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Effectiveness of antimicrobial photodynamic therapy mediated by butyl toluidine blue in preventing medication-related osteonecrosis of the jaws in rats.

Effectiveness of antimicrobial photodynamic therapy mediated by butyl toluidine blue in preventing medication-related osteonecrosis of the jaws-like lesions in rats

ABSTRACT

Background: Medication-related osteonecrosis of the jaws (MRONJ) is difficult to treat, therefore, prevention would be the ideal clinical approach. This study evaluated the effectiveness of antimicrobial photodynamic therapy (aPDT), mediated by butyl toluidine blue (BuTB) in the prevention of MRONJ-like lesions after tooth extraction in rats. **Methods**: Twenty-eight senescent female rats were distributed in groups: VEH and VEH-aPDT, treated with vehicle, ZOL and ZOL-aPDT, treated with 100 µg/Kg of zoledronate, both treatments every three days over seven weeks. After three weeks from the commencement of treatment, the mandibular first molar was extracted. For the VEH and ZOL groups, no local treatment was performed, while with theVEH-aPDT and ZOL-aPDT groups, photodynamic treatment was carried out at 0, 2, and 4 days after extraction. For aPDT, 500µl of BuTB solution was deposited on the dental extraction site (0.5 mg/ml; 60s), followed by irradiation with low-level laser (InGaAIP; 660nm; 35mW; 74.2J/cm²; 60s). After 28 postoperative days, euthanasia was performed. The hemimandibles were processed to: 1) histological analysis of tissue repair; 2) histometric analysis of the percentage of new-formed bone tissue (PNFBT) and percentage of non-vital bone tissue (PNVBT); 3) immunohistochemical analysis for tartrate-resistant acid phosphatase (TRAP). **Results**: The ZOL and ZOL-aPDT groups showed less TRAP-positive cells when compared with VEH and VEH-aPDT. The ZOL group demonstrated great compromise in the tissue repair process, consistent with MRONJ-like lesions. VEH, VEH-aPDT and ZOL-aPDT presented a favorable tissue repair process. PNFBT in the ZOL group was lower than in the VEH, VEH-aPDT and ZOL-aPDT groups, whereas PNVBT in the ZOL group was higher than in the VEH, VEH-aPDT and ZOL-aPDT groups. **Conclusion:** aPDT mediated by BuTB prevented the occurrence of MRONJ-like lesions after tooth extraction in rats.

KEYWORDS: Bisphosphonates. Osteonecrosis. Jaws. Prevention. Photodynamic therapy. Butyl toluidine blue.

INTRODUCTION

Medication-related osteonecrosis of the jaws (MRONJ) is an adverse effect caused by the use of antiresorptive drugs (RUGGIERO *et al.*, 2022; MARX, 2003). The criteria for defining MRONJ are: 1) current or previous treatment with antiresorptive or antiangiogenic agents; 2) exposed bone or bone that can be probed through an intraoral or extraoral fistula(e) in the maxillofacial region that has persisted for more than eight weeks; 3) no history of radiation therapy to the jaws or metastatic disease of the jaws (RUGGIERO *et al.*, 2022). The incidence of MRONJ is low when antiresorptive agents are used at osteoporotic dosage (<0.05%), in contrast, it is much higher when such drugs are used at oncological dosage (<5%) to complement antineoplastic therapy (RUGGIERO *et al.*, 2022).

The etiopathogenesis of MRONJ is partially understood, and is supposedly of a multifactorial nature. Among the etiopathogenic factors are: the potent suppression of the resorptive activity of osteoclasts, which would result in the accumulation of microfractures in bone tissue and, consequently, in areas of non-vital bone tissue, favoring the establishment of infection with subsequent increase in bone necrosis; the potent antiangiogenic effect, which would result in avascular necrosis of bone tissue; favoring infection induced by bisphosphonates, which would increase the ability of bacteria to adhere and colonize exposed bone tissue; the cytotoxic effect of these drugs on various cell lines, which would result in decreased repair capacity, both for soft and hard tissues in the maxillofacial region, and lack of resilience in the local immune response, which would compromise the protective response and tissue repair, concomitantly (KUROSHIMA *et al.*, 2019; AGHALOO *et al.*, 2015; BADEL *et al.*, 2013; ALLEN and BURR, 2009).

Treatment is based on MRONJ staging and consists of the use of drug therapy, predominantly prolonged use of antimicrobial agents associated with surgical procedure. Surgical therapy ranges from conservative to aggressive and consists of curettage and/or sequestrectomy to partial resection of the jaws (RUGGIERO *et al.*, 2014; MADEIRA *et*

al., 2020; KHAN *et al.*, 2017; KHAN *et al.*, 2015). However, the ideal clinical conduct would be one capable of preventing the onset of MRONJ, that is, preventive treatments. Although no individual strategy eliminates all MRONJ risks, some preventive procedures have been recommended: using preoperative and postoperative antibiotics and antimicrobial mouth rinses; primarily closing extractions sites, and maintaining good oral hygiene; smoking cessation and diabetes optimization (RUGGIERO *et al.*, 2022).

