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Photodynamic therapy repairs medication-related osteonecrosis of the jaw by reducing NF-kB protein in rats

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Abstract

Objective: To evaluate whether antimicrobial photodynamic therapy (aPDT) repairs bisphosphonate-related osteonecrosis of the jaw (BRONJ) modulated by the reduction of NF-kB protein in a murine model. Methodology: Male Wistar rats (N=30) were divided into the following groups (n=6/group): negative control (NC); experimental osteonecrosis (ONE); ONE + photosensitizer (PS); ONE + photobiomodulation (PBM); and ONE + aPDT. Over 8 weeks, ONE was induced by zoledronic acid 250 µg/kg injections, except in the NC group, which received sterile 0.9% saline, followed by extraction of the lower left first molar. Red light laser irradiation (wavelength ~660 nm, power 50 mW, energy of 2 J, energy dose of 66.67 J/cm2 for 40 s) was performed once a week for 4 weeks. Methylene blue 0.3% was used as PS. The animals were euthanized and examined macroscopically for the presence of exposed bone and epithelial repair and microscopically by histochemical (hematoxylin-eosin and Masson's trichrome staining) and immunohistochemical (anti-NF-kB) methods. Macroscopic and histomorphometric data were analyzed by one-way ANOVA and Tukey's post-test ($p<0.05$). Results: Mucosal repair, viable osteocytes, and NF-kB immunostaining were observed in the NC, ONE+PS, ONE+PBM, and ONE+aPDT groups. The ONE group showed no mucosal repair, showing empty lacunae and multifocal immunostaining for NF-kB. The ONE+PBM and ONE+aPDT groups had greater deposition of extracellular matrix and less necrotic bone tissue ($p<0.05$). Conclusion: PBM and aPDT treatments for BRONJ were effective for bone and epithelial repair, in addition to reducing inflammation mediated by the decrease of NF-kB protein in the irradiated regions.

Keywords: Osteonecrosis. Bisphosphonates. Dentistry. Antimicrobial Photodynamic Therapy. NF-kappa B.

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Introduction

Medication-related osteonecrosis of the jaw (MRONJ) is described as any lesion with apparent bone or that can be probed through an intraoral or extraoral fistula in the maxillofacial region, present for more than 8 weeks, in individuals who have been exposed to antiresorptive or antiangiogenic drugs and who have not undergone radiation therapy or developed metastatic disease in the jaw[1].

Among the drugs associated with MRONJ, the class of bisphosphonates (BP) has been extensively described in the literature[2,-4] These drugs have an affinity for hydroxyapatite crystals (HAP), accumulating in bone tissue.[5] During bone resorption, the acidic environment favors the release of BPs from HAP and osteoclasts phagocytose degraded bone matrix along with BP molecules. Thus, BPs inhibit osteoclast function by acting as potent inhibitors of the enzyme farnesyl diphosphate synthase in the cholesterol/mevalonate biosynthetic pathway. This inhibition is responsible for a decrease in the activity of GTPases (enzymes that hydroxylate nucleotide guanosine triphosphate [GTP] to guanosine diphosphate [GDP]) in cytoskeletal rearrangement and vesicular trafficking in osteoclasts, promoting cellular apoptosis.[5-7]

More than 90% of MRONJ cases occur in individuals undergoing cancer treatment.[8] The prevalence of MRONJ in patients with cancer treated with zoledronic acid (ZA), the most potent BP, ranges from 0.4% to 1.6%, 0.8% to 2.1%, and 1.0% to 2.3% after 1, 2, and 3 years of ZA exposure, respectively.[9]

Patients undergoing cancer treatment with pamidronate or zoledronate can develop lesions in the maxilla and mandible, and MRONJ is characterized by bone exposure, whether painful or not.[10] MRONJ lesions can be associated with infection, which can affect the soft tissues of the face, interfering with patients' speech and eating and directly affecting their quality of life.[11]

The gold standard treatment for MRONJ ranges from the use of 0.12% chlorhexidine mouthwashes, oral or parenteral antibiotics, and debridement to surgical resection of the lesion area, depending on the diagnostic stage.[12] The development of new treatment modalities is being encouraged due to concerns about the development of microbial resistance to antibiotics, allowing for the investigation of preventive and curative methods for MRONJ lesions, of which antimicrobial photodynamic therapy (aPDT) is an appealing option.[13,14]

The aPDT method involves the use of a light source with a specific wavelength (~630-904 nm) that promotes the activation of molecules called photosensitizers (PS), which are previously absorbed by microbial cells. When irradiated in the presence of oxygen, PS generate high levels of reactive oxygen species (ROS). ROS are cytotoxic and inactivate microorganisms that absorb PS without causing tissue damage.[15] Among existing PS, methylene blue stands out for its broad spectrum of action, associated with light in the red and infrared wavelength ranges.[13]

The aPDT method has antimicrobial and biostimulatory effects, and MRONJ has been extensively described in terms of staging and treatment.[1,13,16,17] However, the mechanisms related to bone cell inhibition in MRONJ are not yet fully understood.[11] Thus, it should be considered that conservative treatment of MRONJ with topical, oral, or systemic antimicrobials rarely cures stage 2 and above MRONJ.[1,18] For this reason, there is interest in developing new, effective, and less invasive techniques for the treatment of MRONJ lesions, such as the use of aPDT. Therefore, this study aimed to evaluate whether aPDT can repair MRONJ and reduce inflammation modulated by the decrease of nuclear factor-kappa B (NF-kB) protein in a rat model of tooth extraction-related MRONJ.

