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ORIGINAL ARTICLE

Low-level laser therapy (LLLT) improves alveolar bone healing in rats

Larissa Nogueira Soares Ribeiro[1] · Fellipe Augusto Tocchini de Figueiredo[2] · Paôla Caroline da Silva Mira[1] · Maya Fernanda Manfrin Arnez[1] · Mirian Aiko Nakane Matsumoto[1] · Luciane Macedo de Menezes[3] · Erika Calvano Küchler[1] · Maria Bernadete Sasso Stuani[1]

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Abstract
The main objective of the present study was to evaluate the effect of low-level laser therapy (LLLT) in enhancing bone healing in irradiated alveolus post-tooth extraction. Sixty male Wistar rats (180 ± 10 g) were used in the present study. The left maxillary first molars were extracted, and the alveolar region was irradiated by diode laser device (GaAlAs) immediately after extraction and for more 3-day daily applications. The animals were randomly assigned into two groups: control group (n = 30, with left maxillary molar extraction—CG) and experimental group (n = 30, with tooth extraction and low-level laser therapy applied to the dental alveolus for 42 s—EG). These groups were divided into subgroups (five rats per subgroup) according to the observation time point—1, 2, 3, 5, 7, and 10 days—post-tooth extraction. The maxillary bone was separated, and the specimens were stained with hematoxylin and eosin, Masson's trichrome, and picrosirius red and immunohistochemistry for RUNX-2. Parametric and nonparametric tests were used with a significance level of 5%. LLLT accelerated bone healing with mature collagen fiber bundles and early new bone formation. Histomorphometric analysis revealed an increase of osteoblast (RUNX-2) and osteoclast (TRAP) activity and in the area percentage of cancellous bone in the lased alveolus compared to the control group. This increase was statistically significant ($p < 0.05$). Application of LLLT with a GaAlAs diode laser device enhanced bone healing and mineralization on alveolar region.

Keywords LLLT · Bone repair · Dental alveolus healing · GaAlAs diode laser · Laser biostimulation

Introduction

The physiological process of an alveolar repair after tooth extraction occurs in four stages, cell proliferation, connective tissue development, connective tissue maturation, and bone differentiation or mineralization [1, 2]. Under normal conditions, bone repair mechanism starts with an increase in osteoblastic activity, 2 days after the injury, quickly forming immature bone tissue, organic matrix, followed by the deposition of calcium salts, being completely filled by compact bone after 4 weeks [3, 4].

Researchers, in all areas of dentistry, seek the ways to induce more efficient bone repair after post-operative tooth extraction. These researchers enhance fast recovery of subjects submitted to this intervention. Dental implants and surgeries are the main aims of these authors [5]; the use of scaffolds and biomaterials have greater results during the regeneration of the alveolar bone [6, 7], but all of these interventions are invasive and need high-cost treatments. As Sancho and collaborators described in their article, the use of invasive interventions of tissues is the main objective of all alveolar bone intervention for regeneration, but in the future, the authors described that "minimal intervention" during the alveolar bone regeneration should be elucidated in new clinical trials [8].

Biostimulatory effects of low-level laser therapy (LLLT), a noninvasive treatment for inflammatory and pain issues, have been evaluated for alveolar bone repair, in particular,

✉ Fellipe Augusto Tocchini de Figueiredo
fellipe.figueiredo@usp.br

[1] Department of Pediatric Dentistry, School of Dentistry of Ribeirao Preto, University of Sao Paulo (USP), Av. Do Café S/N, Ribeirao Preto, SP CEP: 14040-904, Brazil

[2] Department of Dental Materials and Prosthodontics, School of Dentistry of Ribeirao Preto, University of Sao Paulo (USP), Av. Do Café S/N, Ribeirao Preto, SP, CEP 14040-904, Brazil

[3] Dental Program - School of Health and Life Sciences, Pontifical Catholic University of Rio Grande Do Sul, PUCRS, Porto Alegre, RS, Brazil

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the acceleration of bone regeneration [1] in different treatments of the maxillofacial complex [9], due to the increase of mitotic activity of the epithelial cells [10] and changes in capillary density [11] increasing the local microcirculation [12], and, mainly, it increases the synthesis of collagen [13, 14]. Numerous laser devices with different wavelengths, including helium–neon (HeNe 632.8 nm), gallium-aluminum-arsenide (GaAlAs 805 nm), and gallium-arsenide (GaAs 904 nm), have been used with different treatment protocols [2, 15–17].

