Comparative effects of different phenothiazine photosensitizers on experimental periodontitis treatment

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ABSTRACT

Aim: The aim of the present study was to compare the effects of the phenothiazine photosensitizers methylene blue (MB), toluidine blue O (TBO) and butyl toluidine blue (BuTB) in antimicrobial photodynamic therapy (aPDT), as adjuvant therapy to scaling and root planing (SRP) in the treatment of experimental periodontitis (EP) in rats.

Materials and Methods: 120 Wistar rats underwent ligation around the lower left molar. After seven days, the ligature was removed. The animals were separated into the following groups (n=15): EP, no treatment; SRP, SRP and irrigation with saline solution; MB, SRP and deposition of MB; TBO, SRP and deposition of TBO; BuTB, SRP and deposition of BuTB; MB-aPDT, SRP and aPDT with MB; TBO-aPDT, SRP and aPDT with TBO; BuTB-aPDT, SRP and aPDT with BuTB. The aPDT session was performed after SRP with deposition of the photosensitiser and irradiation with a diode laser (DL; InGaAlP, 660 nm, 40 mW, 60 s, 2.4 J). Histological and histometric analysis was performed.

Results: BuTB-aPDT group had a lesser extent of the inflammatory process compared to the EP, SRP, MB and TBO at all experimental periods ($p < 0.05$). At 15 days, the aPDT treated groups had a greater bone tissue structure than groups EP and SRP ($p < 0.05$) The BuTB showed lower Alveolar Bone Loss (ABL) compared to the TBO-aPDT group at 30 days ($p < 0.05$).

Conclusion: aPDT using the photosensitizer BuTB proved to be the adjuvant therapy that most favored the reduction of inflammatory infiltrate in the furcation area and ABL.

1. Introduction

Periodontitis is a multifactorial oral disease characterized by the destruction of dental insertion tissue, triggered by the host's immune-inflammatory response in the presence of dysbiotic biofilm [1-3]. The pathological process is determined by the action of different microbial species, along with the modulation of local and systemic factors that alter the host's immune response [3,4].

The standard treatment for periodontitis is scaling and root planing (SRP), based on the mechanical removal of dysbiotic biofilm and their toxins from the tooth surface [5,6]. In situations where an improvement in the clinical picture is not achieved with conventional mechanical therapy, adjuvant treatments to SRP and surgical periodontal therapies are necessary [7].

The literature mentions several adjuvant therapies used in periodontal treatment, such as systemic and local antibiotic therapy, the use of antioxidants (such as melatonin supplementation and systemic acetylcysteine therapy), chlorhexidine mouthwashes, photobiomodulation (PBM) and antimicrobial photodynamic therapy (aPDT) [7,8-13]. These therapies have been used in recent years to modulate the host's immune-inflammatory process, improve the tissue repair process, reduce the periodontopathogenic microbiota and, consequently, improve the periodontal clinical parameters [7,8-13].

A recent systematic review has shown that the use of systemic antibiotics, especially amoxicillin associated with metronidazole (AMX + MTZ) as an adjunct therapy to SRP, leads to a decrease in probing depth (PD), a gain in the clinical attachment level (CAL) and a decrease in bleeding on probing (BOP), in addition to a decrease in the frequency of periodontal pockets [14]. However, the excessive and/or indiscriminate use of antibiotics can lead to bacterial resistance and a consequent growth of resistant strains, which suggests the need for a new antimicrobial approach [15]. Because of the increasing impact of drug

resistance on public health, the routine prescription of systemic antibiotic therapy as an adjunct to SRP in the treatment of periodontitis is not recommended [16].

Taking into account the time required for the creation of a new antibiotic until its commercialization, the importance of conscious prescription of these drugs only in cases where they are necessary is emphasized. Therefore, the search for adjuvant therapies to replace antibiotics in the treatment of periodontitis, that do not cause bacterial resistance, is needed. Against this background, aPDT has been studied as a promising adjuvant therapy for controlling bacterial infections [12,17,18].

aPDT is a local treatment that consists of using a photosensitizer (PS) which requires a pre-irradiation time for its biodistribution in the tissue, followed by irradiation with visible or near-infrared light at a wavelength compatible with the absorption spectrum of the PS [19]. In the presence of oxygen, reactive oxygen species such as singlet oxygen, superoxide anions and hydroxyl radicals are produced, which can reach various molecular targets including proteins, lipids and nucleic acids in less complex microorganisms like bacteria, viruses, fungi and protozoa. The large number of molecular targets makes it very unlikely that resistance to photodynamic therapy can develop [20].

