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Effects of red and infrared laser on post extraction socket repair in rats subjected to alendronate therapy

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Abstract: This study evaluated the effect of photobiomodulation therapy (PBMT) with a red or infrared laser on the repair of post extraction sockets in rats administered alendronate (ALN). Forty male rats were randomly allocated into four groups: Control Group (CTR): subcutaneous administration of saline solution throughout the experimental period; Alendronate Group (ALN): subcutaneous administration of alendronate during the entire experimental period; Alendronate/Red Laser Group (ALN/RL): administration of ALN and irradiation with a GaAlAs laser (λ 660 nm); and Alendronate/Infrared Laser Group (ALN/IRL): administration of ALN and irradiation with a GaAlAs laser (λ 830 nm). The first lower molars were extracted 60 days after the beginning of the administration of the drugs. The PBMT was applied after tooth extraction (7 sessions with intervals of 48 hours between sessions). Thirty days after tooth extraction, the animals were euthanized. Micro-CT and histometric analysis were performed to assess the bone healing and soft tissue repair of the tooth socket. The ALN group presented with more bone than the CTR; however, most of this bone was necrotic. ALN does not affect the bone microarchitecture. On the other hand, PBMT with IRL enhances the bone density due to the increase in the number and reduction in the spacing of the trabeculae. The amount of vital bone and connective tissue matrix was higher in the ALN/RL and ALN/IRL groups than in the ALN and CTR groups. PBMT enhanced the healing of the post extraction sockets in rats subjected to ALN administration. Furthermore, IRL improved the new bone microarchitecture.

Keywords: Diphosphonates; Low-Level Light Therapy.

Introduction

Bisphosphonates (BFs) are a group of drugs that have been widely indicated for the treatment of diseases related to bone metabolism (*e.g.*, osteoporosis and bone metastases) since these medications have an inhibitory action on bone resorption.[1,2] BFs inhibit the enzyme farnesyl pyrophosphate synthase, which blocks the mevalonate pathway, consequently reducing osteoclastic activity.[3] This effect is enhanced depending on the time of application, the presence of amino groups and the route of administration of this drug.[4]

However, the stronger the effect of the BFs is, the greater the possibility of complications related to their use. Among the possible complications, bisphosphonate-related osteonecrosis of the jaw (BRONJ) is one of the most worrisome conditions due to its difficult resolution.[5] BRONJ is characterized by the presence of exposed bone in the jaws for more than eight weeks in patients undergoing BF therapy without a history of head and neck radiotherapy.[4,5] This complication is usually triggered by local trauma and is associated with surgical procedures (*e.g.*, tooth extractions and implant placement).[4,6]

Some therapies or combinations of therapies have been proposed for the treatment or prevention of BRONJ, such as systemic antibiotic therapy,[7] local irrigation with bioactive agents,[8] surgical debridement and resection,[9] platelet concentrates,[10] and hyperbaric oxygen therapy.[11] However, there is still no agreement on a definitive and completely effective treatment for BRONJ.[7-11] Photobiomodulation therapy (PBMT) has shown promising outcomes in the treatment of conditions that require modulation of bone tissue healing, such as promoting improvement of the healing of bone fractures[12] and post extraction sockets,[13] and promoting implant osseointegration into native bone[14] and grafted areas.[15]

The application of PBMT has been shown to have positive effects on fibroblasts, osteoblasts and keratinocytes that were previously treated with BFs,[16-18] and in this way, PBMT can be a promising treatment for the regeneration of bone tissue in BF users. Indeed, clinical studies have shown that PBMT also enhances the healing of BRONJ lesions.[19,20] However, the number of sessions, the energy density and the wavelength used in these studies are quite variable, and standardization of the irradiation parameters is necessary to improve the predictability of the PBMT treatment. The wavelength used for PBMT is an important factor since this parameter influences the irradiation penetrability and absorption into the tissues.[21-23] Therefore, it is necessary to determine which PBMT wavelengths can exert better effects on bone regeneration under these challenging conditions. Thus, the aim of this study was to evaluate the effect of PBMT with a red or infrared laser on the repair of post extraction sockets in rats subjected to alendronate (ALN) administration.