Other studies described that LLLT is effective in modulating inflammation, accelerating cell proliferation, increasing the number of viable osteocytes [18, 19], stimulating fibroblasts, and collaborating in the production of more ordered collagen fibers, improving the process of bone repair and wound healing [20], but no study has evaluated the differences in many stages of alveolar bone regeneration with gold-standard biomarkers.

Based on these studies, the authors of the present study can assume that low-level laser can improve bone tissue remodeling, and after tooth extraction. The aim of the present study was to evaluate the influence of in vivo 4-day daily application of LLLT on the bone remodeling process after tooth extraction, through descriptive and immunohistochemical analysis.

Material and methods

Experimental protocol

The present study was approved by the Animal Ethics Committee at the University of Sao Paulo, Ribeirao Preto, Brazil (Protocol No.—09.1.1449.53.2) which complies with the international guidelines of animal use on science. Sixty male Wistar albino rats (mean weight 180 ± 10 g) were assigned by blind randomization methods to two groups: control group (n = 30, with left maxillary molar extraction—CG), and experimental group (n = 30, with tooth extraction and low-level laser therapy applied to the dental alveolus—EG). These groups were divided into subgroups (n = 5) according to the observation time point—1, 2, 3, 5, 7, and 10 days—post-tooth extraction (post-operative (P.O.)). The summary of the group separation is presented below:

1 day CG—control group, 1 day P.O. (n = 5);
1 day EG—experimental group, 1 day P.O. (n = 5);
2 days CG—control group, 2 days P.O. (n = 5);
2 days EG—experimental group, 2 days P.O. (n = 5);
3 days CG—control group, 3 days P.O. (n = 5);
3 days EG—experimental group, 3 days P.O. (n = 5);
5 days CG—control group, 5 days P.O. (n = 5);
5 days EG—experimental group, 5 days P.O. (n = 5);
7 days CG—control group, 7 days P.O. (n = 5);
7 days EG—experimental group, 7 days P.O. (n = 5);
10 days CG—control group, 10 days P.O. (n = 5);
10 days EG—experimental group, 10 days P.O. (n = 5).

The animals were housed three per cage in a room with a 12/12-h day/night cycles, temperature of 24 °C ± 2 °C, and humidity of 45–55%, and they received a standard diet and water ad libitum during the entire experimental period.

Anesthetic procedures and tooth extraction

Tooth extractions were performed under sterile conditions and anesthesia: For all procedures, all rats were intramuscularly anesthetized with a combination of xylazine hydrochloride (Dopaser®, Laboratorio Calier do Brasil, Ltda, Osasco, Brazil; 0.025 mL/100 g body weight) for muscular relaxation associated with ketamine hydrochloride (Ketamin®; Cristália Produtos Quimicos Farmaceuticos Ltda, Itapira, Brazil; 0.05 mL/100 g body weight). Under general anesthesia, the maxillary left first molar of all animals (n = 60) were extracted. The dental alveolus was curetted and abundantly irrigated, aiming for removal of residual fragments of hard and soft tissues that could interfere with healing. The molar was extracted in each rat with a dental explorer adapted to rat molars with a gentle buccolingual movement [9].

Laser devices and laser irradiation procedures

After tooth extraction and bleeding control, the extraction alveolus of all the rats were irradiated with a gallium-aluminum-arsenide (GaAlAs) diode laser device (Photon Laser, DMC® Equipamentos, São Carlos, SP, Brazil; λ 830 nm, 30 mW, θ 1 mm, CW and spot of 0.00785-cm^2 area). The irradiation was administered under anesthesia by placing the end of the optical fiber tip in contact with the alveolus of the left side of the maxillary bone. Irradiation was performed immediately after tooth extraction and daily for 3 consecutive days more, corresponding to the dosage of 54 J/cm^2 per day (total dosage of 216 J/cm^2 which corresponds to 4 dosages), lasting 42 s on each application with close contact with the cervical region of the dental alveolus surface. These conditions had been determined by previous experiments [9, 21]. The treatment protocol for the sham groups (control group) was the same as for the experimental group, except that the laser device was not switched on [21].

Surgical procedure

At the end of the experimental periods, the induction of death was performed by anesthetic overdose. The maxilla

was sectioned to obtain the posterior portion of the left hemimaxilla containing the dental alveolus of the extracted tooth.