Antimicrobial photodynamic therapy (aPDT) has positive effects at the cellular and tissue level, which suggests its use as a potential therapy for MRONJ (WAINWRIGHT, 2019; WAINWRIGHT, 1998; MAHMOUDI *et al.*, 2018; CIEPLIK *et al.*, 2018; HAMBLIN, 2016; CARRERA *et al.*, 2016). aPDT consists of the use of light with a wavelength coinciding with the absorption band of a photoantimicrobial (PA), which is selectively pre-absorbed by microbial cells, and which in the presence of oxygen is activated and generates high levels of reactive oxygen, highly cytotoxic and lethal to the microbial cells that absorbed the PA (WAINWRIGHT, 2019; WAINWRIGHT, 1998; MAHMOUDI *et al.*, 2018; CIEPLIK *et al.*, 2018; HAMBLIN, 2016; CARRERA *et al.*, 2016). In addition to the antimicrobial action, studies show that aPDT, when using low power laser as a light source, has associated photobiomodulatory effects, such as modulation of the inflammatory response, angiogenesis, proliferation, migration, differentiation and cellular activity, essential events to promote the tissue repair process (DOMPE *et al.*, 2020).

One of the important factors that determine the efficacy of aPDT is the PA itself, as well as its dosage and its contact time with the target region (KLAUSEN *et al.*, 2020; GHORBANI *et al.*, 2018; WAINWRIGHT *et al.*, 2016). In general, a PA must: provide a high quantum yield of $^1O_2$; be photostable; have a broad spectrum of antimicrobial action; express high binding affinity for microorganisms and low binding affinity and toxicity for normal eukaryotic cells; not show mutagenic action; present a narrow therapeutic window, that is, have photodynamic inactivation parameters necessary for bacteria to be killed efficiently without damage to normal eukaryotic cells (KLAUSEN *et al.*, 2020; GHORBANI *et al.*, 2018; WAINWRIGHT *et al.*, 2016).

Among photoantimicrobials, phenothiazine derivatives, such as methylene blue and toluidine blue, are among the most widely-used. The improvement of photoantimicrobial efficacy may be achieved via changes to the molecular structure. Butyl toluidine blue

(BuTB) is a phenothiazine-based PA produced by chemical modification of the toluidine blue structure. Such modification led to improvements in both its physical properties and photodynamic effects, which include increased lipophilicity and reduced molecular aggregation behavior, which provides a greater capacity for cellular interaction and greater efficiency in the production of reactive oxygen species (WAINWRIGHT *et al.*, 2016). Nuernberg *et al*. (2020) performed the first *in vivo* study using aPDT mediated by BuTB that proved to be extremely effective as an adjunctive treatment to scaling and root planing of experimental periodontitis. Another study compared aPDT mediated by traditional phenothiazine PA with aPDT mediated by BuTB as adjunctive therapy to scaling and root planning (THEODORO et al., 2021). The effectiveness of aPDT mediated by BuTB proved to be superior to other phenothiazine Pas (methylene blue and toluidine blue) in the treatment of experimental periodontitis in rats (THEODORO et al., 2021). Our research group was the first to propose the use of aPDT as a preventive strategy to prevent MRONJ after tooth extraction (ERVOLINO *et al.*, 2019). It was found that aPDT mediated by methylene blue, improved the tissue repair process and prevented the occurrence of MRONJ. Since the physical-chemical modifications of PA can enhance their photodynamic effects, aPDT mediated by BuTB may be an excellent way to prevent MRONJ. Therefore, the present study aims to evaluate the effectiveness and safety of aPDT mediated by a new PA, BuTB, at the site of dental extraction of rats with the main risk factors for MRONJ-like lesions.

MATERIAL AND METHODS

Experimental design

Animals

Twenty-eight senescent female rats (*Rattus norvegicus* - *Wistar*, 20 months), with body weight between 350-450 g, were used. The experimental protocol followed the rules established by the “Guide for the Care and Use of Laboratory Animals” and was approved by the Ethics Committee on the Use of Animals (CEUA) (FOA PROCESS No. 562-2018).

Anesthesia

All experimental procedures (installation of the ligature, extraction, aPDT and euthanasia) were performed under general anesthesia using the combination of ketamine hydrochloride (80 mg/Kg, Francotar®, Virbac, SP, Brazil) and xylazine hydrochloride (10 mg/kg), Rompum®, Bayer, RS, Brazil) intramuscularly.

Ligature-induced experimental periodontitis

One day before the start of drug treatment, a cotton ligature (cotton thread # 24; Coats Corrente, SP, Brazil) was installed around the lower left first molar (Figure 1) of all animals, which was maintained for three weeks, with the intention to promote the accumulation of biofilm and, consequently, the induction of experimental periodontitis (ERVOLINO *et al.*, 2019; TORO *et al.*, 2019; STATIKIEVICZ *et al.*, 2018).