Methodology

Ethical statement

The experimental procedures were performed following the Brazilian Guide for the Production, Maintenance or Use of Animals in Teaching or Scientific Research Activities from the Conselho Nacional de Controle de Experimentação Animal. This study was approved by the Ethics Committee on the Use of Animals of the Federal University of Paraiba (CEUA/UFPB) (protocol no. 5164120121).

Animals

The sample size was calculated based on data described in the literature. The sample consisted of 30-day-old male Wistar rats (N=30; *Rattus norvegicus*) with a mean weight of 300 g, obtained from the animal production unit (UPA) of the Instituto de Pesquisa em Fármacos e Medicamentos

(IPeFarM) of the Universidade Federal da Paraíba and maintained under temperature-controlled conditions (23±2 °C), 50% relative humidity and a 12-hour light-dark cycle, with free access to food (Pellets, Nuvilab®, Quimtia s/a, Paraná, Brazil) and filtered tap water.[14] Five experimental groups (n=6/group) were defined: negative control (NC); experimental osteonecrosis (ONE); ONE + photosensitizer (PS); ONE + photobiomodulation (PBM); and ONE + antimicrobial photodynamic therapy (aPDT).

Osteonecrosis induction

Minas Gerais, Brazil). Before each irradiation in the ONE+aPDT group, 20 µL of MB were administered topically with a volumetric pipette and maintained over the extraction site for 60 s. Irradiation was performed with the TF Premier Plus (MM Optics, São Paulo, Brazil) LED laser at a wavelength of 660 nm (visible red light), power of 50 mW, energy of 2 J, and energy dose of 66.67 J/cm^2 for 40 s at each point (3 mm²). The laser tip was positioned in close contact with the center of the extraction site so that the beam was directed parallel to the long axis of the dental socket, totaling one irradiation point per animal.

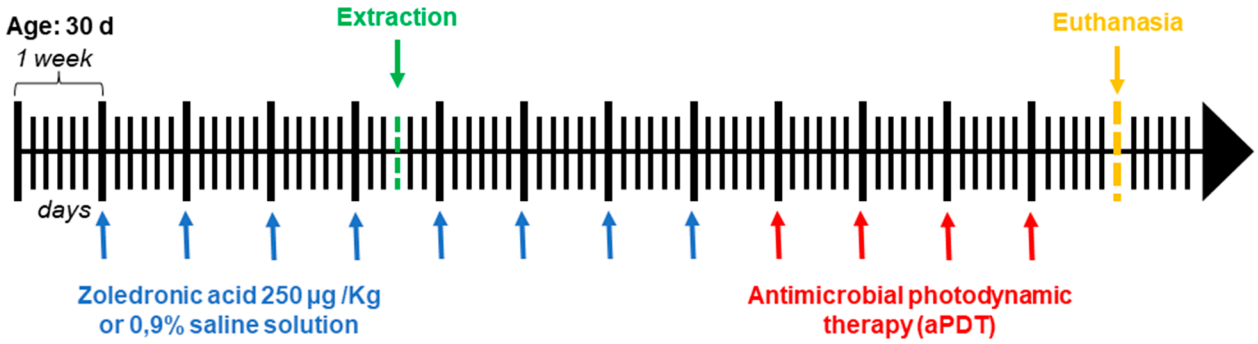

Figure 1- Schematic timeline for the control and ZA groups (animals treated with 0.9% saline and 250 µg/kg zoledronic acid, respectively), starting 4 weeks before tooth extraction

Bisphosphonate administration

In the ONE, ONE+PS, ONE+PBM, and ONE+aPDT groups, 250 µg/kg ZA (Blau Farmacêutica S.A., São Paulo, Brazil) was administered intraperitoneally (IP), totaling 100 µL. In the NC group, 100 µL of sterile 0.9% saline solution was administered IP. The animals received IP injections of ZA and sterile 0.9% saline once a week for 4 weeks prior to extraction of the lower left first molar[19] (Figure 1).

Extraction

After 4 weeks of drug administration, all animals underwent extraction of the lower left first molar. The animals were anesthetized with ketamine (75 mg/kg) + xylazine (10 mg/kg) (Syntec, São Paulo, Bazil) IP[20] and then underwent dental luxation and extraction without root fractures.[14] BP administration continued until week 8 (Figure 1).

Photodynamic therapy

The aPDT sessions started at week 9, once a week for 4 weeks. The anesthetic protocol employed was the same as that used for tooth extraction. The PS used was Methylene blue 0.3% (MB) (RenyLab, Barbacena, (Figure 1).[14] The ONE+PS group received the same MB volume at the same time and interval, except for laser irradiation. The PBM group received the same irradiation protocol at the same time and interval, without MB administration. Rats in the NC and ONE groups received IP injections of anesthetic, but not laser irradiation.