Histotechnical processing

The specimens were fixed in 4% paraformaldehyde buffered with 0.1 M sodium cacodylate (pH 7.4) for 24 h at 4 °C. After 48 h, the specimens were demineralized in a solution of 0.05 M ethylenediaminetetraacetic (EDTA), with pH of 7.4 for approximately 20 days. Specimens were dehydrated in ascending grades of alcohol and embedded in paraffin following histological routine method to obtain tissue blocks. Histological sections of 6 µm thickness were cut in transverse section to the alveolus in a microtome (Microm, model HM 340 E, Germany). The slides were stained with hematoxylin–eosin (H&E) for histological evaluation of inflammation, Masson trichrome stain to observe new bone formation, and picrosirius red (PSR) for the detection of maturation of collagen fibers, according to the conventional technique [22]. H&E- and Mason-stained sections were analyzed under the light microscope, whereas PSR-stained sections were observed under circularly polarized light. Photomicrographs were captured using the Olympus System Microscope BX51, with ProgRes CT3 camera and Image Pro software. Morphometric assessment, i.e., the calculation of the area and perimeter, was made using ImageJ software [23]. The TRAP and RUNX-2 analyses were made in accordance of previous studies published by the present research group [24, 25].

Histomorphometric and microscopic analysis

The bone formation into dental alveolus was more visible in apical and middle thirds. The dental alveolus was divided and analyzed in 3 thirds—cervical, middle, and apical. The cervical third was not evaluated due to the invagination of epithelial and connective tissue. The following criteria were established for the histological and histomorphometry. Bone formation rate (BFR) was evaluated during 2–5 days P.O., the score was measured on the maturation of collagen fiber (using SCORES) during the formation period (FP), and the bone structural unit (B.St.U) was presented at 7 day P.O. when there was a clear mineral deposition due to the red-colored collagen.

Statistical analysis

The percentages of inflammatory process and maturation of collagen fibers obtained in the control and LLLT groups were analyzed by the Mann–Whitney nonparametric test. The data of new bone formation were compared among groups and periods by a two-way analysis of variance (ANOVA) and Bonferroni's post hoc test. Statistical analyses were performed using the GraphPad Prism version 5.0 software (GraphPad software®, Inc.; La Jolla, CA, USA). A significance level of 5% was set for all analysis.

Results

a) Bone formation

The amount of bone formation was obtained from the percentage of neoformed bone in ratio to the total area of the dental alveolus. In the initial periods (1, 2, and 3 days), there was no bone formation rate in none of the groups. At 5 and 7 days, the histometric analysis indicated that bone formation rate with new B.St.U present on the 7 and 10 days post-tooth extraction, in the alveolar area, was significantly higher in the experimental groups (EG) than in the control group (CG). In these periods, the experimental groups showed better results than the CG (13.29% ± 0.83% versus EG 26.17% ± 0.70%; 5 days and CG 21.48% ± 1.41% versus EG 30.74% ± 11.36%—7 days). At 10 days, there was no difference between the control and experimental groups (CG 77.13% ± 2.08% versus EG 82.77% ± 2.43%) (*$p < 0.05$) (Fig. 1).

b) Inflammatory process

The intergroup analysis demonstrated a statistical difference in the periods from 3 to 7 days. The EG demonstrated a reduction in the number of inflammatory cell profiles compared to the control group (*$p = 0.0413$, **$p = 0.0209$, and ***$p = 0.0149$) (Fig. 2).

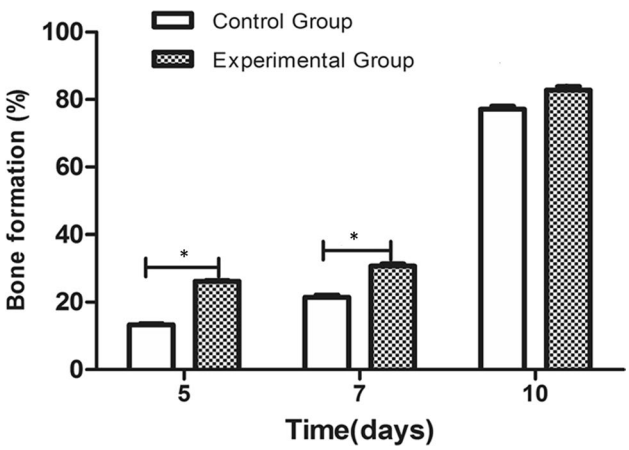

Fig. 1 Expression of the average percentage and standard deviation of bone formation in the control and experimental groups. Greater bone formation in groups exposed to low-power laser. ***$p < 0.001$