Methodology

Animals and treatment protocols

This study used forty 10-week-old male rats (Rattus norvegicus, Albinus Holtzman) weighing 280 to 300 g. The animals were kept in plastic cages with controlled temperature, humidity and light cycles and access to food and water ad libitum. The experimental protocol was approved by the Ethical Committee on Animal Experimentation of our institution (protocol-27/2015). The ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines were followed by the authors.

Experimental groups

The animals were randomly allocated into four groups according to the treatment applied during the experiments: Control Group (CTR): subcutaneous administration of saline solution throughout the experimental period; Alendronate Group (ALN): subcutaneous administration of alendronate (ALN) throughout the experimental period; Alendronate/Red Laser Group (ALN/RL): daily administration of ALN for 60 days and irradiation with a GaAlAs laser (λ 660 nm; Therapy X, DMC Equipment, São Carlos, Brazil); Alendronate/Infrared Laser Group (ALN/IRL): daily administration of ALN for 60 days and irradiation with a GaAlAs laser (λ 808 nm; Therapy X, DMC Equipment, São Carlos, SP, Brazil). The first lower molars were extracted 60 days after beginning drug administration.

Drug administration

The animals in the alendronate (ALN), alendronate/red laser (ALN/RL) and alendronate/infrared laser (ALN/IRL) groups received subcutaneous 1 mg/kg/day alendronate (ALCON Laboratório, São Paulo, Brazil). The animals in the control group received subcutaneous administration of saline solution. The drugs or saline solution was administered throughout the entire experimental period.

Photobiomodulation (PBMT) protocol

Immediately after tooth extraction, the ALD/RL and ALD/IRL animals were submitted to PBMT (Figure 1). The laser used was a gallium aluminium-arsenide (Therapy XT, beam divergence 0.37 rad, CW, DMC Equipment, São Carlos, Brazil) with a power of 100 mW, spot size of 0.06 cm and spot area of 0.0028 cm2 with a continuous operation mode. The red laser (λ 660 nm) and the infrared laser (λ 808 nm) were emitted for 10 seconds at each point. The surgical site was irradiated at 4 equidistant points that encompassed the post extraction sockets (mesial, distal, buccal and lingual regions) in contact with the tissue. Each laser was applied over 7 sessions with intervals of 48 hours between sessions for 14 days. The energy of irradiation at each point was 1 J, which amounted to 4 J of irradiation energy per session at each surgical site.[22] A detailed description of the irradiation parameters used for the PBMT is shown in Table 1. To perform PBMT, the animals were anaesthetized with isoflurane (Isoforine - 100% isoflurane, CRISTÁLIA - Produtos Químicos Farmacêuticos LTDA, Itapira, Brazil).