Euthanasia

At the end of the 12th week, the animals were euthanized according to the "Guidelines for the Practice of Euthanasia of the National Council for the Control of Animal Experiments" with a combination of ketamine (180 mg/Kg) + xylazine (30 mg/Kg) (Syntec, São Paulo, Bazil) IP[20]. Death was confirmed by the absence of corneal reflex.[21]

Histological processing

The samples were fixed in 10% buffered paraformaldehyde at room temperature (23 °C ± 2 °C) for 48 h. Fixed samples were demineralized in 5% nitric acid for 7 days at room temperature, with the solution changed every 48 h. The ON lesions were excised (in the mesial-distal direction of the dental socket), dehydrated with ascending alcohol concentrations

(70%, 80%, 90%, and absolute), cleared in xylene, and embedded in paraffin (maximum temperature 60º C). Coronal sections of 4 µm were made, mounted on histological slides, dewaxed in xylol, and hydrated in decreasing alcohol concentrations. Hematoxylin-eosin (H&E) and Masson's trichrome staining were performed on the sections at room temperature.[22]

Immunohistochemical analysis

Samples subjected to immunohistochemical reactions were reactivated with Trilogy solution (Sigma-Aldrich, St. Louis, Missouri, EUA) in a Paschal pressure chamber (DakoCytomation, Denmark), followed by endogenous peroxidase blocking for 30 min. Specific protein blocking was then performed and the monoclonal antibody for the NF-kB antigen (1:100, CLOUDE-ONE, USA) was applied. The biotinylated secondary antibody (biotinylated goat anti-rabbit and anti-mouse antibodies at 1:100 dilution [DAKO-LSAB 2 System, Peroxidase, K0675]) was then applied. The reaction was developed using 0.024% diaminobenzidine (DAB) solution.[23]

Histological and histomorphometric analysis

Histological sections stained with H&E, Masson's trichrome staining, or NF-kB immunostaining were visualized with a 40x objective under an Eclipse Ci-L light microscope (Nikon, Tokyo, Japan). Assessment and photographic recording were performed using NIS Elements D software (Version 4.00, Nikon, Tokyo, Japan) and a DS-Ri2 microcamera (Nikon, Tokyo, Japan).[22]

To calculate the area of bone necrosis, H&E-stained histological sections were visualized. The necrotic bone area was defined as an area of alveolar bone containing five or more empty lacunae per 1 µm^2 (Figure 2).[24] All pixels of the involved lesions were selected to create a binary image, and the area was calculated in µm^2 and expressed as mean ± standard error of the mean. The area of extracellular matrix (ECM) or NF-kB immunostaining was calculated using algorithms built into the software**.** In each image, all pixels with shades of blue (Masson's trichrome staining) or brown (NF-kB immunostaining) were selected to create a binary image and calculate the area in µm^2, expressing it as mean ± standard error of the mean.[22,23]

Statistical analysis

The data obtained are expressed as mean ± standard error of the mean (per group) and were analyzed using the statistical program GraphPad Prism version 5.0 (GraphPad Software, Inc, California, USA). The Shapiro-Wilk normality test was applied, and data from the histomorphometric analyses were analyzed using one-way analysis of variance (ANOVA) and Tukey's post-test, with a statistical significance level of 5% ($p<0.05$) to determine differences between the experimental groups.

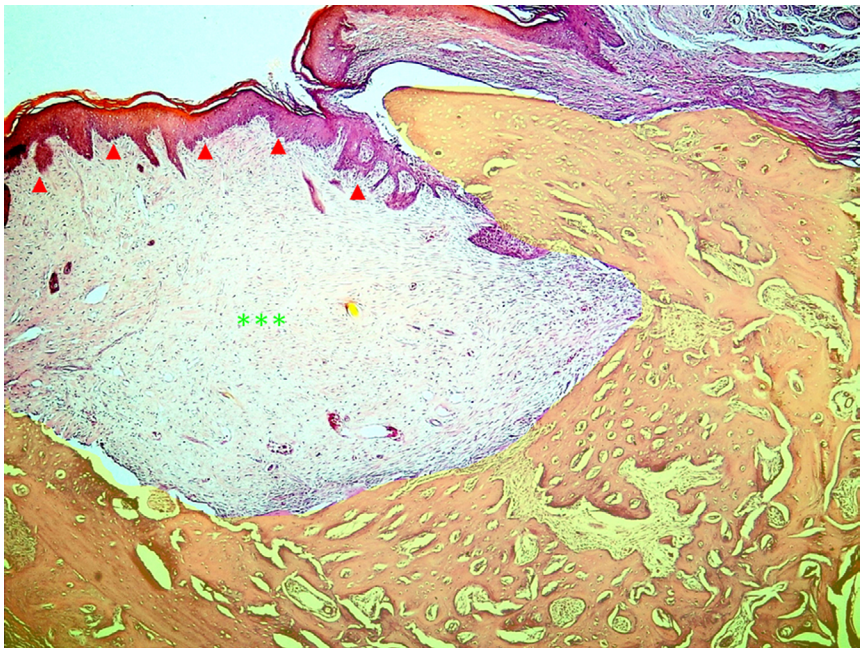

Figure 2- Histological section of the mandible stained with H&E stain showing the alveolar bone (4 weeks post-extraction). Yellowish area: alveolar bone analyzed for histomorphometric area calculation. Green star: granulation/connective tissue. Red arrowhead: epithelial tissue. Note the presence of epithelial lining over the extraction site and nucleated osteocytes and connective tissue islands within the bone matrix