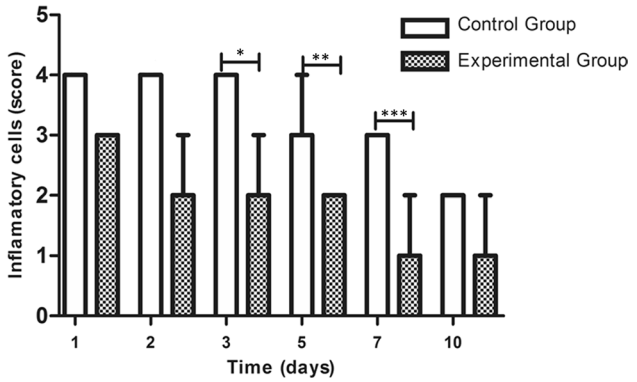

Fig. 2 Expression of the median number of inflammatory cell profiles in the control and experimental groups. Higher expression of inflammatory cells in the control groups compared to the group exposed to low-level laser. *p < 0.05; **p < 0.01; ***p < 0.001

c) Collagen maturation

There was early collagen maturation in the EG compared to the controls (CG) (Fig. 3) (*p < 0.01). In the comparison between the groups, a difference in the collagen maturation was observed, mainly on days 2, 3, and 5, demonstrating better repair quality of the dental alveolus (Fig. 4). In the present figure, cortical bone indicated as Ct and cancellous bone indicated as Cn are shown. Bone formation rate and new B.St.U are demonstrated in Fig. 4. In 7 days, a dentine tissue (De) in the right corner of the CG as an artifact is demonstrated.

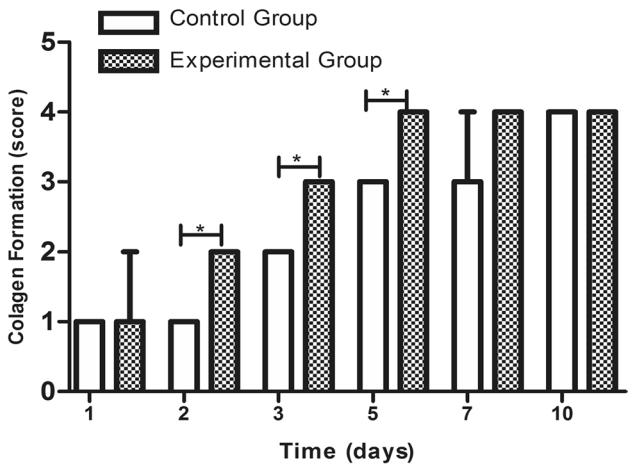

Fig. 3 Expression of the median in the degree of maturation of collagen in the control and experimental groups. The maturation of collagen is evidenced between the periods of 2 and 5 days. *p < 0.05

d) TRAP

The expression of cells immunostained for TRAP (osteoclast expression) was significantly higher (*p < 0.001) in the EG compared to the control (CG) in the period from 1 to 5 days (Fig. 5).

e) RUNX-2

The expression of cells immunostained for RUNX-2 (osteoblast expression) was also significantly higher (p < 0.001) in the experimental group than in the control, in all periods observed showing a new peak in 5 days (Fig. 6).

Discussion

The low-level laser, GaAlAs, influenced the remodeling of the alveolar bone. The laser equipment studied has the following properties: potential for deep penetration, being portable, easy to use, and low cost. In the literature, different protocols for the application of low-power laser are found in in vivo studies regarding wavelength [32, 33], dosimetry [34], exposure time, number of applications, and type of injury produced [15]. It is known that wavelength, total radiated energy, frequency of emission, and dose are directly related to an efficient cellular response to laser therapy, as well as the physiological properties of tissues [35–38]. Therefore, with the diversity of studies in the literature on the use of laser irradiation in bone repair, there is still no standardization as to the type of laser used.

The present study was initially based on the use of infrared light (830 nm) in the alveolus because it is more penetrating with the capability of reaching deep tissues [1]. The single-point laser application protocol was chosen because the top of it is the diameter of the laser tip, facilitating the standardization of specimens. Care was taken to place the optical fiber tip in direct contact with the gingival tissue of the alveolus. In the present study, a daily application was performed for 4 consecutive days, corroborating the present protocol used by previous studies [9, 39]. However, the application protocol used by other authors was a single application [40].

