Effects of butyl toluidine blue photosensitizer on antimicrobial photodynamic therapy for experimental periodontitis treatment in rats

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ABSTRACT

Aim: This study evaluated three concentrations of butyl toluidine blue (BuTB) for antimicrobial photodynamic therapy (aPDT) in experimental periodontitis (EP) in rats.

Material and Methods: EP was ligature-induced at the first mandibular molar in 105 rats. Ligature was removed after 7 days and animals were distributed into the following treatments: SRP, scaling and root planing (SRP) plus saline solution; BuTB-0.1, SRP plus BuTB at 0.1 mg/mL; aPDT-0.1, SRP plus BuTB at 0.1 mg/mL and InGaAlP diode laser (DL) irradiation; BuTB-0.5, SRP plus BuTB at 0.5 mg/mL; aPDT-0.5, SRP plus BuTB at 0.5 mg/mL and DL irradiation; BuTB-2.0, SRP plus BuTB at 2 mg/mL; aPDT-2.0, SRP plus BuTB at 2 mg/mL and DL irradiation. Five animals from each group were submitted to euthanasia at 7, 15 and 30 days post-treatment. The furcation area was submitted to histological, histometric and immunohistochemical (TGF-ß1, OCN and TRAP) analyses.

Results: aPDT-0.5 group presented a better tissue remodeling in all periods, resolution of the inflammatory response and bone neoformation areas at 30 days. aPDT-0.5 also resulted in higher immunolabelling patterns of TGFβ1 at all periods ($p<0.05$) and of OCN at 30 days ($p<0.05$).

Conclusion: aPDT-0.5 showed the best benefits for inflammatory response and periodontal repair process.

Keywords

Periodontitis; Photochemotherapy; Photosensitizers; Dental Scaling; Animal disease model.

1. Introduction

Periodontitis is a chronic multifactorial inflammatory disease. It is a microbially-associated and host-mediated process. The disease is associated with dysbiotic plaque biofilms and is characterized by progressive destruction of the tooth-supporting apparatus (1). Non-surgical treatment of scaling and root planing (SRP) is the initial recommended therapy (2, 3). Despite satisfactory results, some limitations of this mechanical therapy and the better understanding of periodontal disease pathogenesis have led to the development of adjunctive methods for SRP in order to obtain clinical benefits with a low risk of side effects (4).

Antimicrobial photodynamic therapy (aPDT) has been studied as a promising adjuvant therapy (5, 6). aPDT involves the combination of a photoactive agent, called a photosensitizer (PS), associated with light at a wavelength compatible with the PS absorption spectrum, and the presence of oxygen (7). The mechanisms of photochemical action on biomolecules, as a result of excitation of the PS by light, can occur by electron transfer (type I reaction) or by energy transfer (type II reaction), resulting in multiple oxidation-reduction processes. The therapy is based on the generation of free radicals and singlet oxygen ($^1O_2$), which are cytotoxic to cells (7). The development of microbial resistance to this cytotoxic action is unlikely as $^1O_2$ is a primitive molecule and it acts in different molecular sites of the pathogen (8-11).

Based on clinical data, there is evidence that the adjuvant use of aPDT, when compared with conventional SRP treatment, promotes an increase in clinical attachment gain and a reduction in probing depth, especially in the short term (12-14). However, the extent of this statistical clinical attachment gain obtained with the combination of aPDT and SRP does not represent significant clinical relevance (14). Furthermore, the high heterogeneity in light dosimetry parameters adopted among studies represents a challenge in measuring the real efficacy of this therapy (12-14). This scenario highlights the importance of further research to improve the parameters and elements involved in aPDT.