Drug protocol

The treatment protocol lasted seven weeks (Figure 1). The administration of vehicle or zoledronate (Sigma Chemical, St Louis, MO, USA) was performed every three days via the intraperitoneal route. The vehicle consisted of 0.45 ml of 0.9% sodium chloride solution. The dose of zoledronate was 100 µg/kg, which was diluted in 0.45 ml of vehicle. The dose of zoledronate and treatment plan consisted of an adaptation, for the rat, of the protocol used to complement cancer therapy in humans (ERVOLINO *et al.*, 2019; TORO *et al.*, 2019; STATIKIEVICZ *et al.*, 2018; SILVA *et al.*, 2015).

Experimental Groups

After the installation of the ligature, the animals started treatment with vehicle or zoledronate and were distributed randomly into four experimental groups, each composed of 7 animals (Figure 1):

The VEH and ZOL groups received vehicle and zoledronate, respectively, every three days for seven weeks. Three weeks after the start of treatment, the lower left first molar was extracted. Euthanasia was performed 28 days after extraction.

The VEH-aPDT and ZOL-aPDT groups received vehicle and zoledronate, respectively, every three days for seven weeks. Three weeks after the start of treatment, the lower left

first molar was extracted. At 0, 2 and 4 days postoperatively, aPDT was performed at the tooth extraction site. Euthanasia was performed 28 days after extraction.

Dental extraction

For extraction of the lower first molar, the animals were properly positioned on the operating table. Oral cavity antisepsis, sindesmotomy, dislocation and extraction of the lower left first molar were performed using adapted dental instruments (ERVOLINO *et al.*, 2019; TORO *et al.*, 2019; STATIKIEVICZ *et al.*, 2018).

Antimicrobial photodynamic therapy (aPDT)

aPDT sessions were performed at 0, 2, and 4 days after extraction. Butyl toluidine blue at a concentration of 0.5 mg/ml was used as a PA. 500µL of the BuTB was deposited on the extraction site of the lower left first molar (Figure 1) for sixty seconds, and subsequently the laser tip was positioned in the center of the extraction socket, parallel to its long axis and at the level to its opening (Figure 1). The low power laser used in the present study was of the type *Indium Gallium Aluminum Phosphorous* (InGaAlP; Thera Lase®, D.M.C. Equipamentos Ltda®, São Carlos, SP, Brazil) with the following parameters: wavelength: 660 nm (visible laser - red); power: 35 mW; operation mode: continuous; spot size: 0.0283 cm^2; method of application: contact, punctual; energy: 2.1 J/point; application time: 60 seconds; number of application points: 1; energy density per point: 74.2 J/cm^2; irradiance: 1.23 W/cm^2 (ERVOLINO *et al.*, 2019; STATIKIEVICZ *et al.*, 2018). After irradiation, excess BuTB was gently removed from the tooth extraction site with the aid of physiological saline solution and cotton wool balls.

Obtaining samples

At 28 days post-surgery, the animals were deeply anesthetized and euthanasia was performed by transcardiac perfusion with 100ml of physiological saline added 0.1% heparin and then 800ml of 4% formaldehyde (Sigma Chemical®, Saint Louis, MO, USA) in phosphate buffered saline (PBS - Sigma Chemical®), 0.1M, 4°C, pH 7.4. The left hemimandibles were dissected and submitted to post-fixation for 72 hours (ERVOLINO *et al.*, 2019; TORO *et al.*, 2019; STATIKIEVICZ *et al.*, 2018).

Histological processing of samples

The samples were demineralized in 10% ethylenediaminetetraacetic acid (EDTA) (Sigma Chemical®) in PBS, for a period of 60 days, and submitted to conventional histological processing, including paraffin. The microtomy was performed from lingual to buccal, and the histological sections of the portion of the dental alveolus previously occupied by the mesial and distal roots of the lower left first molar and surroundings were collected.

For the histopathological analysis of the dental extraction site and surroundings and for the histometric analysis of the percentage of newformed bone tissue (PNFBT) and the percentage of non-vital bone tissue (PNVBT), the histological sections were stained with hematoxylin-eosin (HE).

For immunohistochemical analysis to detect TRAP, the histological sections were deparaffinized in xylol and hydrated in a decreasing series of ethanol (100º - 100º - 100º - 90º - 70°GL). Antigenic retrieval was performed by immersing the histological slides in citrate buffer (Spring Bioscience, Pleasanton, CA, USA), in a pressurized chamber (Decloaking chamber®, BioCare Medical, Concord, CA, USA) at 95°C, for 20 minutes. At the end of each stage of the immunohistochemical reaction, the histological slides were washed in 0.1M PBS, pH 7.4. Subsequently, the slides were immersed in 3% hydrogen peroxide for 1 hour and 1% bovine serum albumin for 12 hours to block endogenous peroxidase and block nonspecific sites, respectively. The slides containing samples from each experimental group were incubated with the goat TRAP antibody (SC-30833, Santa Cruz Biotechnology®). The sections were incubated with secondary biotinylated antibody for 2 hours and subsequently treated with streptavidin conjugated to horseradish peroxidase - HRP for 1 hour (Universal Dako Labeled HRP Streptavidin-Biotin Kit®, Dako Laboratories, CA, USA). The development was carried out using diaminobenzidine 3,3'-tetrachlorhydrate (DAB chromogen Kit®, Dako Laboratories, CA, USA) as a chromogen. Counterstaining was performed with Harris Hematoxylin, followed by dehydration in ethanol, diaphanization in xylol and coating with mounting medium (Permount, Fisher Scientific, San Diego, CA, USA) and glass coverslips. As a negative control, the specimens were subjected to the procedures described above, eliminating the use of the primary antibody.