In this study, the density value of 54 J/cm^2 was effective in the bioregulation of cellular functions, contributing positively to the regeneration of bone tissue after extraction. The authors further suggest the hypothesis that laser irradiation in the alveolar tissue could stimulate mitochondrial activity and, consequently, increased ATP production [41], with repercussions on the increase in osteoblastic and fibroblastic activity, a fact verified in histological sections during the initial periods of this research.

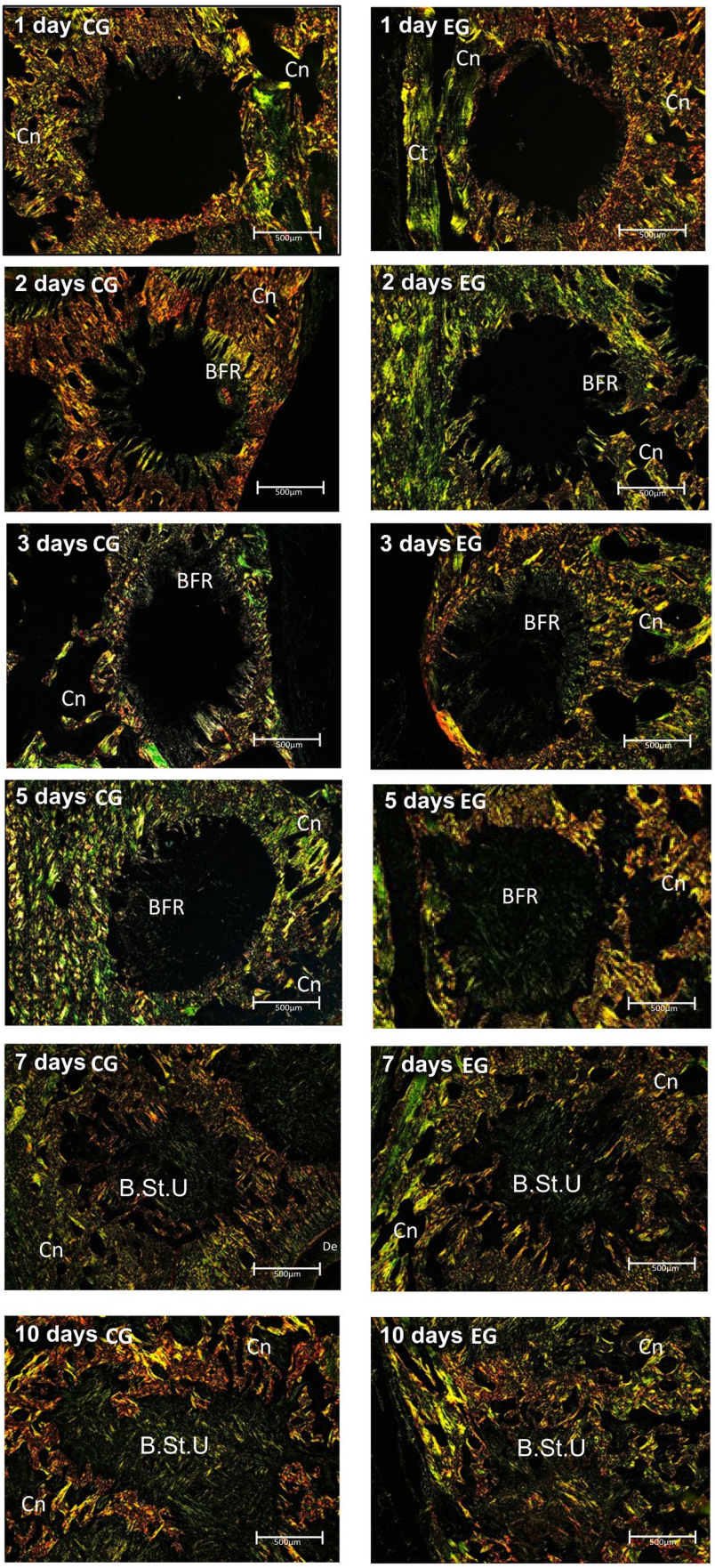

Fig. 4 Polarized light photomicrographs from the control and experimental groups showing the maturation of collagen (original magnification × 200). The left column represents the control group and the right column represents the group exposed to the low-power laser. Statistically significant differences were found between 2 and 5 days, with better values for the percentage of collagen in the groups exposed to the laser ($p < 0.05$), with trends of increased collagen on the 7th day, but not significant. Legend: bone formation rate (BFR), cancellous bone (Cn), new bone structural unit (B.St.U), and cortical bone (Ct)