Results

Macroscopic evaluation of osteonecrosis in treatment with aPDT

After extraction, the samples (Figure 3) from the NC group (A) showed clear, smooth, and homogeneous tissue on the surface of the extraction region, compatible with healthy gingiva (yellow circle). However, those in the ONE group (B) showed no epithelial lining in the extraction region, with the presence of a macroscopic yellow-brownish area compatible with exposed bone (blue circle) in this region. The samples from the other experimental groups—ONE+PS (C), ONE+PBM (D), and ONE+aPDT (E)—showed the same macroscopic pattern as the NC group, i.e., the presence of smooth, clear, and homogeneous tissue over the injured surface, compatible with healthy gingiva (yellow circle).

Histological and histomorphometric evaluations

It was observed (Figure 4) that samples from the NC group (A) had nucleated viable osteocytes (yellow arrowhead) with homogeneous sizes and shapes. On the other hand, samples from the ONE group (B) had empty lacunae (red arrowhead) with a swollen and sometimes granular appearance. However, samples from the ONE+PS group (C) had both necrotic osteocytes (empty lacunae), represented by red arrowheads, and viable osteocytes (yellow arrowhead). Lastly, samples from the ONE+PBM (D) and ONE+aPDT (E) groups had osteocytes that were histologically consistent with those of the NC group.

To confirm the qualitative findings, the area of tissue necrosis (ON), which was the main histological finding for this experimental model, was measured by group. Figure 5 shows the morphometric analysis of ON by experimental condition. The samples from the NC group did not show any areas of ON. However, those in the ONE group showed extensive regions of ON. Samples from the ONE+PS, ONE+PBM, and ONE+aPDT groups showed a reduction in the ON region compared with the ONE group ($p<0.05$).

Histological and histomorphometric evaluations of the extracellular matrix

Samples (Figure 6) from the NC group (A) histologically showed connective tissue, especially collagen fibers, highlighted in blue by Masson's trichrome staining, and fibers with a homogeneous appearance and diffuse distribution (black arrow). However, samples from the ONE group (B) showed fibers with a heterogeneous appearance and focal distribution (red arrow). Samples from the ONE+PS (C), ONE+PBM (D), and ONE+aPDT groups showed

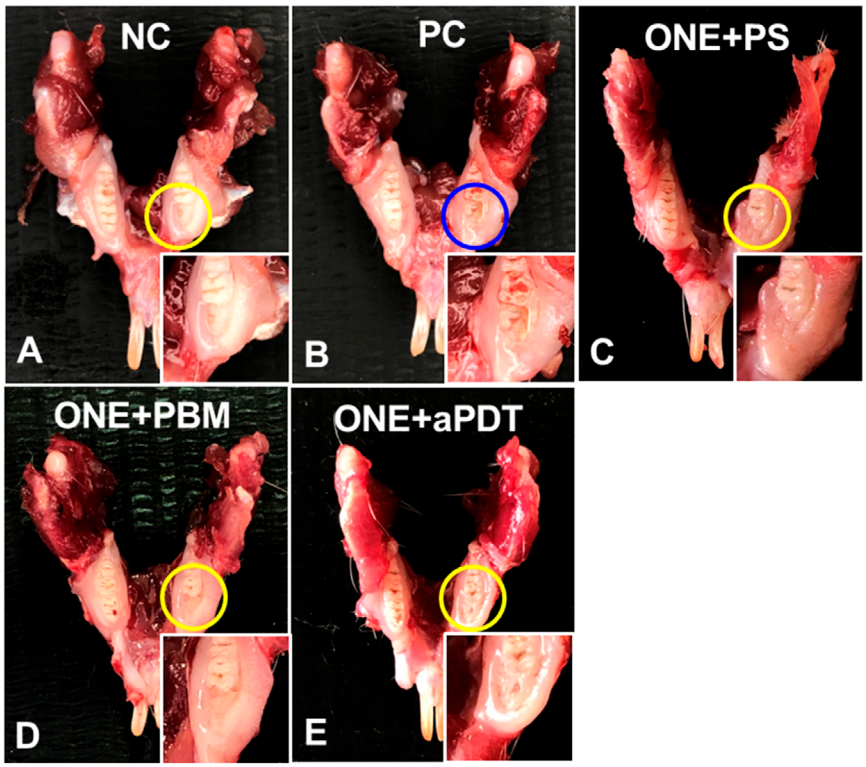

Figure 3- Representative macroscopy of the dissected mandibles of animals by group. Yellow circle: region with gingival tissue covering the alveolar region. Blue circle: exposed bone area. A. NC. B. ONE. C. ONE+PS. D. ONE+PBM. E. ONE+aPDT