Analysis of results

Analysis of general health conditions and intraoral clinical examination

The general health condition of the animals was verified throughout the experimental period and the body weight was monitored. Intraoral clinical examination was carried out, which consisted of a thorough visual inspection of the oral cavity, especially the dental extraction site (Table 1; ERVOLINO *et al.*, 2019; TORO *et al.*, 2019; STATIKIEVICZ *et al.*, 2018).

Microscopic analysis

For histopathological and histometric analysis, three histological sections located in the buccal, middle and lingual portion of the dental alveolus were used. The region of interest consisted of an area of 4mm x 4mm that encompassed the portion of the alveolus previously occupied by the mesial root and the distal root of the lower left first molar and its surroundings. Its distal limit consisted of a line located parallel to the surface of the coronary and root dentin of the lower left second molar, from which it extended to the mesial by 4mm. Its coronary limit consisted of a line located parallel to the amelo-cemental limit of the lower left second molar, from which it extended to the apical by 4mm (ERVOLINO *et al.*, 2019; TORO *et al.*, 2019; STATIKIEVICZ *et al.*, 2018). Microscopic analyzes were performed by a certified histologist, previously calibrated and blind to treatments (EE).

Histological analysis

Optical microscopy was used to evaluate the following in the region of interest: 1) intensity of the local inflammatory response; 2) extension of the inflammatory process; 3) pattern of cellularity and structure of epithelial tissue; 4) pattern of cellularity and structure of connective tissue; 5) pattern of cellularity and structure of bone tissue (ERVOLINO *et al.*, 2019; TORO *et al.*, 2019; STATIKIEVICZ *et al.*, 2018).

Histometric analysis of PNFBT and PNVBT

In the region of interest, images were captured using a digital camera (AxioCam®, Carl Zeiss, Gottingen, Germany) attached to the optical microscope (AxioLab®), Carl Zeiss, Gottingen, Germany) and connected to a microcomputer. With the aid of the image analysis program (Axiovision 4.8.2®, Carl Zeiss, Gottingen, Germany), the total amount of bone tissue was measured, and then the PNFBT and PNVBT were obtained (ERVOLINO *et al.*, 2019; TORO *et al.*, 2019; STATIKIEVICZ *et al.*, 2018). The PNVBT was calculated from regions where more than ten adjacent lacunae were empty or containing necrotic osteocyte remains.

Immunohistochemical analysis of TRAP at the dental extraction site

Images of the samples submitted to immunohistochemical detection of TRAP were captured as previously described. With the aid of the image analysis program (Axiovision 4.8.2®, Carl Zeiss), TRAP-positive cells were quantified per mm^2 of bone tissue (ERVOLINO *et al.*, 2019; TORO *et al.*, 2019).

Statistical analysis

The Bioestat 5.3 program (Instituto Mamirauá, Manaus, AM, Brazil) was used. For histopathological analysis, Kruskal-Wallis Analysis of Variance tests and Student-Newman-Keuls post-test were used. For the histometric analysis of PNFBT and PNVBT and for the immunohistochemical analysis of TRAP, the Shapiro-Wilk, Analysis of Variance (ANOVA) and Tukey post-test tests were used. The level of significance was 5%.

RESULTS

General health conditions and intraoral clinical examination

The general conditions and body weight of the animals in the VEH, VEH-aPDT and ZOL-aPDT groups remained within a normal range and constant throughout the experimental period. The animals in the ZOL group showed a decrease in body weight, especially after extraction, which remained until the end of the experimental period (Figure 2).

On intraoral clinical examination (Table 1), in the ZOL group, a wider extraction socket was observed than in the other experimental groups, whose overlying mucosa was not reconstituted. In four specimens in this group there was bone exposure and even greater enlargement of the extraction site. In the VEH, VEH-aPDT and ZOL-aPDT groups, there was complete, or almost complete, repair of the dental extraction site, with a much smaller extraction socket and covered totally or partially by soft tissues.

Tissue repair at the dental extraction site and adjacency

The ZOL group showed a great compromise in the tissue repair process, presenting histological characteristics consistent with MRONJ-like lesions. The VEH, VEH-aPDT and ZOL-aPDT groups presented a more favorable tissue repair process of the extraction site and overlying mucosa. The parameters, scores and distribution of the specimens according to the histopathological analysis of the level of tissue inflammation and the structure pattern of the epithelial, connective and bone tissues are shown in Table 2 and Table 3, respectively. The histological aspect of the tooth extraction site and its surroundings in VEH, VEH-aPDT, ZOL and ZOL-aPDT at 28 postoperative days are shown in Figures 3, 4 and 5.

PNFBT at the dental extraction site

For the ZOL group, PNFBT was significantly lower than in VEH ($p<0.01$), VEH-aPDT ($p<0.01$) and ZOL-aPDT ($p<0.01$). There was no statistically significant difference in PNFBT between VEH and VEH-aPDT. PNFBT was significantly higher in VEH-aPDT when compared with ZOL ($p<0.01$) and ZOL-aPDT ($p<0.05$) (Figure 4).

