experimental studies on the effect of LLLT on peri-implant bone healing have shown improved osseointegration and a shorter implant rehabilitation period.[8–17]

The rabbit has been the preferred species for research on the process of osseointegration after LLLT. An advantage of rabbits over other animal models is that they are less aggressive, relatively inexpensive to purchase, and easy to house and maintain. So far, studies using rabbits have evaluated the ability of LLLT to improve osseointegration only in implants placed in the tibia.[8,10,13,17,18] However, this may not be representative of what is encountered in clinical practice because, in humans, implants are placed in the mandible or maxilla, which exhibit cortical and medullary structures different from those of the tibia.[19]

This prompted us to investigate the possibility that LLLT may likewise improve bone repair around implants placed in

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the rabbit mandible. The current study was, therefore, designed to test the hypothesis that in the rabbit mandible, LLLT irradiation would also improve peri-implant bone regeneration compared with unirradiated controls.

The objective of our study was to assess the local effects of LLLT on the peri-implant healing process after placement of titanium implants into fresh extraction sockets in the rabbit mandible by measuring bone-to-implant contact (BIC), bone area in rectangle (BAR), bone area within threads (BA), and collagen fiber (CF) area.

Materials and Methods

Animals

Twenty-four 3-month-old male New Zealand rabbits (*Oryctolagus cuniculus*), weighing 3–4 kg, were used in the study. The rabbits were housed in the animal facility at Hospital de Clinicas de Porto Alegre under standard conditions of temperature, humidity, and light intensity and were fed solid chow (Purina; Nestlé Purina Petcare, St. Louis, MO) and water *ad libitum* throughout the experiments. All rabbits underwent surgical extraction of the left mandibular incisor followed by immediate placement of an osseointegrated titanium implant, and this served as the baseline condition for each animal. The study was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre (protocol No. 11-0449/2011), and animals received humane care in compliance with international principles and guidelines for the care and use of laboratory animals.

In vivo *surgical procedure*

The rabbits were anesthetized with intramuscular ketamine/xylazine (Dopalen/ Anasedan; Vetbrands Animal Health Division, São Paulo, SP, Brazil) at a dose of 40/3 mg/kg body weight, and had their left mandibular incisors extracted with #5 pediatric extraction forceps. A conical, self-tapping osseointegrated implant (3.25ø × 11.5 mm, NNT3211; NanoTite; BIOMET *3i*, Palm Beach Gardens, FL) was then placed into the fresh socket (Fig. 1A). To standardize primary stability, the electric drilling motor (Driller, São Paulo, SP, Brazil) was mounted in a contra-angle handpiece and the insertion torque was set at 30 N for all procedures. After implant insertion, a cover screw was placed and the surgical site closed with 4-0 nylon sutures (Ethicon; Johnson & Johnson, São Paulo, SP, Brazil). The long axis of the implant was then marked on the skin to guide later laser irradiation. Tramadol (União Química, Embu-Guaçu, SP, Brazil) was administered intramuscularly (5 mg/kg) immediately after surgery and every 24 h thereafter for analgesia. Enrofloxacin (Zelotril 10%; Agener União, Embu-Guaçu, SP, Brazil) was administered intramuscularly (5 mg/kg) once daily for 3 days for antimicrobial prophylaxis. Sutures were removed on postoperative day 7.

All rabbits were weighed preoperatively to calculate drug doses. After surgery and during laser treatment, the animals were monitored by a veterinarian to ensure the maintenance of good nutritional status until euthanasia.

LLLT protocol

After implant placement, the rabbits were randomly divided into four groups of six animals each: (control), received sham treatment; (E-5), received LLLT with total laser energy density of 5 J/cm^2 per treatment session; (E-10), 10 J/cm^2 per session; and (E-20), 20 J/cm^2 per session. The groups received treatment every 48 h for 13 days, totaling seven treatment sessions.

