



## LJMU Research Online

**Nuernberg, MAA, Wainwright, M, Miessi, DMJ, Scalet, V, Olivo, MB, Ervolino, E, Garcia, VG and Theodoro, LH**

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

<http://researchonline.ljmu.ac.uk/id/eprint/13209/>

### Article

**Citation** (please note it is advisable to refer to the publisher's version if you intend to cite from this work)

**Nuernberg, MAA, Wainwright, M, Miessi, DMJ, Scalet, V, Olivo, MB, Ervolino, E, Garcia, VG and Theodoro, LH (2020) Effects of butyl toluidine blue photosensitizer on antimicrobial photodynamic therapy for experimental periodontitis treatment in rats. Photodiagnosis and Photodynamic Therapy.**

LJMU has developed [LJMU Research Online](http://researchonline.ljmu.ac.uk/) for users to access the research output of the University more effectively. Copyright © and Moral Rights for the papers on this site are retained by the individual authors and/or other copyright owners. Users may download and/or print one copy of any article(s) in LJMU Research Online to facilitate their private study or for non-commercial research. You may not engage in further distribution of the material or use it for any profit-making activities or any commercial gain.

The version presented here may differ from the published version or from the version of the record. Please see the repository URL above for details on accessing the published version and note that access may require a subscription.

For more information please contact [researchonline@ljmu.ac.uk](mailto:researchonline@ljmu.ac.uk)

<http://researchonline.ljmu.ac.uk/>

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

<sup>1</sup>Marta Aparecida Alberton Nuernberg

<sup>2</sup>Mark Wainwright

<sup>1</sup>Daniela Maria Janjacomio Miessi

<sup>1</sup>Vitor Scalet

<sup>1</sup>Mariane Bocalon Olivo

<sup>3</sup>Edilson Ervolino

<sup>1,4</sup>Valdir Gouveia Garcia

<sup>1</sup>Letícia Helena Theodoro

<sup>1</sup>Department of Surgery and Integrated Clinic, São Paulo State University (UNESP), Araçatuba, SP, Brazil, Periodontics Division, School of Dentistry.

<sup>2</sup>School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool, United Kingdom

<sup>3</sup>Department of Basic Sciences, School of Dentistry, São Paulo State University (UNESP), Araçatuba, São Paulo, Brazil

<sup>4</sup>Latin American Institute of Dental Research and Education (ILAPEO), Curitiba, PR, Brazil.

### **Corresponding Author:**

Letícia Helena Theodoro

Address: Faculdade de Odontologia de Araçatuba - UNESP

Rua José Bonifácio, 1193, Centro; CEP: 16015-050, Araçatuba, São Paulo, Brazil.

Telephone number: +55 (18) 36362860

E-mail: leticia.theodoro@unesp.br

## 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- $\beta$ 1, 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 TGF $\beta$ 1 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 ( $^1\text{O}_2$ ), which are cytotoxic to cells (7). The development of microbial resistance to this cytotoxic action is unlikely as  $^1\text{O}_2$  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 analysis and by TGF- $\beta$ 1 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 norvegicus albinus*, Wistar) weighing 180 to 250 g. They were kept in plastic cages with wood shavings, under 12 hours/12 hours light/dark cycles,  $22 \pm 2$  °C ambient temperature, 20 air changes per hour,  $55 \pm 5\%$  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<sup>2</sup>; energy density: 84.8 J/ cm<sup>2</sup>; exposure time: 60 seconds and power density of 1.41 W/ cm<sup>2</sup>. 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- $\beta$ 1 (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<sup>2</sup> 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  $\mu\text{m}$  x 1200  $\mu\text{m}$ , 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  $\mu\text{m}$  (17). For OCN and TGF- $\beta$ 1, 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- $\beta$ 1 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 TGF $\beta$ 1 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 TGF $\beta$ 1 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  $\lambda_{\max}$  values, an increase in  $^1\text{O}_2$  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 TGF $\beta$ 1 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. TGF $\beta$ 1 is involved in the regulation of inflammation and immune response in wound healing (35-37) and in bone resorption control (38-40). Increased TGF $\beta$ 1 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 TGF $\beta$ 1 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 TGF $\beta$ 1 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.