PNVBT at the dental extraction site

In ZOL, PNVBT was significantly higher than in VEH ($p<0.01$), VEH-aPDT ($p<0.01$) and ZOL-aPDT ($p<0.01$). There was no statistically significant difference in PNVBT between VEH and VEH-aPDT. PNVBT was significantly higher in ZOL-aPDT when compared to VEH ($p<0.01$) and VEH-aPDT ($p<0.01$) (Figure 5).

TRAP-positive cells at the dental extraction site

The immunohistochemical technique used for TRAP detection showed high specificity in its detection, which was proven by the total absence of marking in the negative control of the immunohistochemical reaction. The immunoreactive cells presented a brownish color confined exclusively to the cytosolic compartment of osteoclasts (Figure 6).
The amount of TRAP-positive cells in the groups ZOL and ZOL-aPDT was significantly lower than in the group VEH and VEH-aPDT ($p<0.01$) (Figure 6).

DISCUSSION

MRONJ is a pathological condition whose treatment, both preventive and curative, is a huge challenge for dentistry (RUGGIERO *et al.*, 2022). Among the prevention strategies of MRONJ, the standard is prolonged antibiotic administration. However, this may prove to be a failure (HOEFERT and EUFINGER, 2011; LÓPEZ-JORNET *et al.*, 2011). The present study aimed to evaluate the effectiveness and safety of aPDT, using a new PA, BuTB, associated with irradiation with low power laser, in the dental extraction site of rats with the main risk factors for MRONJ-like lesions. In the present study aPDT mediated by BuTB proved to be a safe and effective therapeutic strategy to prevent the onset of MRONJ-like lesions after extraction. Furthermore, the use of the photodynamic approach in place of antibiotics would conserve this valuable resource without contributing to further antibiotic resistance (WAINWRIGHT et al., 2017).

Experimental animal models of MRONJ have contributed significantly to assist in understanding their etiopathogenesis and in proposing and evaluating preventive and/or curative treatment strategies. Human studies concerning the etiopathogenesis of MRONJ have limitations, which makes appropriate animal experimental models of great importance. In such models, it is possible to propose and evaluate the effectiveness and safety of treatment strategies, which is fundamental for the direction of clinical studies. In this study, an experimental animal model of MRONJ-like lesions was established and thoroughly characterized by our research group (ERVOLINO *et al.*, 2019; TORO *et al.*, 2019; STATIKIEVICZ *et al.*, 2018). The main differential of this experimental model is that it was designed based on epidemiological studies that pointed out the main risk factors for the occurrence of human MRONJ (MCGOWAN *et al.*, 2018; OTTO *et al.*, 2012). The

use of senescent female rats (20 months of age) is based on the high frequency with which MRONJ affects women of advanced age (MCGOWAN *et al.*, 2018; OTTO *et al.*, 2012). The use of a high dose of a potent bisphosphonate, zoledronate, took into account that the majority of MRONJ cases occur when this medication is used to complement the antineoplastic therapy for osteotropic tumors, that is, to prevent, mainly the progression of bone metastasis of breast, lung and kidney cancers (MCGOWAN *et al.*, 2018; OTTO *et al.*, 2012). In addition, the present study mimicked an unfavorable local clinical condition capable of considerably increasing the chances of triggering the MRONJ. Clinical studies point to tooth extraction as one of the main local risk factors, especially when associated with the tooth, there is an infectious/inflammatory condition, such as periodontal and/or periapical involvement (MCGOWAN *et al.*, 2018; OTTO *et al.*, 2012). Therefore, initially a ligature was installed around the lower first molar, to induce experimental periodontitis, then extraction of this tooth was performed. In the present study in specimens of ZOL group, there was severe impairment of tissue repair, intense tissue inflammation, presence of biofilm associated with tissues, reduced PNFBT and elevated PNVBT, corroborating previous studies (ERVOLINO *et al.*, 2019; TORO *et al.*, 2019; STATIKIEVICZ *et al.*, 2018).

ZOL is a potent antiresorptive medication capable of inhibiting the bone resorption process acting on osteoclasts by inhibiting the mevalonate pathway, specifically the enzyme farnesyl diphosphate synthase (ROGERS *et al.*, 2020; ROGERS *et al.*, 2011; EBETINO *et al.*, 2011). In osteoclasts, the inhibition of this pathway compromises their migration through the tissues, prevents their proper coupling with the bone matrix, prevents the correct formation of the clear zone and the ruffled zone on the pole facing the resorption lacunae, and blocks the adequate traffic and exocytosis of osteoclastic vesicles essential for the resorptive process. In addition, zoledronate is able to promote the activation of signaling pathways that result in the premature apoptosis of these cells (ROGERS *et al.*, 2020; ROGERS *et al.*, 2011; EBETINO *et al.*, 2011).