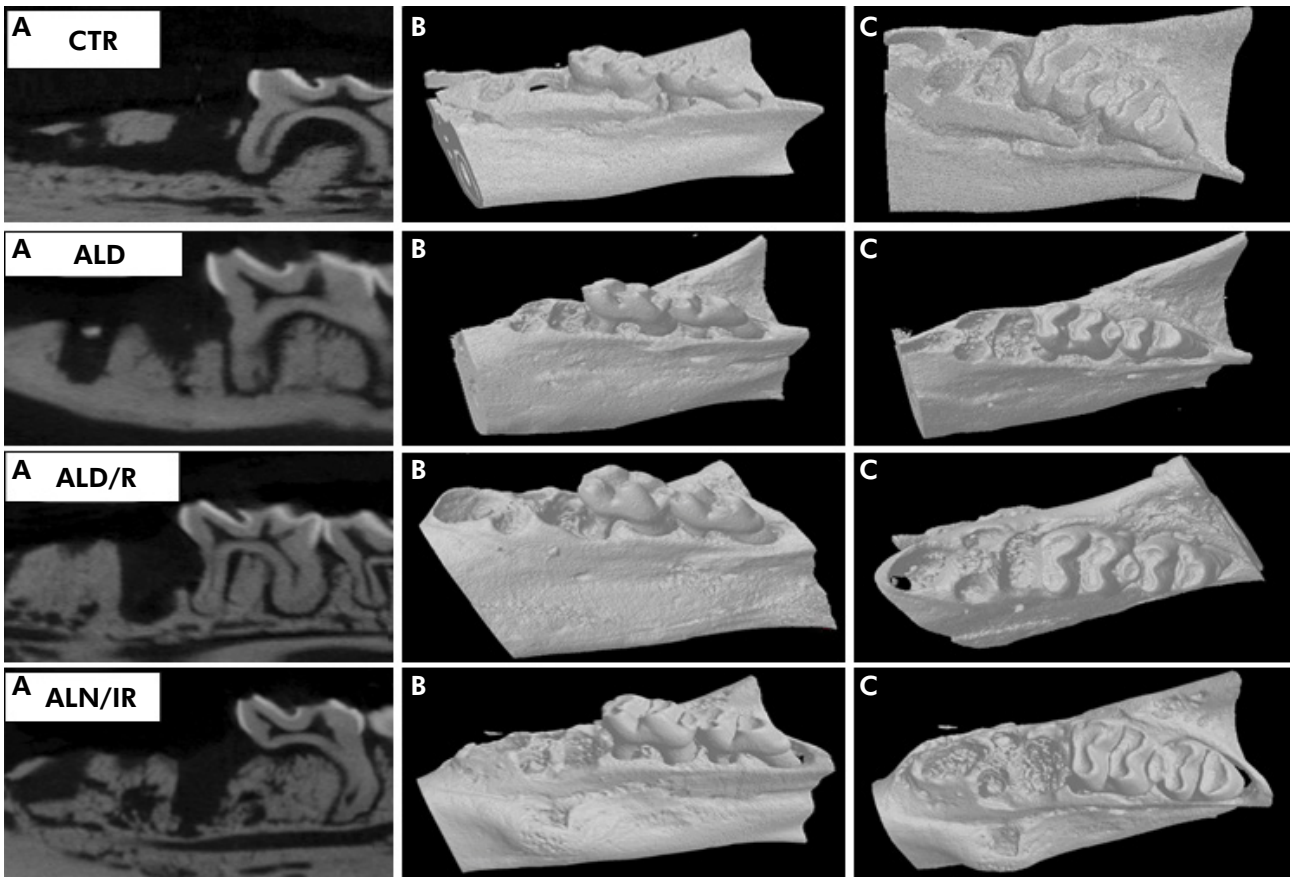

Figure 1. Radiographic (A) and tomographic aspects (B and C) of the alveolus 30 days after the tooth extraction. It can be observed that the animals that received the ALN presented higher bone volume preservation than the CTR.

Table 1. Irradiation parameters of PBMT.

Parameters	Wavelength (nm)	Power (mW)	Spot area (cm^2)	Density (W/cm^2)	Power time (s)	Energy density (J/cm^2)	Energy / point (J)
PBMT RL	660	100	0.0028	3.57	10	357.14	1
PBMT IRL	830	100	0.0028	3.57	10	357.14	1

Surgical procedure

The animals were anaesthetized by a combination of ketamine and xylazine at a proportion of 8 mg/kg of body weight (Francotar - Virbac do Brasil Ind. Com. Ltda., São Paulo, Brazil) to 4 mg/kg body weight (Virbaxyl 2% - Virbac do Brasil Ind. E Com. Ltda., São Paulo, Brazil), respectively. The lower first bilateral molars were extracted with the aid of a Hollenbeck carver, and the roots were dislocated, separated and then removed.[24] The surgical procedures were performed by a single trained professional.

After 30 days, the animals were euthanized by a lethal dose of anaesthetic. The mandibles were removed and fixed in 4% paraformaldehyde for 48 hours and subsequently stored in 70° alcohol.