As noted above, the criteria for successful antimicrobial photodynamic therapy require consideration of light delivery, oxygen availability and photosensitizer administration. Since

the established photoantimicrobials methylene blue and toluidine blue are non-optimal, improving the success rate for photodynamic inactivation of pathogens requires optimization of both the molecular structure and dosage of the photosensitizer for increased uptake, penetration and efficacy (15). The present study demonstrates for the first time the *in vivo* effects of three concentrations of a new PS. Butyl toluidine blue (BuTB) was developed by physicochemical modifications of the molecular structure of the established phenothiazine dye toluidine blue O (TBO) (16). Previously evaluated for photoantimicrobial activity (16), BuTB was evaluated here as a photosensitizing agent for *in vivo* aPDT, as an adjuvant to SRP, in the treatment of experimental periodontitis (EP) in rats. The effectiveness of BuTB concentration was evaluated on alveolar bone loss by histometric analysis, local regulation of osteoclastogenesis and osteoclastic activity by RANKL and OPG immunolabelling and local recruitment of osteoclasts using TRAP immunolabeling. The local inflammatory response and periodontal repair process were evaluated by histological analyisis and by TGF-β1 and osteoblastic activity using OCN immunolabeling.

2. Material and Methods

2.1 Animals

This study was conducted on 105 healthy three-month-old male rats (*Rattus novergicus albinus*, Wistar) weighing 180 to 250 g. They were kept in plastic cages with wood shavings, under 12 hours/12 hours light/dark cycles, 22 ± 2 ˚C ambient temperature, 20 air changes per hour, 55 ± 5% humidity, receiving feed and water *ad libitum.* For all experimental procedures, the animals received general anesthesia with the combination of ketamine hydrochloride (70 mg/kg of body weight) and xylazine hydrochloride (6 mg/kg of body weight) applied intramuscularly in the biceps femoris of the right leg. Procedures for experimental manipulation were carried out according to the guidelines established by the "Guide for the Care and Use of Laboratory Animals" (ARRIVE) and the experimental protocol was approved by the Ethics

Committee on Animal Use (2015-00586, São Paulo State University, UNESP, School of Dentistry, Araçatuba, Brazil).

2.1.2 Induction of experimental periodontitis and experimental groups

EP was induced by placing a number 24 cotton thread (Corrente algodão No. 24, Coats Corrente, São Paulo, SP, Brazil) around the mandibular left first molar for a seven-day period (17). After 7 days, the ligature was removed, and the animals were numbered sequentially from 1 to 105. Simple randomization of the animals (1:1 allocation ratio) was performed using a computer-generated table to 7 different groups: SRP (n = 15), animals treated with SRP followed by irrigation of physiological saline solution; BuTB-0.1 (n = 15), animals treated with SRP followed by irrigation of BuTB at 0.1 mg/mL; aPDT-0.1 (n = 15), animals treated with SRP followed by irrigation of BuTB at 0.1 mg/mL and irradiation with InGaAlP diode laser (DL) (660 nm, 40 mW , 60 s, 2.4 J); BuTB-0.5 (n = 15), animals treated with SRP followed by irrigation of BuTB at 0.5 mg/mL; aPDT-0.5 (n = 15), animals treated with SRP followed by irrigation of BuTB at 0.5 mg/mL and DL irradiation; BuTB-2.0 (n = 15), animals treated with SRP followed by irrigation of BuTB at 2 mg/mL; aPDT-2.0 (n = 15), animals treated with SRP followed by irrigation of BuTB at 2 mg/ mL and DL irradiation.

2.1.3 Scaling and root planing treatment

All animals received SRP treatment with mini-five 1-2-hand manual curettes (Hu-Friedy Co. Inc., Chicago, IL, USA) performing 10 disto-mesial traction movements on the buccal and lingual surfaces of the mandibular left first molars with EP. The interproximal and furcation areas were scaled with the same curettes by cervical-occlusal traction movements (17). The SRP procedures were performed by the same experienced operator, who was trained and blinded to the experimental groups (MAAN).