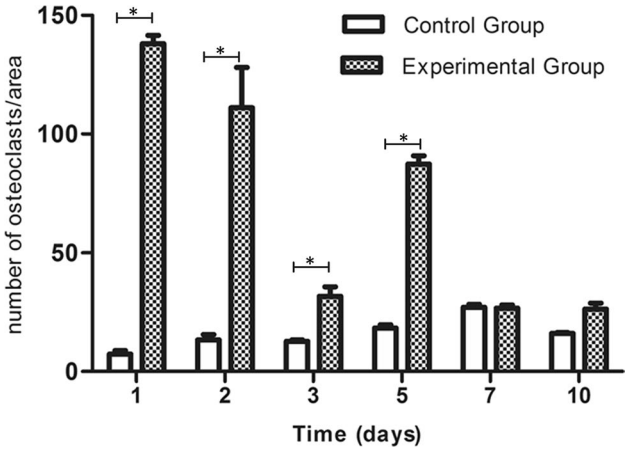

Fig. 5 Expression of the median number of cell profiles immunostained for TRAP in the control and experimental group. This figure shows a higher osteoclast expression value in the groups exposed to the laser. ***p < 0.001

Low-intensity laser used in the present study has been associated to an increase in cell proliferation and stimulates fibroblasts, collaborating in the production of more ordered collagen fibers, determining a better healing pattern in the lesions [35]. The results with staining with the picrosirius red showed better collagen organization in the irradiated group (EG) than in the control group, mainly after 5 days. Laser therapy can also provide anti-inflammatory, analgesic, and biostimulant effects, increasing the microcirculation of the irradiated area, promoting better conditions for tissue repair [11]. Other researchers with a similar experimental model demonstrated that the laser would have stimulating

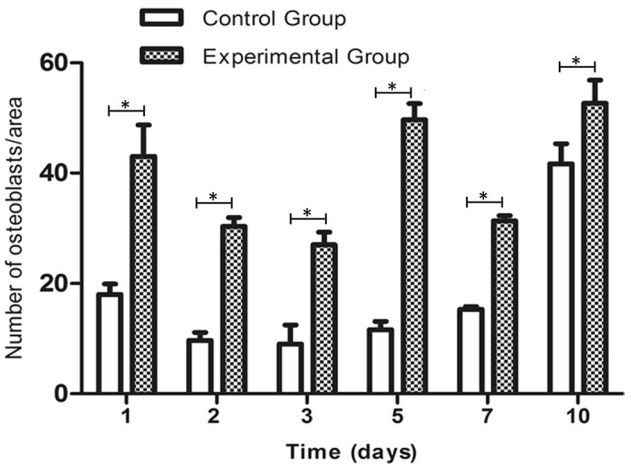

Fig. 6 Expression of the mean and standard deviation in the number of cell profiles immunostained for RUNX-2 in the control and experimental groups. The expression of osteoblast is shown in the figure, demonstrating greater expression of this cell in groups exposed to low-power laser. **p < 0.01; ***p < 0.001

effects on the synthesis of collagen fibers, elastic fibers, and on the proliferation of myofibroblasts [42], in addition to reducing the inflammatory response [43] resulting in contraction and faster epithelialization [44, 45]. In the present study, there was significant reduction in the number of inflammatory cells in the irradiated group, demonstrating a tendency of accelerated connective tissue repair. In the initial 24-h period, the dental alveolus was completely filled with blood clot, with remains of the periodontal ligament rich in fibroblasts and blood vessels in both groups. After 2 and 3 days, the wounds of the EG showed rapid organization and resorption of the blood clot, as well as proliferation of fibroblasts and blood vessels more evident than in the control. Physiologically, after an injury to local blood vessels, a fibrin-rich clot is formed, and in this study, after 2 days, an acute inflammatory reaction was observed, as well as formation of granulation tissue with new capillaries, macrophages, and fibroblasts. In addition to this effect, the laser also promotes greater regulation of the enzyme cyclooxygenase that converts arachidonic acid into prostanoids, modulating the inflammatory response [46].