Microcomputed tomography (micro-CT) analyses

The mandibles were scanned by a μCT scan microtomography (Skyscan, Aartselaar, Belgium) with the following parameters: camera pixel: 12.45; X-ray tube potency: 65 kVP, X-ray intensity: 385 μA, integration time: 300 ms, filter: Al-1 mm, voxel size: 18 μm3. The images were reconstructed, spatially repositioned, and analysed by specific software (NRecon, Data Viewer, CTAnalyser, Aartselaar, Belgium). The region for analysis was obtained by defining a rectangular area of interest in the region of the post extraction socket of the first molars (ROI) with dimensions of 1.20 mm×3.00 mm in the mesio-distal and corono-apical directions and 80 sections in the vestibular-lingual direction. A greyscale threshold of 65-255 was used, which made it possible to analyse the following parameters: a) BV/TV: Bone volume fraction in the ROI; b) BS: Bone surface; c) BS/TV: Bone surface density; d) Tb. Th: Trabecular thickness; e) Tb. Sp: Trabecular separation; f) Tb. N: Trabecular number. The images were analysed by a trained examiner blinded to the treatment groups.

Histological processing

The samples were decalcified in 7% EDTA (Sigma Chemical) for 8 weeks. The specimens were submitted to routine laboratory processing and embedded in paraffin. Serial 4 μm sections were made in the sagittal plane in the vestibule/lingual direction along the axis of the second molar, yielding 3 sections and 8 histological slides per animal. For each animal, three histological slides were randomly stained using haematoxylin-eosin (HE).

Histopathological and histomorphometric analysis

Photomicrographs of three equidistant sections (24 μm apart) located on the vestibular, middle and lingual portions of the dental alveolus were obtained by a digital camera (DP-71, Olympus, Tóquio, Japão) connected to a light microscope (DIASTAR—Leica, Reichert & Jung products, Wetzlar, Germany) with 25×, 50× and 100× magnification. Histopathological analysis was performed to describe the aspect of the new bone and of the inflammatory infiltrate, granulation tissue, and necrotic bone. The analysis was performed by a trained examiner blinded to the treatment group.

For histomorphometric analysis, the region of interest (ROI) was established as a standard rectangular area, extending from the level of the cemento-enamel junction (CEJ) of the lower second molar to the apex of the root and between the mesial and distal alveolar bone walls of the post extraction sockets of the first molar. The measurements were performed on the distal alveolus of the lower first molar in three different regions (ROI 1-superior, ROI 2-mean, ROI 3-apical). Then, for each ROI, an image was obtained (100x magnification) using ImageJ software (ImageJ, 64-bit Java 1.8.0.112), and a grid containing 252 intersection points was placed on this image. The distance between the selected sections was 48 μm, and three measurements were taken for each specimen. The intersection points that coincided with bone tissue were counted, and those of the 3 ROIs were added. Bone volume (%) was calculated as bone volume (mm3) per total tissue volume (mm^3) in the ROIs.[24] The stereometric analysis was performed by a trained examiner blinded to the treatment group.

Statistical analysis

Each animal provided both sides of the post extraction sockets for the analysis, and the mean of each animal was used for the statistical analysis of each parameter. GraphPad Prism 6 software (San

Diego, USA) was used to perform the statistical tests used in this study. The normality of the distribution of the numerical data was evaluated using the Kolmogorov–Smirnov test. The data from the microtomographic analysis were normally distributed, and thus, parametric one-way ANOVA complemented by the post hoc Tukey test was used for the inferential analysis of the data. On the other hand, the data from the histomorphometric analysis were not normally distributed, so the nonparametric Kruskal–Wallis test complemented by Dunn's post hoc test was applied for inferential analysis of the data. All tests were applied at a 5% significance level.

Results

The sample size calculation was performed based on the histomorphometric data of vital bone from a previous study that evaluated the effect of PBMT on post extraction socket repair in rats submitted to radiotherapy.[25] Considering a 30% difference between groups as being statistically relevant with an expected standard deviation of 20.28%, and by fixing the type I error at 0.05 and the beta power at 0.75, the need for ALN maintained the bone volume of the post extraction sockets.