2.1.4 BuBT and antimicrobial photodynamic therapy (aPDT)

For the aPDT treatment and PS in the absence of light, irrigation with 0.3 mL BuTB was performed at three concentrations: 0.1 mg/mL, 0.5 mg/mL and 2 mg/mL. The photosensitizer BuTB was synthesized as previously reported (16). Irrigation was carried out with the aid of an insulin syringe, carefully directing the tip of the needle into the tooth / gingival tissue following homeostasis of the area. In the SRP group, irrigation was performed with 0.3 mL of physiological saline solution.

The laser used was the Indium-Gallium-Aluminum-Phosphorus (InGaAlP) with a wavelength of 660 nm (Photon Lase III, DMC Equipamentos Ltda, São Carlos, São Paulo, Brazil). The laser light was directed to the gingival tissue at the center of the buccal surface and perpendicular to the long axis of the tooth, according to the following treatment protocol: power: 40 mW; application mode: continuous; energy: 2.4 J; spot area: 0.0283 cm^2; energy density: 84.8 J/ cm^2; exposure time: 60 seconds and power density of 1.41 W/ cm^2. DL irradiation was performed one minute after addition of BuTB.

2.2 Laboratory processing for histological, histometric and immunohistochemical analysis

After 7, 15 and 30 days post-treatment, five animals from each group were submitted to euthanasia by lethal dose of thiopental (150 mg/ kg) Cristália, Produtos Químicos Farmacêuticos Ltda., Itapira, SP, Brazil) associated with lidocaine hydrochloride (10mg/kg) (Novafarma Indústria Farmacêutica Ltda, Anápolis, GO, Brazil). The left hemimandibles were dissected and fixed with 4 % formaldehyde in 0.1 M buffered solution for 48 hours. After decalcification, they were processed and embedded in paraffin. Semi-serial histologic sections (4μm thick) were obtained of the central furcation region in a mesial-distal direction. Five equidistant sections were stained with hematoxylin and eosin (H&E) for histological and histometric analysis. For the indirect immunoperoxidase method, three sections were subjected following primary antibodies: goat anti OCN (Osteocalcin, Santa Cruz Biotechnology, Santa

Cruz, CA), goat anti TRAP (Tartrate-resistant acid phosphatase, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti TGF-β1 (Transforming growth factor beta 1, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Histological, histometric and immunohistochemical processing followed the protocol reported by Garcia et al (17).

2.2.1 Histological analysis

A single blinded certified histologist (EE) performed the histological analysis. The following parameters were evaluated: nature and level of inflammation; extent of the inflammatory process; presence and extent of tissue necrosis; structural pattern of extracellular matrix of periodontal tissues and cellularity pattern of periodontal tissues (18).

2.2.2 Histometric analysis

The area of alveolar bone loss in the furcation region, i.e., the area between the bone crest and cementum surface, of the mandibular left first molar was histometrically determined in mm^2 using an image analysis system (Axiovision 4.8.2, Carl Zeiss MicroImaging GmbH, 07740 Jena, Germany) (17) . After excluding the first and last sections in which the furcation region was evident, three equidistant sections from each specimen block were selected and imaged using a digital camera coupled to a light microscope (AxioStar Plus; Carl Zeiss MicroImaging GmbH, 37030 Gottingen, Germany), according to the method of Garcia et al (17). One trained examiner, who was blinded to the treatments, selected the sections for histometric and histological analyses (EE). Another calibrated examiner, who was blinded to the treatments, conducted the histometric analysis (MAAN). The area of alveolar bone loss in the furcation region of each section was measured two times by the same examiner on different days to reduce variations in the data (17). The mean values were averaged and compared statistically.

2.2.3 Immunohistochemical analysis

A treatment-blinded, trained examiner selected the sections (MAAN) and certified and blinded histologist (EE) performed the immunohistochemical analyzes. TRAP-immunolabeled cells located at the center of the interradicular septum of the mandibular left first molar of an area of 1600 µm x 1200 µm, with an increase of 400 x were quantified (17). The coronal limit of this area was the alveolar ridge crest, from which it extends apically by a distance of 1200 µm (17). For OCN and TGF-β1, a semi-quantitative analysis of the immunolabeling was performed throughout the furcation area based on the scores of (17).