The LLLT protocol was performed as previously described.[20] Briefly, laser irradiation was performed with an aluminum-gallium-arsenide (AlGaAs) diode laser at a wavelength of 830 nm (infrared), average power of 50 mW, spot area of 0.002827 cm^2, and in continuous wave (CW) mode (TheraLase; DMC Equipamentos, São Carlos, SP, Brazil). The laser probe was held perpendicular to the long axis of the implant without touching the skin surface (Fig. 1B), and laser was applied sequentially to two points overlying the course of the long axis of the implant (medial and lateral). Three treatment regimens were used: In group E-5, each point was irradiated with an energy density of 2.5 J/cm^2 for 51 sec; therefore, rabbits received a total energy density of 5 J/cm^2 per treatment session. In group E-10, each point was irradiated with 5 J/cm^2 for 101 sec; therefore, rabbits received a total energy density of 10 J/cm^2 per session. In group E-20, each point was irradiated with 10 J/cm^2 for 201 sec; therefore, rabbits received a total

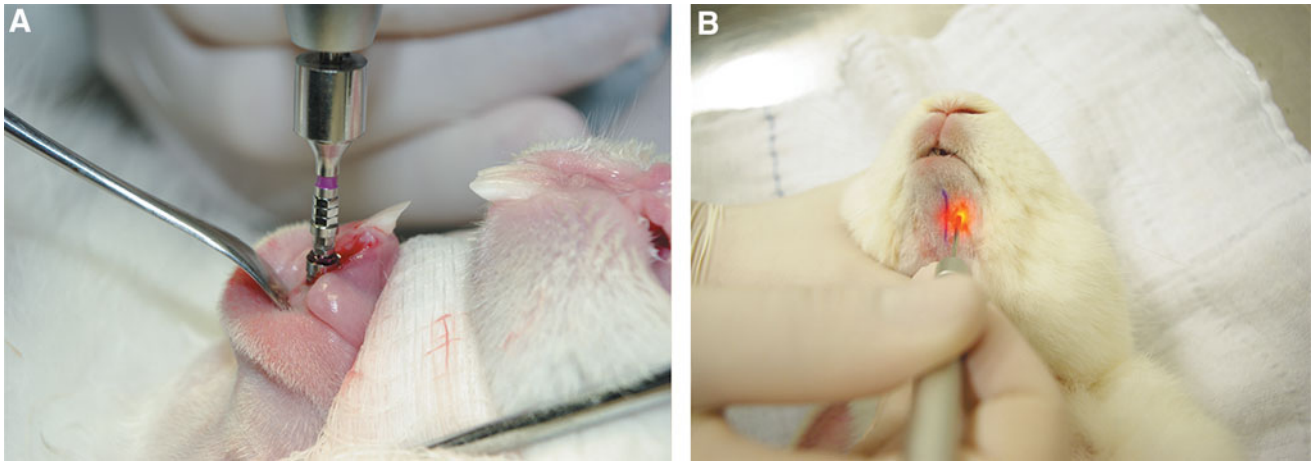

FIG. 1. **(A)** Placement of a 3.25ø × 11.5 mm osseointegrated implant (NanoTite) into the fresh extraction socket. **(B)** Administration of low-level laser therapy (LLLT).

energy density of 20 J/cm^2 per session. At the end of the study period, each rabbit had received a cumulative energy density of 35 J/cm^2 in group E-5, 70 J/cm^2 in group E-10, and 140 J/cm^2 in group E-20. Control animals underwent sham irradiation following the same procedures performed in the experimental groups, but with the laser device unpowered.