The BV/TV evaluated in the micro-CT analysis showed that the post extraction sockets of the animals in the CTR group presented less mineralized tissue volume in the ROI (3.38 ± 1.28%) than the animals in the other experimental groups ($p < 0.05$) (Figure 1). There was no difference regarding the BV/TV among the ALN (8.07 ± 0.89%), ALN/RL (6.69 ± 3.12%) and ALN/IRL (7.43 ± 1.86%) groups (Table 2).

The bone microstructure was affected by PBMT with the IRL laser

The bone microarchitecture was affected by the PBMT, especially with the use of the ILR laser. The ALN/IRL laser group (49.26 ± 14.63 mm^2) presented higher BS than the CTR group (33.10 ± 7.16 mm^2) ($p<0.05$), higher BS/TV (5.80 ± 1.30 mm^2/mm^3) than the CTR (3.97 ± 0.54 mm^2/mm^3) and ALN groups (4.29 ± 0.49 mm^2/mm^3) ($p < 0.05$), lower Tb. Th (0.27 ± 0.07 mm vs. 0.37 ± 0.05 – CTR; 0.39 ± 0.03 mm – ALN; 0.38 ± 0.04 mm ALN/RL) and Tb. Sp (0.57 ± 0.16 mm vs. 0.69 ± 0.04 – CTR; 0.69 ± 0.08 mm – ALN; 0.63 ± 0.05 mm ALN/RL) and higher Tb. N than the other groups (1.77 ± 0.56 mm vs. 1.19 ± 0.15 – CTR; 0.98 ± 0.13 mm – ALN; 1.14 ± 0.10 mm ALN/RL) ($p < 0.05$) (Table 2).

ALN impaired the healing of the bone and soft tissues in the post extraction sockets

The healing of the post extraction sockets in the CTR presented with structured granulation tissue, alveolar bone tissue with intact osteocytes and the presence of osteoblasts in their basal portions. In the ALN, the alveoli presented without epithelial tissue cover, and the interradicular and interdental septa of the extraction region were necrotic, characterized by the absence of osteocytes and, consequently, empty gaps and a lack of resorption (Figure 2). The histomorphometric analysis showed that the ALN presented more necrotic bone than the other groups (24.60% vs. 0.00% – CTR and ALN/IRL; 8.73% ALN/RL) ($p < 0.05$) (Table 3).

Table 2. Mean and standard deviation of the microstructure analysis assessed by the micro CT.

Parameter / Group	CTR	ALN	ALN/RL	ALN/IRL
BV/TV (%)	3.38 ± 1.28^{b}	8.07 ± 0.89^{a}	6.69 ± 3.12^{a,b}	7.43 ± 1.86^{a}
BS (mm^2)	33.10 ± 7.16^{b}	42.07 ± 8.11^{a,b}	43.55 ± 2.37^{a,b}	49.26 ± 14.63^{a}
BS/TV (mm^2/mm^3)	3.97 ± 0.54^{b}	4.29 ± 0.49^{b}	4.57 ± 0.30^{a,b}	5.80 ± 1.30^{a}
Tb.Th (mm)	0.37 ± 0.05^{a}	0.39 ± 0.03^{a}	0.38 ± 0.04^{a}	0.27 ± 0.07^{b}
Tb.Sp (mm)	0.69 ± 0.04^{a}	0.69 ± 0.08^{a}	0.63 ± 0.05^{a}	0.57 ± 0.16^{b}
Tb.N (1/mm)	1.19 ± 0.15^{b}	0.98 ± 0.13^{b}	1.14 ± 0.10^{b}	1.77 ± 0.56^{a}

BV/TV: Bone volume fraction in the ROI; BS: bone surface; BS/TV: bone surface density; Tb.Th: trabecular thickness; Tb.Sp: trabecular separation; Tb.N: trabecular number.
Different letters represent different levels of statistical differences between the groups – One-Way anova complemented by the Tukey´s Test – $p < 0.05$