### **Acknowledgments**

This study was financial in part by “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES)” – Finance Code 001 through a PhD fellowship. The authors of this study thank the Departments of Diagnostic and Surgery and Basic Sciences of the Faculty of Dentistry of Araçatuba – UNESP for material support for the study.

### **References**

1. Tonetti MS, Greenwell H, Kornman KS. Staging and grading of periodontitis: Framework and proposal of a new classification and case definition. *J Periodontol*. 2018;89 Suppl 1:S159-S72. PubMed PMID: 29926952. eng.
2. Smiley CJ, Tracy SL, Abt E, Michalowicz BS, John MT, Gunsolley J, et al. Evidence-based clinical practice guideline on the nonsurgical treatment of chronic periodontitis by means of scaling and root planing with or without adjuncts. *Journal of the American Dental Association (1939)*. 2015 Jul;146(7):525-35. PubMed PMID: 26113100. Epub 2015/06/27. eng.
3. Fang H, Han M, Li QL, Cao CY, Xia R, Zhang ZH. Comparison of full-mouth disinfection and quadrant-wise scaling in the treatment of adult chronic periodontitis: a systematic review and meta-analysis. *Journal of periodontal research*. 2016 Aug;51(4):417-30. PubMed PMID: 26477533. Epub 2015/10/20. eng.
4. Fang H, Han M, Li QL, Cao CY, Xia R, Zhang ZH. Comparison of full-mouth disinfection and quadrant-wise scaling in the treatment of adult chronic periodontitis: a systematic review and meta-analysis. *J Periodontal Res*. 2016;51(4):417-30. PubMed PMID: 26477533. Epub 10/19. eng.
5. Meisel P, Kocher T. Photodynamic therapy for periodontal diseases: state of the art. *Journal of photochemistry and photobiology B, Biology*. 2005 May 13;79(2):159-70. PubMed PMID: 15878121. Epub 2005/05/10. eng.
6. Kikuchi T, Mogi M, Okabe I, Okada K, Goto H, Sasaki Y, et al. Adjunctive Application of Antimicrobial Photodynamic Therapy in Nonsurgical Periodontal Treatment: A Review of Literature. *International journal of molecular sciences*. 2015 Oct 13;16(10):24111-26. PubMed PMID: 26473843. Pubmed Central PMCID: PMC4632741. Epub 2015/10/17. eng.
7. Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). *J Antimicrob Chemother*. 1998 Jul;42(1):13-28. PubMed PMID: 9700525. Epub 1998/08/13. eng.
8. Hamblin MR, Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? *Photochemical & photobiological sciences : Official journal of the European Photochemistry Association and the European Society for Photobiology*. 2004 May;3(5):436-50. PubMed PMID: 15122361. Pubmed Central PMCID: PMC3071049. Epub 2004/05/04. eng.
9. Maisch T. Resistance in antimicrobial photodynamic inactivation of bacteria. *Photochemical & photobiological sciences : Official journal of the European Photochemistry Association and the European Society for Photobiology*. 2015 Aug;14(8):1518-26. PubMed PMID: 26098395. Epub 2015/06/23. eng.