Thirty days after the last treatment session, all animals were euthanized by injection of ketamine/xylazine (Dopalen/Anasedan, 40/3 mg/kg body weight) intramuscularly, with an additional overdose (1 mL/kg body weight) of propofol (Lipuro 1%, 10 mg/mL; B. Braun S.A. Laboratories, São Gonçalo, RJ, Brazil), followed by cardiac arrest induced by injection of potassium chloride 10% (Isofarma Pharmaceutical Industrial Ltda.; Precabura Eusebius, CE, Brazil) at a dose of 1 mL/kg body weight. Propofol and potassium chloride are injectable drugs widely used in Brazil as a valid method for euthanasia in animals. Propofol is an ultra-short-acting, intravenously administered hypnotic/sedative agent with an effect similar to that of barbiturates, but without analgesic action. High doses of potassium chloride exert cardiotoxic effects that quickly cause cardiac arrest, and its intravenous administration in unconscious animals or animals under general anesthesia is considered an acceptable procedure to induce cardiac arrest or death in animal experimentation.[21,22]

All rabbits were in good nutritional status at the time of euthanasia.

Histological preparation

The 24 mandibles were dissected by total removal of soft tissues, disarticulation at the base of the skull, and midline incision, separating the side containing the dental implant. Each mandible was sectioned at the distal end of the lower first molar with a diamond-coated steel disc mounted on a low-speed motor, without touching the implant. The specimens were dehydrated at different alcohol concentrations for 4 weeks and then embedded in different resin concentrations for an additional four weeks (LR White® Embedding Resin Kit: Medium Grade; EM Science, Hatfield, PA). During resin polymerization, the specimens were kept in an oven at 60°C for 24 h. Thin sections were obtained, without prior decalcification, using a cutting system (Exakt; Norderstedt, Schleswig-Holstein, Germany) with calibrated speed to reach the region of interest. The resulting sections were mounted onto 25 × 75 mm acrylic slides and polished using a grinding system (Exakt).

Half of the prepared sections were stained with hematoxylin and eosin (H&E) and the other half were stained with Picrosirius red (PR). All slides were analyzed quantitatively and comparatively under a light microscope at × 100 magnification (Olympus BX51; Olympus Corporation, Tokyo, Japan). Images were captured with a digital camera (Olympus U-TV0.5XC-3) and stored as TIFF (.tif) files using QCapture Pro Imaging (Media Cybernetics Inc., Bethesda, MD) at a resolution of 2560 × 1920 (full-frame) for H&E-stained slides and 1280 × 960 for PR-stained slides.

Histomorphometry

Images were obtained from each implant at the buccal and lingual surfaces and then combined into a single buccal-surface image and lingual-surface image, respectively, using Adobe Photoshop CS6 13.0.1 Extended (Adobe Systems Brasil, São Paulo, SP, Brazil). The image scale was set at 1500 pixels/mm for H&E-stained images and 750 pixels/mm for PR-stained images to be consistent with their original resolution. After compositing, each buccal and lingual-surface image was histomorphometrically evaluated using an image-analysis system (ImageJ 1.46r; NIH, Bethesda, MD).

H&E-stained images. The following parameters were calculated for three consecutive threads of each implant: BAR (%), total area of bone formation from the fifth to the seventh thread of the implant measured in a rectangle; BA (%), amount of bone present within each thread from the fifth to the seventh thread of the implant; and BIC (%), amount of bone in direct contact with the implant surface from the fifth to the seventh thread of the implant.

For assessment of BAR, the region of interest was delimited as a rectangular area (2.5 × 1.2 mm) covering the fifth, sixth, and seventh threads of the implant, and the amount of bone tissue inside the rectangle was calculated as the percentage area of bone formation (%BAR = BAR × 100/area of delimited rectangle) in both implant surfaces (buccal and lingual).

For BA and BIC, two previously calibrated examiners measured the width, area, and perimeter of the fifth, sixth, and seventh threads of each implant and then calculated percent BIC values (%BIC = BIC × 100/perimeter) and BA values (%BA = BA × 100/total thread area). The final %BIC and %BA values for each implant were defined as the average of values determined by the two examiners.