Several studies have presented positive reports of aPDT use as adjuvant treatment to SRP in rats [21] and in humans with periodontitis [12, 22-24]. On the other hand, some studies have shown no additional clinical benefits [25-27]. The studies currently available have a number of limitations that make comparison difficult. The type of PS, its concentration, the pre-irradiation time, type of laser used, wavelength, power and irradiation time have varied between studies. This may be an explanation for the discrepancy in results.

In relation to PSs, phenothiazines are among the subgroups most used in aPDT as an adjunct to SRP, with methylene blue (MB) and toluidine blue-O (TBO) belonging to this same family. Due to their cationic (positive) charge, they have the ability to bind to both Gram-positive and Gram-negative bacteria [28]. Widely used in dentistry, TBO reaches and acts against different microbial species. In order to improve the photodynamic effects of this PS, butyl toluidine blue (BuTB) was developed through physical-chemical modifications in the structure of TBO, exhibiting an increased lipophilic capacity and a lower molecular aggregation behavior; characteristics that promote both the production of reactive oxygen species and the efficiency of cell interaction [28]. A recent study by our group has proven the efficacy of BuTB in controlling bone loss in experimental periodontitis [7].

Due to the variety of photosensitizers and different irradiation protocols used in periodontal treatment, comparative studies are needed to clarify and encourage the standardization of these clinical and physical parameters, reducing the discrepancy in the results of aPDT in the treatment of periodontitis. In addition, the use of cationic photosensitizers in adequate concentrations, which are well absorbed by microorganisms efficient in the generation of reactive oxygen species, can also promote a greater efficiency of aPDT. Thus, the present study aimed to compare the effects of a new photosensitizer, BuTB, with MB and TBO, as photosensitizers in aPDT used as an adjuvant in the treatment of EP in rats.

2. Material and Methods

2.1 Animals

In total, 120 male Wistar rats (*Rattus novergicus*) 3 months of age with a body weight between 180 - 250g were used in this study. The animals were kept in plastic cages (4 animals per cage) with controlled light/dark cycle (12-h light/12-h dark) and access to feed and water ad libitum and maintained in an environment with stable temperature ($22°C \pm 2°C$). The experimental procedures were carried out in accordance with the rules established by the "Guide for the Care and Use of Laboratory Animals" [29], and the experimental protocol was approved by the FOA Animal Use Ethics Committee - UNESP (CEUA Process No. 00729-2019).

2.1.2 Anesthesia

For all procedures that required animal handling, the animals were anesthetized intramuscularly with ketamine hydrochloride (80 mg/kg, Francotar®, Virbac, SP, Brazil) and xylazine hydrochloride (10 mg/kg, Rompum®, Bayer, RS, Brazil).

2.1.3 Induction of experimental periodontitis

On the first day of the experimental protocol, the rats were placed on an adapted surgical table, where a cotton ligature (cotton thread # 24, Coats Corrente®, SP, Brazil) was installed around the first left lower molar of each animal the in order to induce EP [30]. The ligature was stabilized in a sub-gingival position with a surgical knot [30].

2.1.4 Experimental groups

Seven days after EP induction, the ligature was removed and the animals were randomly assigned to eight experimental groups: EP group (n = 15), no treatment; SRP group (n = 15), SRP and irrigation with 0.3 mL of saline solution; MB group (n = 15), SRP and deposition of 0.3 mL MB photosensitizer 100 µg/mL in the periodontal pocket; TBO group (n = 15) SRP and deposition of 0.3 mL TBO photosensitizer at 100 µg/mL in the periodontal pocket; BuTB group (n = 15), SRP and deposition of 0.3 mL BuTB photosensitizer at 100 µg/mLin the periodontal pocket; MB-aPDT group (n = 15), SRP and deposition of 0.3 ml of MB at 100 µg/mLin the periodontal pocket for 60 seconds and diode laser (DL) irradiation (InGaAlP, 660 nm, 40 mW, 60 s , 2.4 J); TBO-aPDT group (n = 15), SRP and deposition of 0.3ml TBO at 100µg/mLin the periodontal pocket for 60 seconds and DL irradiation (InGaAlP, 660 nm, 40 mW, 60 s, 2.4 J); BuTB-aPDT group (n = 15), SRP and deposition of 0.3 ml BuTB at 100 µg/mLin the periodontal pocket for 60 seconds and DL irradiation (InGaAlP, 660 nm, 40 mW, 60 s, 2.4 J; Figure 1).