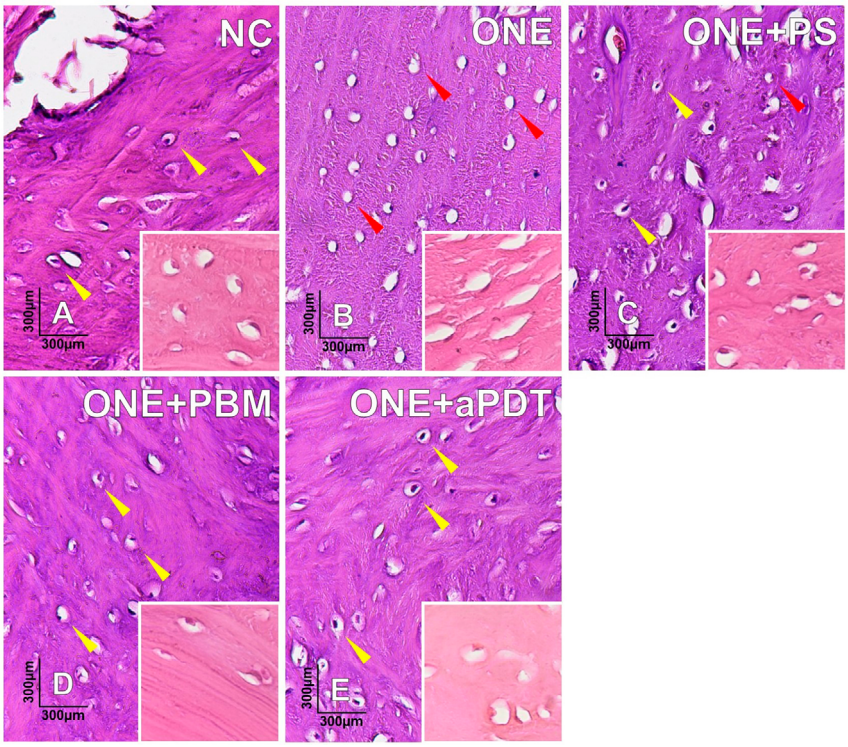

Figure 4- Histological section of mandibles stained with H&E, showing bone tissue (A-E). A. NC. B. ONE. C. ONE+PS. D. ONE+PBM. E. ONE+aPDT. Yellow arrowhead: nucleated osteocytes. Red arrowhead: empty lacunae

fibers with an appearance similar to that of the fibers in the NC group (black arrows).

Under all experimental conditions, the morphometric analysis of the ECM area (Figure 7) was based on the observed marking area in the histochemical reaction by Masson's trichome staining. Samples from the NC group showed large areas of intense deposition of cholanogenic ECM. However, animals in the ONE group showed few areas of ECM deposition. Samples from the other experimental groups—ONE+PS, ONE+PBM, and ONE+aPDT—showed an increase in ECM deposition compared with samples from the ONE group ($p<0.05$).

Evaluation immunohistochemistry for NF-kB

Samples from all experimental groups histologically showed connective tissue with a region of bone tissue with brownish or brown markings, indicating positive immunohistochemical reaction for NF-kB (Figure 8). In the NC group (A), focal labeling was observed only in connective tissue cells and spaces (black arrowhead). However, in the ONE group (B), multifocal markings were found in multiple tissue regions, especially those rich in granulation tissue (red arrowhead). Samples from the ONE+PS (C) and ONE+PBM (D) groups showed immunohistochemical staining patterns similar to that of the NC group. In the ONE+aPDT group (E), multifocal markings were present in regions rich in granulation tissue (black arrowhead).

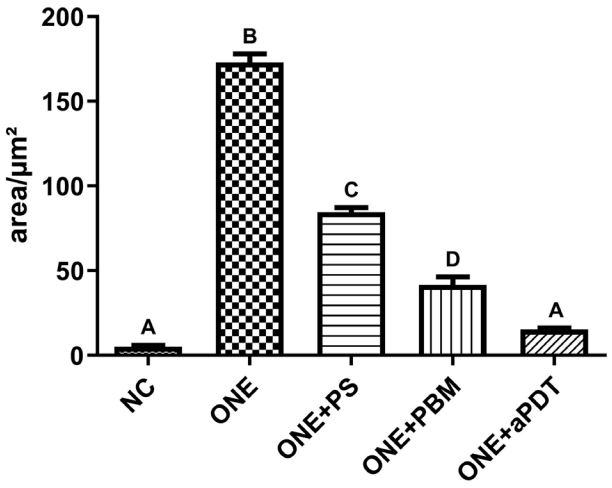

Figure 5- Morphometric analysis of ON area under different experimental conditions. Results are presented as mean ± standard error of the mean, n=6 animals per group. Different letters=$p<0.05$ when compared with the ONE group. One-way ANOVA followed by Tukey's test

Under all experimental conditions, the morphometric analysis of the NF-kB deposition area (Figure 9) showed that the NC group had a low positivity for NF-kB, while the ONE group showed an increase in the area. The samples from the other experimental groups—ONE+PS, ONE+PBM, and ONE+aPDT—showed a significant decrease compared with those from the ONE group ($p<0.05$).