PR-stained images. A polarizing filter was used for the analysis of CF. Total area of CF formation was determined

Table 1. Results of Histomorphometric Analysis After Low-Level Laser Therapy at Different Energy Densities

Parameters	*Control* (n = 6)	*E-5* (n = 6)	*E-10* (n = 6)	*E-20* (n = 6)	p *Value*
%BAR	17.25 ± 5.73a	21.23 ± 5.09a	19.21 ± 7.65a	23.22 ± 9.5a	0.528
%BA	28.93 ± 1.58a	32.45 ± 8.03a	31.92 ± 3.48a	33.85 ± 2.32a	0.892
%BIC	54.5 ± 9.79b	52.2 ± 12.31b	50.19 ± 17.5b	81.19 ± 6.52a	0.001
%CF	8.36 ± 1.58b	8.39 ± 2.25b	10.01 ± 2.32b	16.38 ± 2.57a	0.001

Control = sham treatment; E-5 = 5 J/cm^2 per treatment session; E-10 = 10 J/cm^2 per treatment session; E-20 = 20 J/cm^2 per treatment session.

Values are expressed as mean ± SD. Values in the same row with different letters (a, b) are significantly different from each other [$p < 0.05$, analysis of variance (ANOVA)].

BAR, bone area in rectangle; BA, bone area within threads; BIC, bone-to-implant contact; CF, collagen fiber.

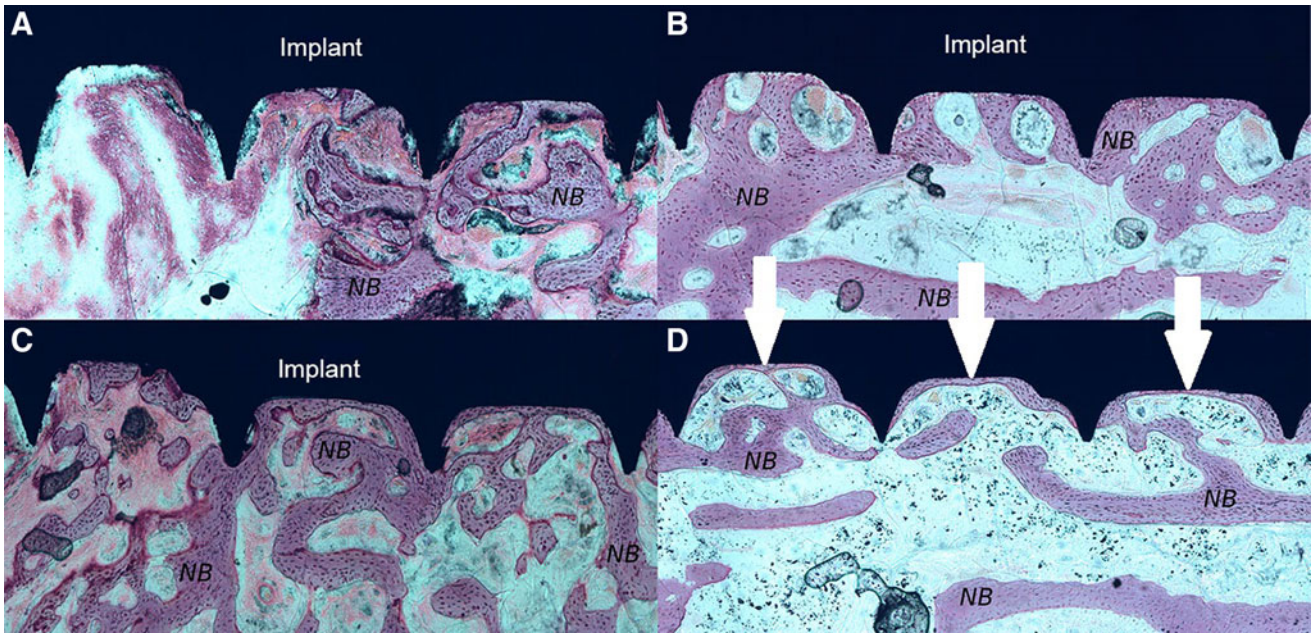

FIG. 2. Histological appearance of bone-to-implant contact in the fifth, sixth, and seventh threads of the implant at the buccal surface: **(A)** control group, **(B)** group E-5, **(C)** group E-10, **(D)** group E-20. A greater amount of newly formed bone is observed in group E-20 (arrow). Hematoxylin and eosin stain, magnification ×100, resolution 1500 pixels/mm.