2.1.5 Scaling and root planing

For scaling and root planing the cotton ligatures were removed and antisepsis of the oral cavity was performed on the buccal and lingual surfaces, consisting of 10 tensio-mesial traction movements and, in the interproximal and furcation areas, 10 cervical-occlusal traction movements, using mini-five 1-2 hand curettes (Hu-Friedy Co. Inc., Chicago, IL, USA) [7,30].

2.1.6 Antimicrobial photodynamic therapy (aPDT)

A single session of supporting treatments was performed right after SRP. For this, 0.3 ml of MB, TBO or BuTB photosensitizer (PS) in the same concentration of 100 µg/ml was deposited inside the periodontal pocket of the lower first left molars. In the MB-aPDT, TBO-aPDT and BuTB-aPDT groups, after the PS had been in contact with the periodontal tissues for sixty seconds (pre-irradiation time), irradiation with a low-powered diode laser (DL) was performed. The DL used in the present study was an InGaAlP with a wavelength of 660 nm (PHOTON LASE III® D.M.C. Equipamentos Ltda, São Carlos, SP, Brazil) with the following parameters: power: 40 mW; operation mode: continuous laser; spot diameter: 0.0283 cm^2; method of application: punctual contact; energy: 2.4 J; exposure time: 60 seconds; energy density: 84.8 J/cm²; irradiance: 1.41 W/cm². Laser irradiation was performed

on the buccal bone plate of the lower first molar, with the tip positioned perpendicular to the root [7]. In the MB, TBO and BuTB groups, only the photosensitizers corresponding to each group were deposited, and DL irradiation was not performed.

2.2 Laboratory processing for histological and histometric analysis

After the experimental protocol was concluded, the animals were euthanized with an anesthetic overdose of Tiopental (Cristália, Produtos Químicos Farmacêuticos Ltda., Itapira, SP, Brazil), at a concentration of 150 mg/kg intraperitoneally at 7, 15 and 30 days post-treatment.

The hemi-mandibles were dissected and submitted to demineralization in PBS plus 10% ethylenediamine tetraacetic acid (EDTA) for 2 months. After that period, the samples went through conventional histological processing, included in paraffin and sectioned into a 4 µm thick slides using a microtome. For the histopathological analysis of periodontal tissues and for the histometric analysis of alveolar bone loss (ABL), the histological sections were stained with hematoxylin-eosin (HE). Histopathological and histometric analyses were performed according to the protocol described by Garcia *et al.*, 2014 [31].

2.2.1 Histological analysis

Histological analysis was performed under light microscopy by a certified histologist (EE), calibrated and blinded to the treatments. The images were captured by a digital camera (AxioCam®, Carl Zeiss) attached to an optical microscope (AxioLab®) and connected to a microcomputer. In an area of 1mm² (1mm x 1mm) located in the center of the furcation region, the quantification of the inflammatory infiltrate present in the connective tissue and bone tissue margins was performed. The predominant inflammatory cells were mononucleated cells (lymphocytes and macrophages) and some polymorphonuclear cells (especially neutrophils) were also present. The following parameters were evaluated using the score system: *intensity of the local inflammatory response* (score 1, absence of inflammation, that is, in the evaluated area, cells responsible for structuring tissues, fibroblasts, in the case of connective tissue, and osteoblasts and osteocytes, in the case of bone tissue, and rare cells were found. inflammatory; score 2, mild inflammation up to 1/3 of the cells are inflammatory of the total cells observed in the visual field; score 3, moderate inflammation from 1/3 to 2/3 of the cells are inflammatory of the total cells observed in the visual field; score 4, intense