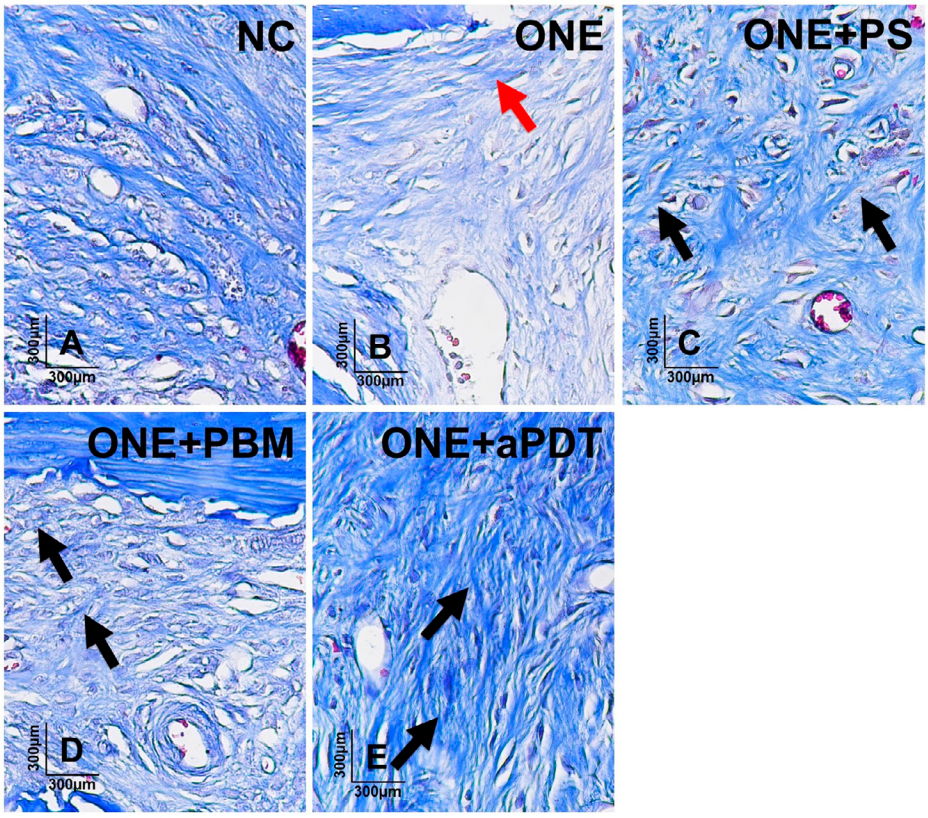

Figure 6- Histological section of mandibles stained with Masson's trichrome staining, showing the connective tissue (A-E). A. NC. B. ONE. C. ONE+PS. D. ONE+PDT. E. ONE+aPDT. Black arrow: extracellular matrix with fibers with a homogeneous appearance and diffuse distribution. Red arrow: extracellular matrix with fibers with a heterogeneous appearance and focal distribution

Discussion

This study evaluated the efficacy of aPDT in the treatment of MRONJ in a rat model of tooth extraction. For this purpose, the experimental model of ON induction in rats proposed by Biguetti, et al.[19] (2019) was adapted, inducing ON lesions in Wistar rats (*Rattus norvegicus)* after exposure to 250 µg/kg ZA for 7 weeks. It was observed that aPDT minimized the negative effects of ZA on the soft and hard tissues of the extraction site, modulating tissue repair and adaptation as a potential treatment for MRONJ.

The physical evaluation of the lesions showed bone exposure in the ONE group, in which a large area of necrosis was found, which can be explained by the absence of or decrease in the activation of growth and differentiation factors (bone morphogenetic protein and insulin growth factor-1 and -2) that stimulate the differentiation of osteoblasts and the secretion of bone matrix. These factors are expressed by the action of osteoclasts in contact with bone during the remodeling process. However, these cells are impaired by the use of ZA, which prevents the expression of receptor activator of nuclear factor-kB (RANK) by osteoclast precursor cells and inhibits binding

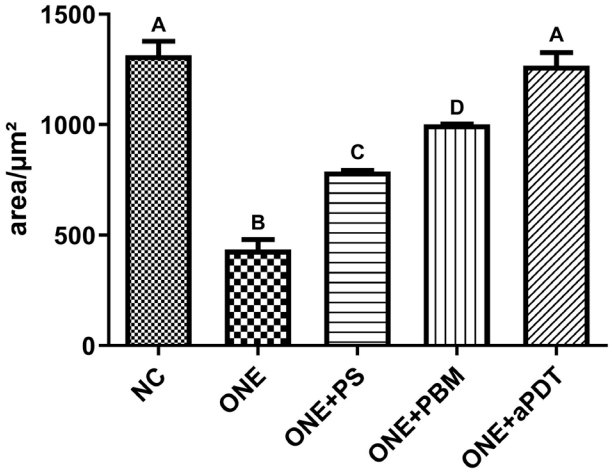

Figure 7- Morphometric analysis of the ECM deposition area under different experimental conditions. Results are expressed as mean ± standard error of mean, n=6 animals per group. Different letters=p<0.05 when compared with the ONE group. One-way ANOVA followed by Tukey's test

to RANK ligand expressed by osteoblasts, which is necessary for osteoclastogenesis and the regulation of bone remodeling.[25,26] These molecular events cause a decrease in the deposition of new bone matrix, preventing the repair and maintenance of this tissue.[27]

Chronic use of ZA also increases the number of inflammatory cells and the production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and