10. Wainwright M, Maisch T, Nonell S, Plaetzer K, Almeida A, Tegos GP, et al. Photoantimicrobials-are we afraid of the light? *The Lancet Infectious diseases*. 2017 Feb;17(2):e49-e55. PubMed PMID: 27884621. Pubmed Central PMCID: PMC5280084. Epub 2016/11/26. eng.
11. Hu X, Huang YY, Wang Y, Wang X, Hamblin MR. Antimicrobial Photodynamic Therapy to Control Clinically Relevant Biofilm Infections. *Frontiers in microbiology*. 2018;9:1299. PubMed PMID: 29997579. Pubmed Central PMCID: PMC6030385. Epub 2018/07/13. eng.
12. Atieh MA. Photodynamic therapy as an adjunctive treatment for chronic periodontitis: a meta-analysis. *Lasers in medical science*. 2010 Jul;25(4):605-13. PubMed PMID: 20024665. Epub 2009/12/22. eng.
13. Azaripour A, Dittrich S, Van Noorden CJF, Willershausen B. Efficacy of photodynamic therapy as adjunct treatment of chronic periodontitis: a systematic review and meta-analysis. *Lasers in medical science*. 2018 Feb;33(2):407-23. PubMed PMID: 29177555. Epub 2017/11/28. eng.
14. Chambrone L, Wang HL, Romanos GE. Antimicrobial photodynamic therapy for the treatment of periodontitis and peri-implantitis: An American Academy of Periodontology best evidence review. *Journal of periodontology*. 2018 Jul;89(7):783-803. PubMed PMID: 30133749. Epub 2018/08/23. eng.
15. Maisch T. Anti-microbial photodynamic therapy: useful in the future? *Lasers in medical science*. 2007 Jun;22(2):83-91. PubMed PMID: 17120167. Epub 2006/11/23. eng.
16. Wainwright M, O'Kane C, Rawthore S. Phenothiazinium photosensitisers XI. Improved toluidine blue photoantimicrobials. *Journal of photochemistry and photobiology B, Biology*. 2016 Jul;160:68-71. PubMed PMID: 27093001. Epub 2016/04/20. eng.
17. Garcia VG, Longo M, Gualberto Junior EC, Bosco AF, Nagata MJ, Ervolino E, et al. Effect of the concentration of phenothiazine photosensitizers in antimicrobial photodynamic therapy on bone loss and the immune inflammatory response of induced periodontitis in rats. *Journal of periodontal research*. 2014 Oct;49(5):584-94. PubMed PMID: 24206053. Epub 2013/11/12. eng.
18. Zuza EP, Garcia VG, Theodoro LH, Ervolino E, Favero LFV, Longo M, et al. Influence of obesity on experimental periodontitis in rats: histopathological, histometric and immunohistochemical study. *Clin Oral Investig*. 2018;22(3):1197-208. PubMed PMID: 28929308. Epub 09/19. eng.

19. Festing MF. The design and statistical analysis of animal experiments. *ILAR journal*. 2002;43(4):191-3. PubMed PMID: 12391393. Epub 2002/10/23. eng.
20. Dereka XE, Markopoulou CE, Vrotsos IA. Role of growth factors on periodontal repair. *Growth Factors*. 2006;24(4):260-7. PubMed PMID: 17381067. eng.
21. Sodek J, McKee MD. Molecular and cellular biology of alveolar bone. *Periodontology* 2000. 2000 Oct;24:99-126. PubMed PMID: 11276877. Epub 2001/03/30. eng.
22. Roberts FA, Darveau RP. Microbial protection and virulence in periodontal tissue as a function of polymicrobial communities: symbiosis and dysbiosis. *Periodontol* 2000. 2015;69(1):18-27. PubMed PMID: 26252399. eng.
23. Graves DT, Fine D, Teng YT, Van Dyke TE, Hajishengallis G. The use of rodent models to investigate host-bacteria interactions related to periodontal diseases. *Journal of clinical periodontology*. 2008 Feb;35(2):89-105. PubMed PMID: 18199146. Pubmed Central PMCID: PMC2649707. Epub 2008/01/18. eng.
24. Kilian M, Chapple IL, Hannig M, Marsh PD, Meuric V, Pedersen AM, et al. The oral microbiome - an update for oral healthcare professionals. *British dental journal*. 2016 Nov 18;221(10):657-66. PubMed PMID: 27857087. Epub 2016/11/20. eng.
25. de Souza JA, Nogueira AV, de Souza PP, Cirelli JA, Garlet GP, Rossa C, Jr. Expression of suppressor of cytokine signaling 1 and 3 in ligature-induced periodontitis in rats. *Archives of oral biology*. 2011 Oct;56(10):1120-8. PubMed PMID: 21511249. Epub 2011/04/23. eng.
26. de Molon RS, Park CH, Jin Q, Sugai J, Cirelli JA. Characterization of ligature-induced experimental periodontitis. *Microscopy Research and Technique*. 2018;81(12):1412-21.
27. Alberton Nuernberg MA, Janjacomio Miessi DM, Ivanaga CA, Bocalon Olivo M, Ervolino E, Gouveia Garcia V, et al. Influence of antimicrobial photodynamic therapy as an adjunctive to scaling and root planing on alveolar bone loss: A systematic review and meta-analysis of animal studies. *Photodiagnosis Photodyn Ther*. 2019;25:354-63. PubMed PMID: 30658106. Epub 01/15. eng.
28. Garcia VG, Longo M, Fernandes LA, Gualberto EC, Jr., Santinoni Cdos S, Bosco AF, et al. Treatment of experimental periodontitis in rats using repeated adjunctive antimicrobial photodynamic therapy. *Lasers in medical science*. 2013 Jan;28(1):143-50. PubMed PMID: 22526974. Epub 2012/04/25. eng.
29. Bacellar IO, Pavani C, Sales EM, Itri R, Wainwright M, Baptista MS. Membrane damage efficiency of phenothiazinium photosensitizers. *Photochemistry and photobiology*. 2014 Jul-Aug;90(4):801-13. PubMed PMID: 24571440. Epub 2014/02/28. eng.