2.3 Examiner calibration

Before the histometric and immunohistochemical analysis were performed, an examiner was trained by double measurements of thirty samples of bone loss and TRAP, with one-week interval between them. The measurements were statistically analyzed using the Pearson correlation coefficient (significance level at 5%), which demonstrated a high correlation level (0.95) for both the histometric and immunohistochemical analyses.

2.4 Statistical analysis

The sample calculation was performed considering the bone loss in the furcation region as primary outcome variable. The secondary outcome was to describe the immunolabeling patterns and histological characteristics in the furcation area. Calculation of sample size n=5 showed an 85% study power ($p<0.05$)(19).

Statistical analysis of all data was performed using Bioestat software (version 5.3, Bioestat, Mamirauá Institute, Manaus, AM, Brazil) with a 5 % significance level. The normality of all quantitative data was previously analyzed using the Shapiro Wilk test. Intra and intergroup analyzes of alveolar bone loss and TRAP were performed by one-way analysis of variance, followed by Tukey's test. The evaluation of TGF-ß1 and OCN scores was performed

using the non-parametric Kruskal-Wallis test. This test was followed by the non-parametric Student-Newman-Keuls test when the Kruskal-Wallis test demonstrated significant difference between groups.

3. Results

3.1 Histological analysis

The aPDT-0.5 group showed lower magnitude for local inflammatory response, which reduced throughout the experimental periods, improving periodontal tissue repair. The other experimental groups presented local inflammatory response and similar periodontal tissue repair process. However, they differed from the SRP groups, where an inflammatory response of greater magnitude and compromised periodontal tissue repair capacity were observed (Figure 1 and 2). The scores and distribution of specimens according to histological analysis are presented in table 1.

3.2 Histometric analysis

The results of the histometric analysis are presented in figure 3. There was greater bone loss in the furcation region of the animals of the SRP group when compared to the specimens of the other groups at 7 and 15 days ($p < 0.05$). At 30 days, alveolar bone loss was statistically higher in the SRP group when compared to BuTB-2.0, aPDT-2.0, aPDT-0.5, BuTB-0.5 and BuTB-0.1 ($p < 0.05$) and there was no statistically significant difference in relation to the aPDT-0.1 group ($p > 0.05$).

3.3 Immunohistochemical analysis

In the TGFβ1 analysis, the SRP group presented a low immunolabeling pattern (score 1) in all evaluated periods. At 7 days, the aPDT-0.1 and aPDT-2.0 groups presented statistically significant differences in relation to the SRP group ($p < 0.05$); whereas the aPDT-0.5 group

showed a higher immunolabeling pattern than SRP, BuTB-0.1, BuTB-0.5 and BuTB-2.0 groups ($p< 0.05$). At 15 days, all aPDT treatment groups remained with higher immunolabeling pattern compared to the SRP group ($p< 0.05$) and the aPDT-0.5 group also presented statistical differences in relation to BuTB-0.1 and BuTB-2.0 groups ($p< 0.05$). At 30 days, statistically significant differences were observed in the aPDT-0.5 group compared to SRP, BuTB-0.1, BuTB-0.5 and BuTB-2.0 groups ($p< 0.05$) (Figure 4).

Regarding OCN, the evaluated treatment groups did not show statistically significant differences in the immunolabeling pattern at 7 and 15 days after treatment. At 30 days, a higher immunolabeling pattern was observed in the aPDT-0.5 group compared to SRP, BuTB-0.1, BuTB-0.5 and BuTB-2.0 groups ($p< 0.05$) (Figure 5).

Regarding TRAP, there was a lower number of TRAP-positive cells at 7 and 15 days in BuTB-0.1 and aPDT-0.1, BuTB-0.5, aPDT-0.5 BuTB-2.0 groups compared to the SRP group ($p< 0.05$). The aPDT-2.0 group had a low number of TRAP-positive cells only at 15 days ($p< 0.05$) (Figure 6).