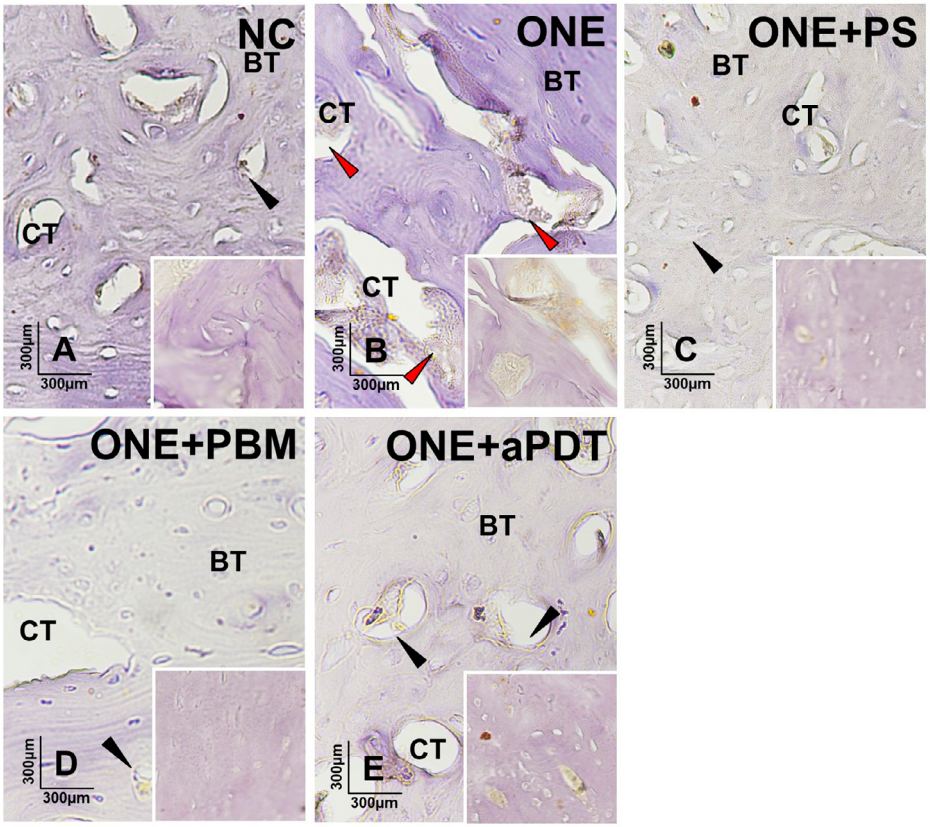

Figure 8- Histological sections of mandibles in immunohistochemical reaction for NF-kB stained in Hematoxylin (A-E). A. NC. B. ONE. C. ONE+PS. D. ONE+PBM. E. ONE + aPDT. CT: connective tissue. BT: bone tissue. Red arrowhead: positive staining for NF-kB in connective gaps. Black arrowhead: positive staining for NF-kB in connective tissue

interleukin-1β (IL-1β), which are associated with the progression of MRONJ.[28] These cytokines act on the chemotaxis of neutrophils, increasing their number and causing greater local damage via ROS production.[28] Depending on the dose of ZA, neutrophil death may occur, which also releases ROS that contribute to increased oxidative stress.[29] This increased oxidative stress activates the NF-kB inflammatory pathway.[30]

The physical evaluation of the NC, ONE+PS, ONE+PBM, and ONE+aPDT groups showed complete epithelial lining over the alveolus, with homogeneous appearance and diffuse distribution of ECM fibers. In the ONE group, the bone remained clinically exposed and the ECM fibers showed a heterogeneous appearance with focal distribution. Furthermore, the area of ECM deposition was significantly larger in the experimental and NC groups than in the ONE group. This was because exposure to ZA is associated with decreased migration and proliferation of epithelial, endothelial, and fibroblast cells and impairs the expression of vascular endothelial growth factor by decreasing vascularization.[31] In this case, the homeostasis of the injured tissues was compromised, making wound repair difficult due to decreased vascularization and inflammation, thereby favoring

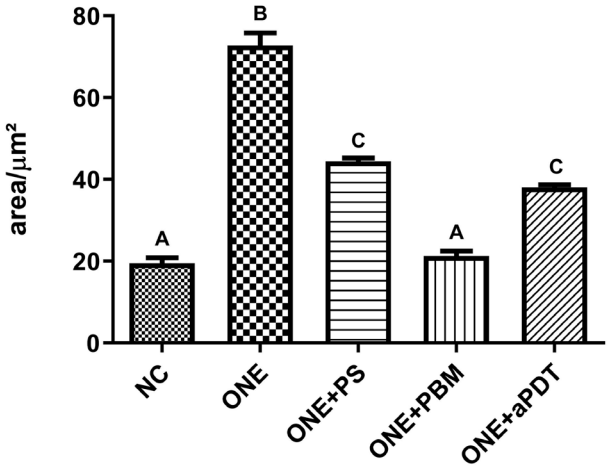

Figure 9- Morphometric analysis of the NF-kB immunolabeling area under different experimental conditions. Results are expressed as mean ± standard error of the mean, n=6 animals per group. Different letters=p<0.05 when compared with the ONE group. One-way ANOVA followed by Tukey's test

the progression of ON.[31,32]

In the immunohistochemical analysis, a higher distribution and labeling of NF-kB was observed in the ONE group than in the other experimental groups, confirming the presence of an active inflammatory process mediated by this protein. NF-kB regulates the expression of genes related to chronic inflammation, such as TNF-α, IL-1β, and interleukin-6; the first two

are responsible for the chronicity of inflammation, as they recruit and activate neutrophils and promote the expression of more NF-kB, feeding the transcription of inflammatory cytokines by this pathway.[33] The decrease in NF-kB labeling in the other experimental conditions and the presence of nucleated osteocytes, especially in the ONE+PBM and ONE+aPDT groups, was due to the use of aPDT, which reduced the oxidative stress promoted by neutrophils and the inflammatory process in the experimental lesions of ON, thereby mediating their repair.