from the fifth to the seventh thread of the implant measured in the same rectangle as before (2.5 × 1.2 mm). Image segmentation was based on local thresholding using gray scale morphology (ImageJ 1.46r), and the percentage of CF (%CF) was calculated as the amount of CF inside the rectangle (%CF = CF area in rectangle × 100/area of delimited rectangle) in both implant surfaces (buccal and lingual).

Statistical analysis

The data were entered into Excel spreadsheets for analysis with PASW Statistics for Windows, version 18.0. One way analysis of variance (ANOVA) with Tukey's multiple comparison test was used to analyze the histomorphometric data. The level of significance was set at 5% ($p < 0.05$).

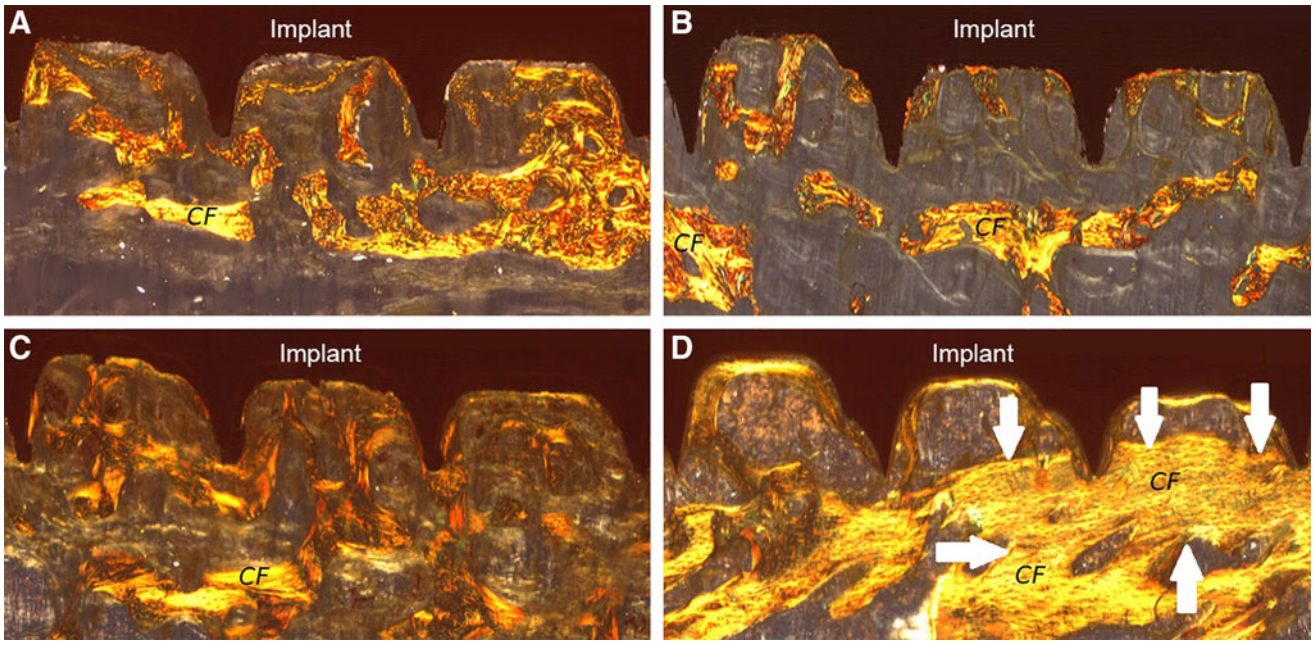

FIG. 3. Light micrograph with a polarizing filter of collagen formation in the fifth, sixth, and seventh threads of the implant at the buccal surface: **(A)** control group, **(B)** group E-5, **(C)** group E-10, **(D)** group E-20. A greater amount of collagen fibers is observed in group E-20 (arrow). Picrosirius red stain, magnification ×100, resolution 1500 pixels/mm.