4. Discussion

Results from this study showed that animals treated with aPDT using BuTB at 0.5 mg/mL presented greater control of the inflammatory response and better periodontal tissue repair than animals treated with the other concentrations. Corroborating this data, aPDT-0.5 group presented higher immunolabeling pattern of TGFβ1 at all periods and for OCN at 30 days. One of the main cytokines involved in the periodontal repair (20) and a biomarker of active osteoblast (21), respectively.

Periodontal disease is marked by the action of different microbial species and modulation of local and systemic factors that alter host response (1, 22). In the experimental model used in this study, ligature installation leads to plaque accumulation, which acts as a key factor for the development of a dysbiotic microbiota (23). The dysbiotic microbiota induces

periodontal tissue destruction by means of a dysregulated inflammatory immune response of the host (24). In this experimental model, bone loss occurs predictably over a period of 7 days (23). Ligature-induced periodontitis in rats has been frequently used in periodontal research due to the involvement of live microbes naturally existent in animal species with distinct virulence features, and products of the microbial metabolism (25). Previous histologic results detected after 1 day of the ligature placement show an intense infiltration of inflammatory cells, disrupted epithelial integrity at the dentogingival junction, connective tissue attachment loss, and alveolar bone resorption (26).

Measurement of bone loss as a consequence of the inflammatory response of EP was evaluated by histometric analysis of alveolar bone loss in the furcation region. All groups receiving local irrigation with BuTB, with or without subsequent DL irradiation, demonstrated less significant alveolar bone loss than the group treated with SRP alone. The favorable results of the adjuvant use of aPDT or PS to control alveolar bone loss in EP in rats are in agreement with the literature. According to a meta-analysis of animal studies, aPDT favors the reduction of alveolar bone loss in EP in rats. Most studies used methylene blue (MB) and TBO photosensitizers, at the concentration of 0.1 mg/mL (27).

The bone loss results obtained with the aPDT treatment with BuTB are comparatively better than results obtained in previous studies with similar methodology that used MB and TBO (17, 28). In relation to TBO, BuTB presents an increase in λ_{max} values, an increase in $^1O_2$ quantum yield, a decrease in aggregation behavior and an increase in lipophilicity (16). These characteristics positively influence PS uptake and subcellular distribution (29, 30). Besides the potential for ROS production, the efficacy of a PS agent is determined by the degree of its interaction with the target (31, 32). The decreased molecular aggregation behavior of BuTB results in more single molecules available to interact with the cell and single molecules are more effective in producing ROS due to a simpler interaction with incident light (33). Additionally, the bone tissue response to the BuTB treatment alone, without DL irradiation,

may suggest a cellular interaction of the PS with a cell-critical target or mechanism. Effects against the polysaccharides of the bacterial cell membrane and the biofilm matrix can also be expected, given the cationic nature of BuTB (11, 34). This hypothesis can explain both the increased photodynamic efficacy and increased dark toxicity against microbial cells (16). More studies are needed to understand the cellular interactions of BuTB with prokaryotes and eukaryotes.

Regarding the inflammatory response analysis, the three aPDT experimental groups obtained positive results in relation to the extent and intensity of the inflammatory process and cellularity pattern of the connective and bone tissues. However, the aPDT-0.5 group animals were the only ones that demonstrated total resolution of the local inflammatory response, with presence of dense connective tissue and some bone neoformation areas at 30 days.

The superior results obtained in the treatment of aPDT with BuTB at 0.5 mg/mL in relation to the 2 mg/mL concentration may be related to the aggregation behavior. Although BuTB shows lower aggregation than the parent compound TBO, the increase of PS concentration favors stacking interactions/aggregation (33). Similar results were observed in a previous study on the influence of concentrations of 10 mg/mL and 0.1 mg/mL of photosensitizers MB and TBO in the treatment of EP in rats, in which the smallest concentrations of both PS were the most effective ones (17). In the present study, it can be hypothesized that while the highest concentration of BuTB may have interfered in the phototoxic action of aPDT by aggregation behavior, the antimicrobial effect of the 0.1mg/mL concentration may have been lower than that reached by the 0.5 mg/mL. Further studies with microbiological analysis will provide important elucidations regarding the antimicrobial effect on periodontopathogens.