inflammation with more than 2/3 of the cells are inflammatory of the total cells observed in the visual field), *extent of the inflammatory process* (score 1 - absence of inflammation, score 2 - extended to half a part of the connective tissue in the furcation region, score 3 - extended throughout the connective tissue in the furcation region, and score 4 - extended throughout the connective tissue and bone in the furcation region), *structural pattern of the connective tissue in the furcation region* (score 1, moderate amount of fibroblasts and a large amount of collagen fibers, dense connective tissue; score 2, moderate amount of both fibroblasts and collagen fibers; score 3, small amount of fibroblasts and collagen fibers; and score 4, severe tissue disorganization with necrotic areas), *structural pattern of alveolar bone tissue of the furcation region* (score 1, bone trabeculae with a regular contour and filled with active osteoblasts, including bone neoformation areas; score 2, bone trabeculae with an irregular contour coated with many osteoblasts and active osteoclasts; score 3, bone trabeculae with an irregular contour coated with active osteoclasts, and score 4, areas with necrotic bone and bone trabeculae with an irregular contour filled with active or inactive osteoclasts) [32].

2.2.2 Histometric analysis

The measurement of ABL in the furcation region of the lower left first molar was determined histometrically in mm² with the aid of an image analysis system (Axiovision 4.8.2, Carl Zeiss MicroImaging GmbH, 07740 Jena, Germany). After excluding the first and last slide, where the furcation region was evident, three equidistant slides of the same specimen were selected for histometric analysis. This selection was made by a calibrated and blinded examiner (EE). Another calibrated and blinded examiner performed the histometric analysis of the furcation region (TER). ABL measurement was performed by the same examiner three times in the same specimen on different days to reduce the variation in results [31].

2.3 Examiner calibration

Prior to histometric analysis, the examiners performed the ABL analysis on thirty samples, with an interval of one week between them. Measurements were analyzed statistically using Pearson's correlation coefficient ($\alpha = 5\%$), where a high correlation level was observed (0.97).

2.4 Statistical analysis

The sample was calculated considering ABL in the furcation region as the primary outcome variable. The secondary outcome was described by the histological characteristics in the furcation area. Considering a minimum difference of 0.1 between the treatment means and a standard deviation of 0.01, the results showed that a sample size of 4 animals ($\alpha = 0.05$) would present a study power of 95% [7]. Taking into consideration a loss of 20%, a sample size of 5 animals per group was chosen [7]. The normality of all quantitative data was previously analyzed using the Shapiro Wilk test. The intra- and inter-group ABL analyses were performed by analysis of variance (ANOVA), followed by the Tukey post-test. The semi-quantitative data from the histological analysis were submitted to the Shapiro-Wilk variance analysis, Kruskal-Wallis test followed by the Student-Newman-Keuls post-test at a significance level of 5%.

3. Results

3.1 Histometric analysis

The results of the histometric analysis are presented in figure 2. There was a greater alveolar bone loss (ABL) in the furcation region in the animals of the EP group compared to groups SRP, TBO, BuTB, MB-aPDT and BuTB-aPDT at 7, 15 and 30 days (p <0.05), at 7 and 15 days compared to the MB group (p <0.01) and at 15 days compared to group TBO-aPDT (p <0.01). The SRP group showed a greater ABL than the groups TBO after 30 days (p <0.05), BuTB and MB-aPDT at 15 days (p <0.05), and BuTB-aPDT at 7, 15 and 30 days (p <0.01). The MB group had more ABL than group TBO after 30 days (p <0.05). The TBO group showed more ABL than the BuTB-aPDT group at 7 days p <0.05), and less than group TBO-aPDT at 30 days (p <0.01). Group BuTB had less ABL than group TBO-aPDT at 30 days (p <0.05), and the BuTB-aPDT group showed less ABL than the TBO-aPDT group after 7 (p <0.05) and 30 days (p <0.01). In the intragroup analysis, the TBO group showed a lower rate of ABL after 30 days than on day 7 (p <0.01).