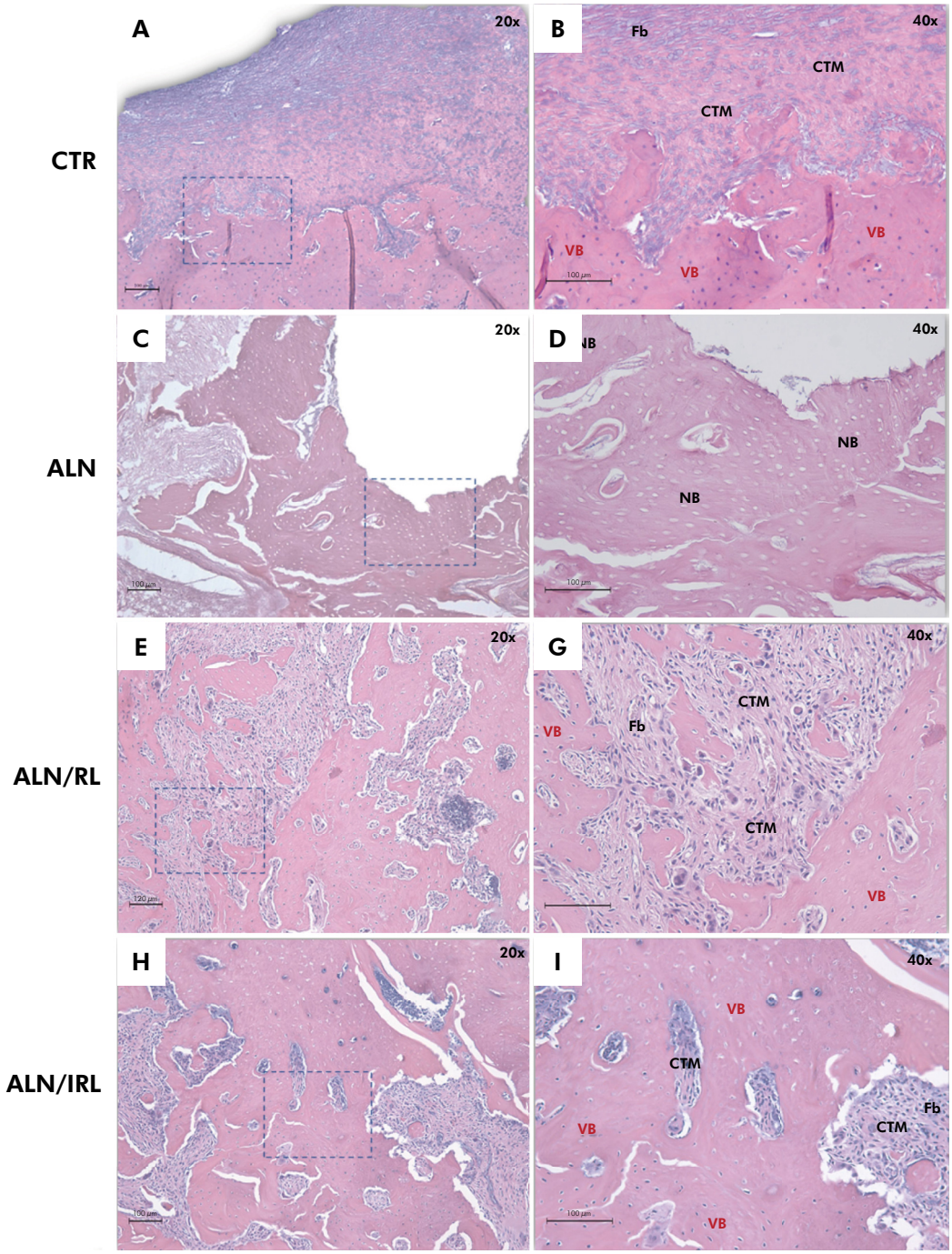

Figure 2. Histological aspects of the post-extraction sockets. A)CTR (200X); B) CTR (400X); C)ALN (200X); D) ALN (400X); E) ALN/RL (200X); F) ALN/RL (400X); G) ALN/IRL (200X); H) ALN/IRL (400X). The CTR presented a normal bone remodeling at the socket after extraction, with the presence of vital bone (VB), characterized by the presence of osteocytes, connective tissue matrix (CTM) and fibroblasts (Fb). The ALN group presented a preservation of the alveolar morphology but the bone tissue present was necrotic (NB). ALN / RL and ALN/IRL groups presented an alveolar preservation with the presence of a repaired tissue inside the alveolus areas with presence of vital bone with the presence of osteocytes (VB), associated with the presence of a well-organized connective tissue (CTM), fibroblasts (Fb), and the presence of epithelial tissue covering the wound. (Hematoxylin-Eosin stain)