A previous study analyzed the *in vitro* photoantimicrobial efficiency of BuTB, demonstrating a significantly increased activity against Gram-negative bacteria, such as *Pseudomonas aeruginosa* (16). The best bone loss control observed in the present study, as well

as the modulation of the inflammatory response and tissue repair stimulation achieved in the aPDT-0.5 group, may be associated with high photoantimicrobial activity of this new PS.

Regarding the TGFβ1 immunohistochemical evaluation, it can be observed that, in a general way, the three treatment groups with aPDT obtained higher immunolabeling pattern in relation to SRP, mainly at 7 and 15 days. TGFβ1 is involved in the regulation of inflammation and immune response in wound healing (35-37) and in bone resorption control (38-40). Increased TGFβ1 levels in the crevicular fluid have been pointed out as a marker of prognosis for the progress of tissue repair (41). The highest immunolabeling patterns observed in the aPDT-0.5 group, in relation to the other groups, are associated with better resolution of inflammation and better tissue repair observed in the histological analysis. Better results were also observed in relation to OCN. Treatment with aPDT-0.5 resulted statistically in a higher immunolabeling pattern compared to SRP treatment and treatments with PS alone during the period of 30 days. OCN is one of the most abundant non-collagenous proteins in the bone matrix and a biomarker of active osteoblasts during the late phase of the bone formation process (21).

The increase in OCN and TGFβ1 immunolabeling, as well as the presence of bone neoformation observed in animals treated with aPDT, may also be associated with the photobiomodulation effect by irradiation of tissues with DL(42). An *in vivo* analysis of human osteoblasts cultured in hypoxia demonstrated that photobiomodulation stimulates osteoblast differentiation and proliferation and increases BMP-2, OCN and TGFβ1 expression (43). In the present study, however, we found that bone neoformation and a significant increase in OCN expression were observed only in the aPDT-0.5 group, suggesting the interference of the higher PS concentrations in the results obtained with aPDT.

Regarding the immunohistochemical analysis on the presence of TRAP-positive cells, it was observed that all treatments with BuTB presented smaller amount of TRAP-positive cells in the first post-treatment periods in relation to the SRP treatment. TRAP is a proteolytic enzyme secreted by osteoclasts during bone resorption (44). The TRAP immunolabeling pattern

is related to the data obtained in the bone loss histometric analysis. Based on these data, it can be suggested that the treatments with BuTB presented a lower bone resorption rate in the initial posttreatment periods, resulting in lower bone loss in the furcation region in all evaluated periods compared to SRP. The effect of the adjuvant use of aPDT on the reduction of TRAP expression has also been demonstrated in previous studies (17, 45-49).

The definition of the most effective BuTB concentration (0.5 mg/mL) will serve as a starting point for future investigations in animals and humans. The absence of analysis of the antimicrobial action of BuTB on the main pathogens involved in periodontal disease can be pointed out as a limitation of this study. Additional *in vivo* analysis of the antimicrobial action of BuTB will generate important evidence and will help to explain the benefits in the inflammatory response and tissue repair observed in the present study.

BuTB as a photosensitizing agent in aPDT, as adjunctive to SRP for treatment of EP, showed promising results on alveolar bone loss control at all concentrations employed. BuTB at 0.5 mg/mL associated with DL showed better control of the local inflammatory response and better tissue repair.

Conflict of Interest Statement

The authors have declared no conflict of interest.

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Table 1. Parameters, scores and distribution of specimens according to histopathological analysis in SRP, BuTB-0.1, aPDT-0.1, BuTB-0.5, aPDT-0.5, BuTB-2.0 and aPDT-2.0 groups at different study time points.