In the present study, the groups ZOL and ZOL-aPDT, showed a significantly lower number of TRAP-positive cells, that is, osteoclasts, which proves the effectiveness of treatment with the anti-resorptive medication. Both histological and immunohistochemical analysis showed that the TRAP-positive cells present in these groups at the dental

extraction site and in its surroundings were presented as large, rounded, hyper-nucleated cells, with no polarization and distant from the bone matrix, or that is, presenting morphological characteristics of osteoclasts that are not active. Such findings are in line with studies that show a significant reduction in osteoclasts at the tooth extraction site (ERVOLINO *et al.*, 2019; TORO *et al.*, 2019; STATIKIEVICZ *et al.*, 2018).

Among the most popular members of the PA class are methylene blue and toluidine blue (KLAUSEN *et al.*, 2020; GHORBANI *et al.*, 2018; WAINWRIGHT *et al.*, 2016). The cationic nature of these PA allows them to be able to interact with both Gram-positive and Gram-negative bacteria, and even with other types of microorganisms, such as fungi and viruses, that is, they have a wide spectrum of action, that allows them to be effective in controlling infections in various locations (KLAUSEN *et al.*, 2020; GHORBANI *et al.*, 2018; WAINWRIGHT *et al.*, 2016). On the other hand, BuTB was developed through physical-chemical modifications in the structure of toluidine blue (WAINWRIGHT, 2016). Such modifications were made in order to further improve its photodynamic effects, among which are, the increase in lipophilic capacity and the reduction of molecular aggregation behavior, which results in greater capacity for cellular interaction and greater efficiency in the production of reactive oxygen species (WAINWRIGHT, 2016). A previous study carried out by our group in animals evaluated the treatment of experimental periodontitis with aPDT, using different concentrations of BuTB, followed by irradiation with low-power laser. The study showed that aPDT mediated by BuTB proved to be an effective adjunctive treatment of experimental periodontitis, especially when it was used at a concentration of 0.5 mg/ml (NUERNBERG *et al.*, 2020). Using this concentration of BuTB followed by irradiation with laser (660 nm), the authors found a reduction in alveolar bone loss, modulation of local inflammation and favoring the tissue repair process (NUERNBERG *et al.,* 2020). Based on this study we used BuTB at a concentration of 0.5 mg/ml in aPDT, and the positive effects of the present study were similar to those obtained by Nuernberg *et al.* (2020), especially with regard to the modulation of local inflammation and favoring of the tissue repair process, as observed in the VEH-aPDT and ZOL-aPDT groups.

In the present study, it was found that the use of aPDT mediated by BuTB proved to be an effective and safe preventive therapy against the occurrence of MRONJ-like lesions

post-extraction, as observed in the ZOL-aPDT group. This preventive treatment was able to promote epithelial regeneration, modulate the local inflammatory response, improve the repair of connective tissue and stimulate alveolar bone neoformation, corroborating previous studies in animals (SILVA *et al.*, 2022; SARKARAT *et al.*, 2019; ERVOLINO *et al.*, 2019). In addition, it caused a significant reduction in PNVBT at the dental extraction site and in its surroundings, a histological characteristic commonly observed in bone tissue when using drugs with a potent anti-resorptive action (ERVOLINO *et al.*, 2019; TORO *et al.*, 2019; STATIKIEVICZ *et al.*, 2018).

These findings corroborate a previous study using the same conditions and experimental model, however, using another phenothiazine photoantimicrobial agent in aPDT. Ervolino *et al.* (2019) evaluated the preventive action of aPDT at the tooth extraction site. The study used aPDT mediated by methylene blue at a concentration of 100 µg/ml and a pre-irradiation time of 60 seconds, followed by irradiation with diode laser (660 nm) in the same parameters of irradiation used in the present study. The study showed that aPDT significantly improved the process of alveolar repair and prevented the occurrence of MRONJ-like lesions. Although aPDT mediated by methylene blue has resulted in positive effects in preventing MRONJ, the use of strategies aimed at improving PA is of paramount importance, given that this can enhance the beneficial effects of aPDT.

The present study is of great importance because it can guide clinical studies and, as mentioned earlier, potentiate the effects of aPDT when used as a preventive therapeutic strategy for MRONJ. Recently, the use of aPDT has started to become one performed in the dental clinic to prevent MRONJ (TARTATORI *et al.*, 2020; POLI *et al.*, 2019; POLI *et al.*, 2018). Human studies have been reported in a series of clinical cases in which aPDT was used preventively after dental extraction, or removal of implants, in patients who were using oral bisphosphonate. In the studies of POLI *et al.* (2019) and POLI *et al.* (2018) used 10 mg/ml methylene thionine chloride (methylene blue) as PA and a pre-irradiation time of 180 seconds, followed by local irradiation with diode laser (660nm, 100mW) using a probe that, with circular movements, remained radiating an area of about 3 cm^2 for 60s with an energy density of 3.6 J/cm^2, and whose total energy density varied according to the size of the surgical wound.