Table 3. Median and interquartile range of the histomorphometric analysis in all the groups, evaluating inflammatory cells, vital bone, necrotic bone and connective tissue matrix (%).

Parameter / Group	CTR	ALN	ALN/RL	ALN/IRL
Inflamatory cells	20.63 (9.32-56.35)[a]	0.00 (0.00 -48.61)[a]	6.34 (1.38 – 16.27)[a]	5.15 (0.00 – 12.10)[a]
Vital bone	0.00 (0.00 – 0.00)[b]	0.00 (0.00 – 0.00)[b]	30.15 (14.08 – 57.54)[a]	23.80 (12.10 – 38.09)[a]
Necrotic bone	0.00 (0.00 - 11.31)[b]	24.60 (9.71 – 39.89)[a]	8.73 (0.00 – 18.05)[a,b]	0.00 (0.00 – 14.88)[b]
Connective tissue matrix	0.00 (0.00 – 0.00)[b]	0.00 (0.00 – 0.00)[b]	0.00 (0.00 – 31.35)[b]	38.49 (19.92 – 59.96)[a]
Empty spaces	66.26 (36.71 – 88.10)[a]	42.08 (21.24 – 84.33)[a,b]	32.90 (16.48 – 47.92)[b]	9.53 (4.22 – 35.33)[a,b]

Different letters represent different levels of statistical differences between the groups – Kruskall -Wallis complemented by the Dunn-´s Test – $p < 0.05$

PBMT reversed the effects of ALN on the healing of the post extraction sockets

The post extraction sockets of the ALN/RL presented with remnants of necrotic bone but were associated with the formation of immature bone filling the basal region of the alveolus and the conservation of osteocytes. In these cases, the presence of granulation tissue and large areas suggestive of bone matrix deposition were verified in the basal portion of the post extraction socket. In the ALN/IRL, the presence of necrotic areas was also observed. However, the basal region presented with bone formation, and the presence of rounded osteocytes was associated with a large number of osteoblasts, with areas suggestive of mineralization (Figure 2).

In the histomorphometric analysis, it was found that the amount of vital bone and connective tissue matrix was higher in the ALN/RL and ALN/IRL (vital bone: 23.80-30.15%; connective tissue matrix 0.00–38.49%) than in the ALN and CTR (0.00%) ($p < 0.05$). There were no significant differences between the groups regarding the number of inflammatory cells (Figure 2, Table 3).

Discussion

The use of BFs has been shown to alter bone tissue metabolism, one effect of which is to slow bone healing.[3,5] Tooth extraction in BF users represents a challenge for clinicians due to the increased time required for the healing process and the possibility of BRONJ occurrence. It was found in this study that PBMT with different wavelengths (λ 660 nm, red laser, and λ 830 nm, infrared laser) improved the healing of bone and the soft tissues in the post extraction sockets of rats that were subjected to BF therapy.

The animals that were treated with ALN showed greater maintenance of mineralized tissues in the alveolar process after tooth extraction compared to the CTR. These results corroborate previous studies that demonstrated the effectiveness of BFs in stabilizing pathological bone loss.[1,2] Aguirre et al.,[26] reported that the application of ALN in rats reduced the loss of bone volume after extraction; however, there was also less formation of new bone and a reduction in the number of blood vessels. Thus, the report of volumetric stabilization in this model does not represent a benefit since the normal course of the healing process in post extraction sockets was related to a reduction in the alveolar process[26,27] and the maintenance of bone tissue provided by the use of BFs may actually be due to nonremodelling of necrotic bone.[24]