PARAMETERS AND RESPECTIVE SCORES	PERCENTAGE OF THE ANIMALS																				
	Experimental groups and time points																				
	SRP			BuTB-0.1			aPDT-0.1			BuTB-0.5			aPDT-0.5			BuTB-2.0			aPDT-2.0		
	7d	15d	30d	7d	15d	30d	7d	15d	30d	7d	15d	30d	7d	15d	30d	7d	15d	30d	7d	15d	30d
INTENSITY OF LOCAL INFLAMMATORY RESPONSE																					
(0) Absence of inflammation (presence of rare inflammatory cells)									20%			20%		40%	100%			20%			20%
(1) Small quantity of inflammatory cells (< 1/3 of cells are inflammatory cells)			20%	40%	40%	80%	80%	80%	60%	60%	80%	80%	100%	60%		40%	40%	60%	80%	100%	80%
(2) Moderate quantity of inflammatory cells (from 1/3–2/3 of cells are inflammatory cells)	60%	100%	80%	60%	60%	20%	20%	20%	20%	40%	20%					60%	60%	20%	20%		
(3) Large quantity of inflammatory cells (over 2/3 of cells are inflammatory cells)	40%																				
INFLAMMATION EXTENSION																					
(0) Absence of inflammation									20%			20%		40%	100%			20%			40%
(1) Partial extension of connective tissue				20%	40%	80%	80%	100%	80%	60%	80%	80%	100%	60%		20%	60%	60%	80%	100%	60%
(2) Entire extension of connective tissue, without reaching bone tissue	100%	100%	100%	80%	60%	20%	20%			40%	20%					80%	40%	20%	20%		
(3) Entire extension of connective tissue and bone tissue																					
CELLULAR PATTERN AND CONNECTIVE TISSUE STRUCTURE OF THE FURCATION REGION																					
(0) Moderate quantity of fibroblasts and large quantity of collagen fibers (dense connective tissue)									20%			20%		60%	100%			20%			20%
(1) Moderate quantity of both fibroblasts and collagen fiber			40%	40%	40%	80%	80%	80%	60%	60%	80%	80%	100%	40%		40%	40%	60%	80%	100%	80%
(2) Small quantity of both fibroblasts and collagen fiber	100%	100%	60%	60%	60%	20%	20%	20%	20%	40%	20%					60%	60%	20%	20%		
(3) Severe tissue disorganization with necrosis areas																					
CELLULAR PATTERN AND BONE TISSUE STRUCTURE OF THE FURCATION REGION																					
(0) Bone trabeculae with regular contour coated with active osteoblasts, including areas of new bone formation														20%	20%						
(1) Bone trabeculae with irregular contour coated with active osteoblasts and osteoclasts			40%	20%	40%	80%	60%	100%	100%	60%	80%	80%	100%	80%	80%	20%	60%	80%	80%	100%	100%
(2) Bone trabeculae with irregular contour coated with active osteoclasts	80%	100%	60%	80%	60%	20%	40%			40%	20%	20%				80%	40%	20%	20%		
(3) Areas of necrotic bone and bone trabeculae with irregular contour coated with active osteoclasts	20%																				

Figure 1. Photomicrographs of the left mandibular first molar with experimental periodontitis showing magnitude of local inflammatory response, level of alveolar bone loss, and alveolar repair process in SRP (a, h), BuTB-0.1 (b, i), aPDT-0.1 (c, j), BuTB-0.5 (d, k), aPDT-0.5 (e, l), BuTB-2.0 (f, m) and aPDT-2.0 (g, n) at 7 days. Note the presence of inflammatory infiltrate and greater alveolar bone loss in the SRP group. In contrast, in the other groups, and especially those treated with aPDT, there were few inflammatory cells and less alveolar bone loss. Abbreviations and symbols: ab, alveolar bone; ct, connective tissue. Original magnification: a-g, 100x; h-n, 250x. Scale bars: a-g, 250 μm; h-n, 100 μm;. Staining: hematoxylin and eosin (H & E).