The study of TARTAROTI *et al.* (2020) used 0.01% (= 1 mg/ml)methylene blue as PA and a pre-irradiation time of 5 min, followed by local irradiation with diode laser (660nm, 0.028cm², 100mW, 3.57W/cm², 90s and 9J per point, 321 J/cm^2 , in three points, using a probe placed at central of the socket and two equidistant points). After aPDT in all studies, patients underwent weekly photobiomodulation sessions until total tissue repair. In these studies it was concluded that aPDT mediated by traditional phenothiazine PAs is a promising prevention strategy to prevent the occurrence of MRONJ in patients undergoing treatment with bisphosphonates and undergoing dental extraction or removal of implants (TARTAROTI *et al.*, 2020; POLI *et al.*, 2018; POLI *et al.*, 2019).

In addition to the importance of the PA in aPDT, another component of great importance in this type of treatment is the light used to promote photodynamic effects. In the present study, in VEH-aPDT and ZOL-aPDT, BuTB was activated by a visible diode laser - red with an InGaAlP emitter, with a wavelength in the range of 660nm, a power of 35mW, an energy density per point 74.2J/cm^2, which was applied for 60 seconds to the center of the surgical wound with a 0.0283 cm^2 tip at 0, 2 and 4 days after extraction. Such parameters, in addition to ensuring the activation of the PA, still produce photobiomodulation effects. Among these effects are the ability to stimulate the repair of soft and hard tissues via modulation of the local inflammatory response, increased cell proliferation, stimulation of the synthesis of extracellular matrix elements and increased neovascularization (DOMPE *et al.*, 2020).

In the present study, aPDT was performed exclusively, which consisted of the deposition of the photoantimicrobial agent followed by irradiation with laser, that is, exclusive photobiomodulation was not performed. Therefore, the observed effects were exclusively due to aPDT. In a previous study by our research group using the same conditions and experimental model photobiomodulation therapy was used exclusively, that is, without the use of a photoantimicrobial agent, and with exactly the same parameters used in the present study (STATKIEVICZ et al., 2018). Since we already had results referring to the exclusive use of photobiomodulation in this same experimental model, we considered that it would not be necessary to include groups treated only with laser in this study, given that our objective was to evaluate the exclusive use of aPDT, specifically the one mediated by butyl toluidine blue (BuTB), which is a photosensitizing agent recently modified from

toluidine blue. .That study showed the importance of adequate laser parameters in the prevention of MRONJ. However, it must be taken into account that local microbial control is also of fundamental importance in the prevention of MRONJ. Therefore, aPDT is a more complete preventive therapeutic strategy for MRONJ, considering its antimicrobial and photomodulatory capacity, when the laser is used.

Such findings demonstrate that each of the components of aPDT, photoantimicrobial agent (encompassing several variables, such as photoantimicrobial class, concentration, physicochemical properties, pre-irradiation time) and light (encompassing several variables, such as type and light and all the parameters used), as well as the establishment of the aPDT protocol (including an adequate indication of the treatment, the moment of its execution, the number of sessions, the training of the operator, among others) are of great importance for the success of this technique. Thus, the constant improvement of each of the elements that make up this technique and its protocols, which tend to be increasingly individualized, are able to significantly enhance their beneficial effects.

The occurrence of MRONJ has a considerable negative impact on patients' quality of life, especially when it occurs concurrently with the treatment of serious diseases, such as cancer (CAMINHA *et al.*, 2020; TENORE *et al.*, 2020). The treatment of MRONJ, although necessary, also negatively affects such patients during a certain period (RUGGIERO *et al.*, 2022; MADEIRA *et al.*, 2020; KHAN *et al.*, 2017; KHAN *et al.*, 2015), especially those undergoing cancer therapy, considering that it is invasive, prolonged, with a doubtful prognosis and can result in serious sequelae. Preventive therapeutic strategies are thus ideal in the case of MRONJ, especially treatments such as aPDT, which have no adverse effect. The use of aPDT mediated by BuTB has proved to be a preventive therapy capable of promoting epithelial regeneration, improving connective tissue repair, stimulating alveolar bone neoformation and modulating the response inflammatory process, thus avoiding the occurrence of post-extraction MRONJ.

The findings of the present study, allowing for the limitations that are inherent in an animal study, further support aPDT as an effective strategy for the prevention of MRONJ. In addition, this is another study that aims to guide future clinical research involving the prevention of MRONJ through the use of aPDT. Controlled and randomized clinical

studies aiming at the establishment of preventive protocols to be used in patients who used or use drugs with anti-resorptive action and who require invasive dental interventions have not been carried out and are extremely necessary. The antimicrobial action, the potent photobiomodulation effect, the absence of adverse effects, the non-promotion of bacterial resistance, the low cost and the ease of execution (WAINWRIGHT, 2019; MAHMOUDI *et al.*, 2018; CIEPLIK *et al.*, 2018; HAMBLIN, 2016; WAINWRIGHT, 1998), suggest aPDT as a very promising preventive strategy to avoid MRONJ.

CONCLUSION

Within the limits and conditions employed in the present study, it is concluded that aPDT mediated by BuTB at the dental extraction site of senescent female rats treated with high-dose zoledronate proved to be an effective therapeutic strategy to prevent the occurrence of MRONJ-like lesions after extraction.