3.2 Histological analysis

The results of the histological analysis are presented in Figure 3. The intensity of the inflammatory response was greater in the EP group on days 7 and 30 than in all other experimental groups, and on day 15 the intensity was higher in the EP group than in the SRP,

MB, TBO, BuTB, MB-aPDT and BuTB-aPDT groups (p <0.05; Figure 4, Figure 5, Figure 6). The extent of the inflammatory process was greater in the EP group compared to groups BuTB, MB-aPDT, TBO-aPDT and BuTB-aPDT after 7 and 30 days (p <0.05); and higher than groups MB-aPDT and BuTB-aPDT on day 15 (p <0.05). At 7 and 15 days, the BuTB-aPDT group had a lesser extent of inflammatory processes than groups SRP, MB and TBO (p<0.05). The same could be observed after 30 days, when the extent continued to be smaller in the BuTB-aPDT group than in groups SRP, MB and TBO (p<0.05).

The connective tissue structure pattern was better in the BuTB-aPDT group compared to EP in all experimental periods (p<0.05) and SRP, MB,TBO and BuTB groups at 7 and 15 days(p<0.05). At 7 days, the bone tissue structure pattern was worse in the EP group than groups MB-aPDT, TBO-aPDT and BuTB-aPDT (p<0.05). At 15 days, the EP and SRP group had a bone tissue structure worse than groups MB-aPDT, TBO-aPDT and BuTB-aPDT (p <0.05). After 30 days, the bone tissue structure was also worse in the EP group than in groups MB, TBO, BuTB, MB-aPDT, TBO-aPDT and BuTB-aPDT (p <0.05).

4. Discussion

The immunoinflammatory response characteristic of periodontitis is directly associated with the presence of dysbiotic biofilm [2]. Therefore, there is a consensus in the literature that periodontal treatment should include the mechanical removal of microbial deposits from the tooth and root surfaces [33]. Knowing the limitations of SRP in the failure to induce the ecological changes necessary to maintain the desired clinical improvements in all cases, adjuvants therapies have been tested to supply cases where no clinical improvement is observed with SRP alone [14]. Systemic antibiotic therapy has been used as adjunctive therapy to SRP in the treatment of periodontitis, and has been recommended in the intervention of Stage III periodontitis in adults [16]. However, due to concerns over public health, especially in relation to microbial resistance, this adjuvant therapy is not recommended for routine use in periodontal treatment [16].

The results of the present study have shown that in animals treated with SRP and with TBO photosensitizer alone (without the presence of light) there was a greater ABL reduction after 30 days, and when using BuTB isolatedly there was a greater reduction on days 15 and 30. Animals that were treated with aPDT using MB as photosensitizer showed less ABL after 15 days (MB-aPDT group), as was the case in all experimental periods of the

BuTB-aPDT group, compared to the groups treated with SRP only. Previous study showed a decreased inflammation and significantly reduced bone loss in the furcation regions of animals treated with SRP, followed by irrigation with MB and TBO without presence of light, compared to findings in animals treated with SRP alone [31].

In vitro studies also showed that MB and TBO, even without the presence of light, had an antimicrobial effect [32,33]. Wilson et al., 1993 [34] observed that MB was more effective than TBO against Fusobacterium nucleatum [34]. Usacheva et al., 2001 [35] observed that MB had a higher photobactericidal activity than TBO against *S. aureus* 6538 [35].

Some recent systematic reviews do not demonstrate additional clinical benefits of aPDT as an adjuvant therapy, compared to SRP monotherapy [36-38], and its clinical use is not suggested following the results analysis [16]. However, several randomized controlled clinical studies have shown clinical advantages when used as an adjunct to initial or maintenance periodontal therapy at the beginning or after 6 months of treatment [12, 39-42].

Positive results in the inflammatory response have been observed with the use of aPDT as an adjuvant treatment of experimental periodontal disease in studies with systemically healthy animals [7,31] and, especially when changed systemically [43-47]. Regarding its antimicrobial effectiveness, *in vitro* and *in vivo* studies have shown that aPDT has a bactericidal effect on some bacteria associated with the etiology of periodontitis, mainly *Porphyromonas gingivalis* [48,49].

In addition to the antimicrobial action, studies have suggested that aPDT, when using a low-powered laser as light source, also has photobiomodulatory effects, such as modulation of the inflammatory response, angiogenesis, and the proliferation, migration, differentiation and activity of cells; all of which are considered essential events in promoting the tissue repair process [50].