30. Benov L. Photodynamic therapy: current status and future directions. *Medical principles and practice : international journal of the Kuwait University, Health Science Centre*. 2015;24 Suppl 1:14-28. PubMed PMID: 24820409. Epub 2014/05/14. eng.
31. Wainwright M. Synthetic, small-molecule photoantimicrobials - a realistic approach. *Photochemical & photobiological sciences : Official journal of the European Photochemistry Association and the European Society for Photobiology*. 2018 Nov 1;17(11):1767-79. PubMed PMID: 29905338. Epub 2018/06/16. eng.
32. Bacellar IOL, Oliveira MC, Dantas LS, Costa EB, Junqueira HC, Martins WK, et al. Photosensitized Membrane Permeabilization Requires Contact-Dependent Reactions between Photosensitizer and Lipids. *Journal of the American Chemical Society*. 2018 Aug 1;140(30):9606-15. PubMed PMID: 29989809. Epub 2018/07/11. eng.
33. Wainwright M, McLean A. Rational design of phenothiazinium derivatives and photoantimicrobial drug discovery. *Dyes and Pigments*. 2017 2017/01/01;136:590-600.
34. George S, Hamblin M, Kishen A. Uptake pathways of anionic and cationic photosensitizers into bacteria 2009. 788-95 p.
35. Dereka XE, Markopoulou CE, Vrotsos IA. Role of growth factors on periodontal repair. *Growth factors (Chur, Switzerland)*. 2006 Dec;24(4):260-7. PubMed PMID: 17381067. Epub 2007/03/27. eng.
36. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society*. 2008 Sep-Oct;16(5):585-601. PubMed PMID: 19128254. Epub 2009/01/09. eng.
37. Koivisto L, Heino J, Hakkinen L, Larjava H. Integrins in Wound Healing. *Advances in wound care*. 2014 Dec 1;3(12):762-83. PubMed PMID: 25493210. Pubmed Central PMCID: PMC4250945. Epub 2014/12/11. eng.
38. Fox SW, Lovibond AC. Current insights into the role of transforming growth factor-beta in bone resorption. *Molecular and cellular endocrinology*. 2005 Nov 24;243(1-2):19-26. PubMed PMID: 16219413. Epub 2005/10/13. eng.
39. Tang E, Khan I, Andreana S, Arany PR. Laser-activated transforming growth factor- $\beta$ 1 induces human  $\beta$ -defensin 2: implications for laser therapies for periodontitis and peri-implantitis. *Journal of periodontal research*. 2017;52(3):360-7.
40. Kasagi S, Chen W. TGF-beta1 on osteoimmunology and the bone component cells. *Cell & bioscience*. 2013 Jan 15;3(1):4. PubMed PMID: 23321200. Pubmed Central PMCID: PMC3565958. Epub 2013/01/17. eng.