In fact, in the histopathological analysis, it was verified that in the animals treated with ALN, the post extraction sockets presented a greater amount of necrotic bone than the animals in the CTR and PBMT groups. The greater presence of necrotic bone in the ALN group is related to its mechanism of action, which consists of blocking the osteoclastic action that reduces bone tissue remodelling.[1] These findings are in accordance with previous reports in the literature that demonstrated that BFs reduce bone tissue remodelling. Therefore, after tooth extraction, BFs can reduce alveolar remodelling,[28] and in severe cases, osteonecrosis of the jaw may occur.[6] It is important to note that tooth extraction may be the main trigger for the occurrence of these complications.[5,6,28] Another important finding of

the histometric analysis was the absence of vital bone and connective tissue matrix in the CTR and ALN groups. The explanation of these findings may be due to different reasons. In the CTR group, the post extraction socket resorption reduced the bone level below the region of interest for the histometric analysis. Therefore, only the inflammatory granulation tissue in the upper portion of the alveoli and empty spaces was observed due to the resorption process. Regarding the ALN group, most of the post extraction sockets were filled with necrotic bone occupying a space that could otherwise have been filled with vital bone tissue or connective tissue.

PBMT with RL or IRL improved the healing pattern of the post extraction sockets of rats that underwent ALN administration. PBMT promoted a reduction in the amount of necrotic bone and increased the amount of vital bone, promoted the reorganization of connective tissue and enhanced the epithelization of the wound. Previous studies have shown that PBMT promotes proliferative effects associated with the increased proliferation of fibroblasts,[18] osteoblasts,[16] and keratinocytes[17] by inducing greater expression of growth factors such as TGFβ1, BMP2 and alkaline phosphatase,[15,22,29] which may have benefited the process of repair observed in our study.

Despite the absence of differences in the post extraction socket repair processes between the RL and IRL lasers, the IRL induced more alterations in the bone microarchitecture. It has been reported that different wavelengths can interfere with the regenerative capacity of lasers.[21,30] RL has been shown to enhance bone and periodontal repair in fewer sessions than used in our study;[23,31] however, the energy produced by RL does not penetrate as efficiently into tissues as IRL,[21] and this may be the reason for the higher impact of the IRL on the bone microstructure. To make a direct comparison between RL and IRL, the protocol used in this study consisted of the application of 7 irradiation sessions for 14 days that proved to be beneficial in the repair of bone defects maintained with a clot or in grafted areas treated with IRL.[22,32] Thus, the absence of huge differences in post extraction socket repair between the two types of lasers observed in this study may be because the experimental model that enables direct irradiation into the wound is not a condition that requires great laser penetrability, and the large number of sessions may have diluted the possible beneficial effect of one type of laser over the other.[33]

Finally, this study has drawbacks that must be taken into consideration in the interpretation of our data. The animals used were healthy, while ALN is typically used for patients with pathological changes in bone metabolism, which may lead to differences in the results. Indeed, a previous preclinical study showed that ALN administration enhanced bone repair in post extraction sockets 14 days after tooth extraction in ovariectomized rats; however, as observed in this study, the ALN group presented with less bone formation than the CTR group (SHAM).[34] The effect of the number of sessions of PBMT should be evaluated in the future to provide PBMT protocols with a potentially reduced number of sessions. It is possible that a reduction in the number of PBMT sessions can make the difference between RL and IRL therapy more evident. In addition, a combination of both wavelengths may improve bone healing. One clinical study showed that the use of PBMT with RL and IRL presented similar good outcomes in periodontal treatment compared with antimicrobial photodynamic therapy.[35] Another clinical study showed that PBMT combined with IR and RL presented good outcomes in the healing of BRONJ lesions associated with the use of systemic antibiotics.[20] The effect of the association of both wavelengths on the prevention and treatment of BRONJ requires more investigation.

Conclusion

It can be concluded that low-level laser therapy performed with a red laser or infrared laser reversed the deleterious effect of alendronate therapy on the tissue healing of tooth extraction sockets. Furthermore, the IRL improved the bone microarchitecture of the new bone.

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