In the ONE+PBM and ONE+aPDT groups, re-epithelialization of the dental sockets was observed, which can be explained by the initial stage of MRONJ in the animals, which favored tissue biostimulation. There was also a decrease in necrotic bone area, an increase in ECM deposition, and preservation of nucleated osteocytes compared with the ONE group. This result was made possible by the use of PBM, which increased osteoblast activity in extraction sockets in healthy subjects, accelerating the deposition of new bone matrix and promoting better healing of the epithelium.[34] These effects have also been described in ON lesions.[35,36]

The ONE+aPDT group had the smallest area of bone necrosis compared with the ONE group and the largest area of ECM deposition, with fibers with a homogeneous appearance and diffuse distribution and a cellular pattern similar to that of the NC group. These results are consistent with the expected effects of aPDT, which combines PBM with antimicrobial activity promoted by the use of a PS that is selective for microbial cells and sensitive to the wavelength of light used for irradiation. This approach has been shown to be effective in the treatment of ON lesions, reducing or preventing infection, mediating the inflammatory response, and stimulating angiogenesis, proliferation, migration, and cell differentiation.[14,36,37]

The ONE+PS group showed areas of ON in the bone tissue, represented by the presence of empty lacunae, but also presented nucleated cells and an increase in ECM deposition. This result shows that PS alone can influence bone repair, but not completely: it requires irradiation with light at the appropriate wavelength, even with clinical re-epithelialization of the alveolus and absence or reduction of inflammation mediated by NF-kB in the connective tissue. This can be explained by the antimicrobial mechanism of MB, which is effective against Gram-negative bacteria due to its hydrophilic capacity, low molecular weight, and positive charge, reducing infection in ON lesions and leading to tissue recovery.[15]

PBM and aPDT have been described separately as therapeutic options for MRONJ lesions,[14,35-37] with few studies comparing the two proposals. In this study, it was observed that the two treatments had positive effects on the extraction sites, such as a decrease in the area of bone necrosis, an increase in the area of ECM, and a decrease in inflammation, represented by the low level of NF-kB labeling in the ONE+PBM and ONE+aPDT groups. These characteristics confirm that PBM and aPDT are effective in the treatment of ON lesions.

The use of aPDT should be highlighted, as the corresponding experimental group showed the greatest decrease in the area of bone necrosis and the greatest increase in the production of ECM, influenced by the use of methylene blue as PS. In addition, it would be interesting to test aPDT in combination with other treatments, such as application of ozonated water[38] or probiotics,[39] in order to test their mutual effects.

There are limitations to developing an animal model of osteonecrosis by tooth extraction. The surgical procedure has technical restrictions, such as the limited time for tooth extraction due to anesthesia-induced apnea and respiratory difficulties, and the limited literature on the extraction procedure in rats. Despite these limitations, our research group was able to achieve a great methodological performance based on the study of Ervolino, et al.[14] (2019).

Conclusion

The use of PBM and aPDT for the treatment of experimental MRONJ lesions was effective for bone and epithelial repair in this experimental model. aPDT was responsible for the greatest reduction in the ON group, increase in the ECM group, and decrease in inflammation mediated by the NF-kB pathway in the regions of irradiation, although the use of PS alone and light alone (PBM) also showed positive effects.

Acknowledgments

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could influence the study reported in this paper.

Data availability statement

All data generated and analyzed during this study are included in this published article.

Authors' contributions

Pontes, Jannerson: Conceptualization (Equal); Data curation (Equal); Formal analysis (Equal); Investigation (Equal); Methodology (Equal); Validation (Equal); Visualization (Equal); Writing – original draft (Equal); Writing – review & editing (Equal). **Figueiredo, Ludmilla:** Formal analysis (Equal); Investigation (Equal); Methodology (Equal). **Lima, Wilson:** Formal analysis (Equal); Investigation (Equal); Methodology (Equal). **Araújo, Rubens:** Formal analysis (Equal); Investigation (Equal). **Santos, Ana Beatriz Rodrigues dos:** Formal analysis (Equal); Investigation (Equal). **Almeida, Leopoldina de Fátima Dantas de:** Conceptualization (Equal); Methodology (Equal). **Alves, Adriano Francisco:** Conceptualization (Equal); Data curation (Equal); Formal analysis (Equal); Funding acquisition (Equal); Investigation (Equal); Methodology (Equal); Project administration (Equal); Resources (Equal); Software (Equal); Supervision (Equal); Validation (Equal); Visualization (Equal); Writing – review & editing (Equal).

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