41. Kuru L, Griffiths GS, Petrie A, Olsen I. Changes in transforming growth factor-beta1 in gingival crevicular fluid following periodontal surgery. *Journal of clinical periodontology*. 2004 Jul;31(7):527-33. PubMed PMID: 15191588. Epub 2004/06/12. eng.
42. de Freitas LF, Hamblin MR. Proposed Mechanisms of Photobiomodulation or Low-Level Light Therapy. *IEEE J Sel Top Quantum Electron*. 2016 May-Jun;22(3):7000417. PubMed PMID: 28070154. eng.
43. Pyo SJ, Song WW, Kim IR, Park BS, Kim CH, Shin SH, et al. Low-level laser therapy induces the expressions of BMP-2, osteocalcin, and TGF-beta1 in hypoxic-cultured human osteoblasts. *Lasers in medical science*. 2013 Feb;28(2):543-50. PubMed PMID: 22552925. Epub 2012/05/04. eng.
44. Hayman AR. Tartrate-resistant acid phosphatase (TRAP) and the osteoclast/immune cell dichotomy. *Autoimmunity*. 2008 Apr;41(3):218-23. PubMed PMID: 18365835. Epub 2008/03/28. eng.
45. Garcia VG, Fernandes LA, Macarini VC, de Almeida JM, Martins TM, Bosco AF, et al. Treatment of experimental periodontal disease with antimicrobial photodynamic therapy in nicotine-modified rats. *Journal of clinical periodontology*. 2011 Dec;38(12):1106-14. PubMed PMID: 22092666. Epub 2011/11/19. eng.
46. Garcia VG, Gualberto Junior EC, Fernandes LA, Bosco AF, Hitomi Nagata MJ, Casatti CA, et al. Adjunctive antimicrobial photodynamic treatment of experimentally induced periodontitis in rats with ovariectomy. *Journal of periodontology*. 2013 Apr;84(4):556-65. PubMed PMID: 22680299. Epub 2012/06/12. eng.
47. Gualberto EC, Jr., Theodoro LH, Longo M, Novaes VC, Nagata MJ, Ervolino E, et al. Antimicrobial photodynamic therapy minimizes the deleterious effect of nicotine in female rats with induced periodontitis. *Lasers in medical science*. 2016 Jan;31(1):83-94. PubMed PMID: 26545755. Epub 2015/11/08. eng.
48. de Oliveira PG, Silveira ESAM, Novaes AB, Jr., Taba M, Jr., Messoria MR, Palioto DB, et al. Adjunctive effect of antimicrobial photodynamic therapy in induced periodontal disease. Animal study with histomorphometrical, immunohistochemical, and cytokine evaluation. *Lasers in medical science*. 2016 Sep;31(7):1275-83. PubMed PMID: 27351664. Epub 2016/06/29. eng.
49. Garcia VG, Gualberto ECJ, Ervolino E, Nagata MJH, de Almeida JM, Theodoro LH. aPDT for periodontitis treatment in ovariectomized rats under systemic nicotine. *Photodiagnosis and photodynamic therapy*. 2018 Jun;22:70-8. PubMed PMID: 29481872. Epub 2018/02/27. eng.





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  $\mu\text{m}$ ; h-n, 100  $\mu\text{m}$ ; 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  $\mu\text{m}$ ; h-n, 100  $\mu\text{m}$ ; Staining: hematoxylin and eosin (H & E).

Figure 3. Mean and standard deviation of the area of alveolar bone loss ( $\text{mm}^2$ ) 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- $\beta$ 1 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- $\beta$ 1. (b-h) Photomicrographs showing immunolabeling pattern for TGF- $\beta$ 1 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- $\beta$ 1 -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;  $\alpha$ , statistically significant difference in relation to 7 days in the same group;  $\beta$ , statistically significant difference in relation to 15 days in the same group. Original magnification: 1000x. Scale bars: 25  $\mu$ m. 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;  $\alpha$ , statistically significant difference in relation to 7 days in the same group. Original magnification: 1000x. Scale bars: 25  $\mu$ m. 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<sup>2</sup> 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;  $\alpha$ , statistically significant difference in relation to 7 days in the same group. Original magnification: 1000x. Scale bars: 25  $\mu$ m. Counterstaining: Harris hematoxylin.