DECLARATIONS OF INTEREST

None

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Table 1: Scores and distribution of specimens according to clinical analysis of tooth extraction site and adjacent sites in VEH, VEH-aPDT, ZOL and ZOL-aPDT at 28 postoperative days.

CLINICAL ANALYSIS				
PARAMETER AND RESPECTIVE SCORES	**NUMBER OF SPECIMENS**			
	EXPERIMENTAL GROUPS			
	VEH (n=7)	**VEH-aPDT (n=7)**	**ZOL (n=7)**	**ZOL - aPDT (n=7)**
CLINICAL ASPECT OF TOOTH EXTRACTION SITE AND ADJACENCIES				
(1) absence of exposed bone and totally repaired mucous membrane	7	7	-	5
(2) absence of exposed bone and partially repaired mucous membrane	-	-	-	2

(3) MRONJ – large extraction site with small area of exposed bone (less than half of the alveolar space) and impairment of mucous membrane repair	-	-	3	-
(4) MRONJ – large extraction site with great area of exposed bone (more than half of the alveolar space) and impairment of mucous membrane repair	-	-	4	-
Median	**1**	**1**	**4†‡**	**1§**

Symbols: †, statistically significant difference in relation to VEH group; ‡, statistically significant difference in relation to VEH-aPDT group; §, statistically significant difference in relation to ZOL group.

Table 2: Parameters, scores and specimen distribution according to the histopathological analysis of the level of tissue inflammation during the post-tooth extraction repair process in VEH, VEH-aPDT, ZOL and ZOL-aPDT at 28 postoperative days.

HISTOPATHOLOGICAL ANALYSIS: LEVEL OF TISSUE INFLAMMATION					
PARAMETERS AND RESPECTIVE SCORES	**NUMBER OF SPECIMENS EXPERIMENTAL GROUPS**			
	VEH (n=7)	**VEH-aPDT (n=7)**	**ZOL (n=7)**	**ZOL – aPDT (n=7)**
INTENSITY OF LOCAL INFLAMMATORY RESPONSE				
(1) absence of inflammation	7	7	-	5
(2) mild quantity of inflammatory cells	-	-	-	2
(3) moderate quantity of inflammatory cells	-	-	3	-
(4) severe quantity of inflammatory cells	-	-	4	-
median	**1**	**1**	**4†‡**	**1§**
INFLAMMATION EXTENSION				
(1) absence of inflammation	7	7	-	5
(2) partial extension of connective tissue	-	-	-	2
(3) entire extension of connective tissue, without reaching bone tissue	-	-	2	-
(4) entire extension of connective tissue and bone tissue	-	-	5	-
median	**1**	**1**	**4†‡**	**1§**

Symbols: †, statistically significant difference in relation to VEH group; ‡, statistically significant difference in relation to VEH-aPDT group; §, statistically significant difference in relation to ZOL group.

Table 3: Parameters, scores and specimen distribution according to the histopathological analysis of the level of tissue inflammation during the post-tooth extraction repair process in VEH, VEH-aPDT, ZOL and ZOL-aPDT at 28 postoperative days.

HISTOPATHOLOGICAL ANALYSIS: TISSUE STRUCTURE PATTERN				
PARAMETERS AND RESPECTIVE SCORES	**NUMBER OF SPECIMENS EXPERIMENTAL GROUPS**			
	VEH (n=7)	**VEH-aPDT (n=7)**	**ZOL (n=7)**	**ZOL – aPDT (n=7)**
CELLULAR PATTERN AND EPITHELIAL TISSUE STRUCTURE				
(1) epithelial tissue with moderate thickness completely recovering extraction site	4	6	-	4
(2) epithelial tissue with thin thickness completely recovering extraction site	3	1	-	3
(3) thin layer of epithelial tissue only in edges of open surgical wound	-	-	3	-
(4) absence of epithelial tissue on open surgical wound	-	-	4	-
median	**1**	**1**	**4†‡**	**1§**
CELLULAR PATTERN AND CONNECTIVE TISSUE STRUCTURE				
(1) moderate quantity of fibroblasts and large quantity of collagen fibers	4	6	-	2
(2) moderate quantity of both fibroblasts and collagen fibers	3	1	-	5
(3) small quantity of both fibroblasts and collagen fibers	-	-	2	-
(4) severe tissue disorganization with necrosis areas	-	-	5	-
median	**1**	**1**	**4†‡**	**2§**
CELLULAR PATTERN AND BONE TISSUE STRUCTURE				
(1) absence of non-vital bone in adjacencies of extraction site and trabecular bone filling more than half of dental alveolus	6	7	-	4
(2) absence of non-vital bone in adjacencies of extraction site and trabecular bone filling less than half of dental alveolus	1	-	-	3
(3) presence of few areas with non-vital bone in adjacencies of extraction site and trabecular bone filling less than a third of dental alveolus	-	-	2	-
(4) presence of many areas with non-vital bone in adjacencies of extraction site and trabecular bone filling less than a third of dental alveolus	-	-	5	-
median	**1**	**1**	**4†‡**	**1§**

Symbols: †, statistically significant difference in relation to VEH group; ‡, statistically significant difference in relation to VEH-aPDT group; §, statistically significant difference in relation to ZOL group.