Figure 2. Photomicrographs of the left mandibular first molar with experimental periodontitis showing the course of the inflammatory response, level of alveolar bone loss, and alveolar repair process in SRP (a, h), BuTB-0.1 (b, i), aPDT-0.1 (c, j), BuTB-0.5 (d, k), aPDT-0.5 (e, l), BuTB-2.0 (f, m) and aPDT-2.0 (g, n) at 30 days. Note a less favorable tissue repair process and the greater alveolar bone loss in the SRP group. In contrast, in groups treated with aPDT, there was no inflammatory infiltrate, less alveolar bone loss and a better pattern of tissue repair. Note that in the aPDT-0.5 group there are even osteoblast concentration and foci of bone neoformation (*). Abbreviations and symbols: asterisks, foci of bone neoformation; ab, alveolar bone; ct, connective tissue. Original magnification: a-g, 100x; h-n, 250x. Scale bars: a-g, 250 μm; h-n, 100 μm;. Staining: hematoxylin and eosin (H & E).

Figure 3. Mean and standard deviation of the area of alveolar bone loss (mm^2) 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 SRP group at 7 days; ‡, Statistically significant difference in relation to the SRP group at 15 days; ¶, Statistically significant difference in relation to the SRP group at 30 days.

Figure 4. Immunolabeling pattern for TGF-β1 in the furcation region of the left mandibular first molar. (a) Median and interquartile deviation of the scores attributed to the immunolabeling pattern for TGF-β1. (b-h) Photomicrographs showing immunolabeling pattern for TGF-β1 in SRP (b), BuTB-0.1 (c), BuTB-0.5 (d), BuTB-2.0 (e), aPDT-0.1 (f), aPDT-0.5 (g), aPDT-2.0 (h), at 7 days. Abbreviations and symbols: arrows, TGF-β1 -immunolabelling cell; ab, alveolar bone; †, statistically significant difference in relation to SRP in the same time point; ‡, statistically significant difference in relation to aPDT-0.5 in the same time point; α, statistically significant difference in relation to 7 days in the same group; β, statistically significant difference in relation to 15 days in the same group. Original magnification: 1000x. Scale bars: 25 µm. Counterstaining: Harris hematoxylin.

Figure 5. Immunolabeling pattern for OCN in the furcation region of the left mandibular first molar. (a) Median and interquartile deviation of the scores attributed to the immunolabeling pattern for OCN. (b-h) Photomicrographs showing immunolabeling pattern for OCN in SRP (b), BuTB-0.1 (c), BuTB-0.5 (d), BuTB-2.0 (e), aPDT-0.1 (f), aPDT-0.5 (g), aPDT-2.0 (h), at 30 days. Abbreviations and symbols: arrows, OCN-immunolabelling cell; ab, alveolar bone; †, statistically significant difference in relation to SRP in the same time point; ‡, statistically significant difference in relation to aPDT-0.5 in the same time point; α, statistically significant difference in relation to 7 days in the same group. Original magnification: 1000x. Scale bars: 25 µm. Counterstaining: Harris hematoxylin.

Figure 6. Immunolabeling pattern for TRAP in the furcation region of the left mandibular first molar. (a) Mean and standard deviation of the number of TRAP-positive cells per mm^2 according to treatments and time points. (b-h) Photomicrographs showing immunolabeling pattern for TRAP in SRP (b), BuTB-0.1 (c), BuTB-0.5 (d), BuTB-2.0 (e), aPDT-0.1 (f), aPDT-0.5 (g), aPDT-2.0 (h), at 30 days. Abbreviations and symbols: arrows, TRAP-immunolabelling cell; ab, alveolar bone; †, statistically significant difference in relation to SRP in the same time point; α, statistically significant difference in relation to 7 days in the same group. Original magnification: 1000x. Scale bars: 25 µm. Counterstaining: Harris hematoxylin.