Based on clinical data, there is evidence that the adjuvant use of aPDT may provide similar clinical improvements in PD and CAL when compared with conventional periodontal therapy [37]. Other studies have demonstrated significant benefits of aPDT adjuvant to SRP with respect to PD reduction and CAL gain after 3 and 6 months [37,51,52], stimulating new research to improve the parameters and elements involved in aPDT. In this experimental study, aPDT acted as a significant supporting therapy compared to SRP monotherapy in the period of 15 days, when using MB and BuTB in all evaluated periods.

Such findings confirm the effect of aPDT mainly in the initial periods after treatment, since the photodynamic therapy in these cases was not repeated and acts only during irradiation, with PS light being absorbed in the tissue. Furthermore, the photodynamic action occurs in a single moment with the release of reactive oxygen species.

aPDT has a set of differentials and peculiarities, with an emphasis on the discovery of improved molecular compositions with selectivity characteristics [19]. The search for a PS that is effective in the photodynamic inactivation of bacteria and fungi began at the outset of the 20th Century, and the selection of an ideal drug remains a challenge [18].

The present study aimed to compare three different phenothiazine photosensitizers (MB, ATO and BuTB) at the same concentration (100 µg/ml) in the adjuvant treatment to SRP in rats with EP. The photosensitizers MB and TBO at a concentration of 100 μg/ml, used isolatedly or in aPDT showed the best the best results in reducing ABL [31].

In order for the PS to be effective it must be cationic (positively charged), which allows for the connection and penetration in different classes of microbial cells [51]. Due to the fact that different PS types are found in the literature, studies that clarify their dosage as well as the time of administration are necessary [53]. The most studied PS are: hematoporphyrin derivatives (action spectrum between 620-650 nm), phenothiazines, (such as toluidine blue O and methylene blue - action spectrum between 620-700 nm) and cyanine (action spectrum between 600 -805 nm) [54-56]. Methylene blue and toluidine-blue O have been used successfully in aPDT against various types of bacteria [35,57]. Due to the cationic charge, these photosensitizers are able to bind to both Gram-positive and Gram-negative bacteria. They attach to negatively charged polysaccharides in these microorganisms, causing molecular damage in the presence of light [57]. To improve the photodynamic effects of TBO, BuTB was developed through physical-chemical modifications in the structure of TBO, so as to improve its lipophilicity characteristics and production of reactive oxygen species [28].

Studies that have evaluated inflammatory molecular interactions and bone tissue repair through immunostaining with TRAP, OCN and TGF-β [7], RANKL and OPG [31] in rats with EP treated with aPDT as adjuvant to SRP, demonstrated less TRAP immunostaining than the SRP monotherapy group [7,31]. Animals treated with aPDT using the photosensitizers MB and TBO showed greater immunostaining for RANKL and OPG [31].

This demonstrates less bone resorption activity and greater osteoblastic activity in the groups treated with aPDT.

On the other hand, animals submitted to aPDT using BuTB showed higher TGF-ß1 immunostaining than animals treated with SRP only [7]. Such findings confirm the results of the present study, where there was less ABL in the animals treated with aPDT using different phenothiazine photosensitizers in the same concentration. However, in the comparative analysis between the different PS, BuTB has proven to be more effective in controlling ABL.

The results of the histopathological analysis of periodontal tissues in the furcation region showed a lesser extent of inflammatory infiltrate in the tissues in all treated groups compared to the EP group, however, animals treated with BuTB alone or with the three PSs in aPDT showed a lesser extent of this infiltrate in periodontal tissues compared to animals treated with SRP only.This demonstrates the effectiveness of PS BuTB in the immunoinflammatory response, even without the presence of light, corroborating with previous study [7]. The data suggest that this phenothiazine PS can exert antibacterial effects even without being exposed to light, because they are cationic and act on both Gram-positive and Gram-negative bacteria. Previous results have demonstrated that MB, TBO and BuTB at a concentration of 0.1 mg/ml and BuTB at 0.5 mg/ml were more effective at reducing the inflammatory infiltrate in experimental periodontitis in rats [7,31].