Results

The histomorphometric results of bone and collagen formation rate in the control and experimental (E-5, E-10, and E-20) groups are shown in Table 1. The mean (SD) total area of newly formed bone (BAR) was 17.25% (5.73%) in controls, 21.23% (5.09%) in E-5, 19.21% (7.65%) in E-10, and 23.22% (9.5%) in E-20. The mean (SD) bone area within threads (BA) was 28.93% (1.58%) in controls, 32.45% (8.03%) in E-5, 31.92% (13.48%) in E-10, and 33.85% (12.32%) in E-20. Although %BAR and %BA values were higher in group E-20, there were no statistically significant differences among groups (Table 1).

The histological appearance of BIC and CF formation in the threaded area of interest in all groups are shown in Figs. 2 and 3, respectively. Histomorphometric evaluation showed significantly higher mean (SD) BIC values (81.19% [6.52%]) and significantly more CF (16.38% [2.57%]) in group E-20 than in all other groups ($p < 0.05$) (Table 1). No significant differences were observed among the control, E-5, and E-10 groups in these two parameters.

Discussion

This study sought to add to the existing literature by determining the effects of LLLT on peri-implant bone regeneration in the rabbit mandible, a model not previously reported but which can provide data that may be more representative of what is encountered in clinical practice. Similar to findings described in previous studies,[8–10,13,14,18] our results showed enhanced bone growth around implants in all irradiated animals, although without significant difference from baseline values in all groups except E-20. This can certainly be attributed to the photobiomodulation action of LLLT. However, we should also consider the nanoimplant surface treatment and the fact that bone healing is three times faster in rabbits than in humans,[8] factors that might have contributed to this outcome.

In the current study, the best BIC results were observed in group E-20, which was exposed to an energy density of 20 J/cm^2 per session. This finding is consistent with previous studies indicating that LLLT exposure increases BIC.[17,18] We also found a statistically significant increase in collagen fiber deposition in group E-20, compared with all other groups, which can be attributed to increased fibroblast activity and proliferation induced by the biomodulatory effects of laser therapy.[10,13,23,24]

Laser irradiation has been shown to have a positive effect on the early stages of osseointegration.[8,15,18,23,25,26] Therefore, in this study, rabbits were exposed to a 13 day LLLT protocol, which corresponds to the initial bone healing period after implantation. However, the multiplicity of models used for this purpose and lack of consensus on dosing protocols prevent adequate comparison among studies.

Variations in energy densities used to enhance implant wound healing are also found in the literature. In the current study, the total energy density delivered at the end of the study period was 35 J/cm^2 for rabbits in group E-5, 70 J/cm^2 for group E-10, and 140 J/cm^2 for group E-20. Total energy densities used in previous studies in rabbits range from 86 to 602 J/cm^2. Nevertheless, similar LLLT effects on osseointegration have been described over this wide range.[10,13] Conversely, some studies have reported that laser therapy using low doses may not be effective, and that high doses could even inhibit healing.[27,28] This study, despite using lower doses than those used previously, had positive results for peri-implant bone regeneration because of laser irradiation. This warrants further investigation using low-dose laser therapy.

In our study, implants were placed in fresh incisor extraction sockets in the rabbit mandible, mimicking the clinical situation in humans. Studies conducted to date, in the rabbit tibia, have limited their evaluation to the cortical bone structure.[19] However, our implants were not subjected to masticatory loads, which may be considered a limitation of the study. Further studies, which take the influence of functional load into account, are needed.

Conclusions

Our findings demonstrated that photobiostimulation with LLLT using an 830 nm AlGaAs laser at an energy density of 20 J/cm^2 per session had a significant positive effect on new bone formation around dental implants inserted into fresh incisor extraction sockets in the rabbit mandible.

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Author Disclosure Statement

No competing financial interests exist.

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