The intense vascular and fibroblast proliferation observed mainly in the EG group in the periods of 3 to 7 days is probably due to the action of the laser in the remaining cells of the fibers of the periodontal ligament that remained adhered to the alveolar wall, similar to the results obtained by other authors [35, 47–50]. This fact was probably responsible for the acceleration in the repair process of the irradiated group, where the evolution was more evident and faster, characterized by faster organization of the blood clot, intense and earlier vascular proliferation, in addition to faster epithelial closure of the alveoli. These phenomena became more intense in the period of 5 days, when the irradiated alveolus was completely filled with neoformed connective tissue, strengthening the evidence that the action of the laser occurs at the vascular and cellular level, with greater intensity in the initial phases of the repair process. These histological findings observed in H/E staining were confirmed in TM and PS staining.

The number of inflammatory cell profiles, degree of maturation of the collagen, and numbers of cancellous bone formed during the experiment were more significant and earlier in the EG than in the control group. These confirms the acceleration in the repair process determined by its action on cell proliferation, also demonstrating that the results of using the laser were less effective in the late times, since, in the final post-operative period (10 days), there was no difference in the repaired bone between the irradiated group and the control. Thus, the role of the laser in the proliferation of osteoblasts, fibroblasts, macrophages, and lymphocytes, further assisting in the differentiation and activation of osteoclasts and pre-osteoblasts observed in this study, justifies an accelerated response in bone repair in the irradiated

groups compared to control, corroborating the reports of other researchers [51–53].

From the literature reports [54] and from the results of the EG, the authors believe that there is not a single parameter that produces the biostimulatory effects of laser therapy, but it is due to the combination of different parameters and their variations according to the adopted experimental model, as the 4 times application of LLLT. The low-intensity laser enhanced the bone remodeling process after tooth extraction in rats. In the post-operative times of up to 7 days, the results in bone repair in the irradiated group were better than in the nonirradiated group.

In the intergroup analysis, laser treatment showed a higher number of immunoreactive cells for the TRAP and RUNX-2 proteins in experimental animals than in controls. The number of inflammatory cell profiles, degree of maturation of the collagen, and numbers of cancellous bone formed during the experiment were more significant and earlier in the irradiated group than in the control group, determined by its action on cell proliferation. Thus, the role of the laser in the proliferation of osteoblasts, fibroblasts, macrophages, and lymphocytes, further assisting in the differentiation and activation of osteoclasts and preosteoblasts observed in this study, justifies an accelerated response in bone repair in the irradiated groups compared to control, corroborating the reports of other researchers [55–61]. However, in the final post-operative time (10 days), there was no difference in bone repair between the EG and C. The number of inflammatory cell profiles, degree of maturation of the collagen, and numbers of cancellous bone formed during the experiment were more significant and earlier in the irradiated group than in the control group, especially in the initial periods. The number of immunoreactive cells for the TRAP and RUNX-2 proteins were more significant in the irradiated group compared to the control and inversely proportional.

The present study demonstrates a limitation when extrapolating the results of the LLLT use in rats to humans as treatments. The inflammatory, immune, and histological responses in these animals are similar to the human response with the limitation of time of response to treatments [62] which is faster in rats. The use of rats in bone experimentations is well-established [63], and the LLLT dose is similar in humans and rats with similar results [64].

From 7 to 10 days post-extraction, the TRAP reactions demonstrated a less immunostaining than the RUNX-2, which means that the osteoblast activity was more stained on late days post-operative of the present study. The osteoblast activity was more evident on the groups exposed to the LLLT; therefore, it demonstrated a better response from the alveolar tissue on long-term post-operative, when there is not any further application of laser.

In the present study, the density value of 54 J/cm^2 was adequate and effective in the bioregulation or normalization of cellular functions, contributing positively to the regeneration of bone tissue after extraction. The LLLT improved bone remodeling process after tooth extraction in rats in the post-operative times of up to 7 days; the results in bone repair in the irradiated group were better than in the nonirradiated group.

Conclusion

One application post-extraction followed with three consecutive daily applications of LLLT improves early repair of dental alveolus post-tooth extraction of molars.

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Declarations

Ethics approval Animal Ethics Committee at the University of Sao Paulo (CEUA-FORP), Ribeirao Preto, Sao Paulo, Brazil (Protocol No.—09.1.1449.53.2).

Conflict of interest The authors declare no competing interests.

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