In the present study, BuTB demonstrated a more structured connective tissue, with more fibroblasts and a greater amount of collagen fibers compared to animals treated with SRP alone in all experimental periods. These data underline *in vivo* the results presented in an *in vitro* study, where the photosensitizer BuTB demonstrates an increase in lipophilic capacity and a lower molecular aggregation behavior compared to TBO, characteristics that promote both the production of reactive oxygen species, and the efficiency of cell interaction, light absorption and lipophilicity [28] which led, in this study, decreases in the inflammatory process and improved repair of the connective tissue

The limitations of the present study are related to the absence of a microbiological analysis of the treated areas in the animals, due to the great difficulty in collecting sufficient bacterial samples from the periodontal pocket of rats. However, the ligature-induced periodontal disease model is already well established in the literature for assessing local or

systemic treatments [7], and the greatly improved photoantimicrobial effects of BuTB relative to those of TBO and MB are well established in vitro [28].

Therefore, within the limits of this study, it can be concluded that the use of BuTB isolatedly as an adjuvant therapy to SRP was effective in reducing ABL. Additionally, aPDT using MB, TBO and BuTB in the same concentration was effective in reducing ABL, but BuTB-aPDT demonstrated greater benefits in reducing ABL and favoring the reduction of inflammatory infiltrate in the furcation area, thus promoting the acceleration of tissue and bone repair.

Declaration of Competing Interest

The authors have declared no conflict of interest.

Acknowledgments

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Figures Legends

Figure 1. Study design

Figure 2. Graphic with mean and standard deviation of the area of alveolar bone loss (mm²) in the furcation region of the first left lower molar, in the different experimental groups and evaluation periods. Abbreviations and symbols: ABL, alveolar bone loss; *, Statistically significant difference in relation to the EP group at same period; †, Statistically significant difference in relation to the SRP group at same period; ‡, Statistically significant difference in relation to the MB group at same period; ¶, Statistically significant difference in relation to the TBO group at same period; α, Statistically significant difference in relation to the BuTB group at same period; β, Statistically significant difference in relation to the TBO-aPDT group at same period; μ, Statistically significant difference in relation to the TBO-aPDT group at 7 days.

Figure 3. Graphics indicate the median (horizontal line or yellow horizontal line), first and third quartiles of the Intensity of local inflammatory response (a), Extension of inflammatory process (b), Structural pattern of connective tissue (c) and Structural pattern of alveolar bone tissue scores (d) in the furcation region of the mandibular first molars according to groups and time points. Symbols: *, Statistically significant difference in relation to the EP group at same period; †, Statistically significant difference in relation to the SRP group at same period; ‡, Statistically significant difference in relation to the MB group at same period; ¶, Statistically significant difference in relation to the TBO group at same period; α, Statistically significant difference in relation to the BuTB group at same period.

Figure 4. Photomicrographs of the left mandibular first molar with experimental periodontitis showing the course of the inflammatory response and tissue repair process in EP (a), SRP (b), BuTB (c), MB-aPDT (d), TBO-aPDT (e), BuTB-aPDT (f) at 7 days. Abbreviations and

symbols: ab, alveolar bone; ct, connective tissue. Original magnification: a-f, 250x. Scale bars: a-f, 250 μm; Staining: hematoxylin and eosin (H & E).

Figure 5. Photomicrographs of the left mandibular first molar with experimental periodontitis showing the course of the inflammatory response and tissue repair process in EP (a), SRP (b), BuTB (c), MB-aPDT (d), TBO-aPDT (e), BuTB-aPDT (f) at 15 days. Abbreviations and symbols: ab, alveolar bone; ct, connective tissue. Original magnification: a-f, 250x. Scale bars: a-f, 250 μm; Staining: hematoxylin and eosin (H & E).

Figure 6. Photomicrographs of the left mandibular first molar with experimental periodontitis showing the course of the inflammatory response and tissue repair process in EP (a), SRP (b), BuTB (c), MB-aPDT (d), TBO-aPDT (e), BuTB-aPDT (f) at 30 days. Abbreviations and symbols: ab, alveolar bone; ct, connective tissue. Original magnification: a-f, 250x. Scale bars: a-f, 250 μm; Staining: hematoxylin and eosin (H & E).