Marta Aparecida Alberton Nuernberg

Efeito do fotossensibilizador butyl azul de toluidina na terapia fotodinâmica antimicrobiana para o tratamento da periodontite experimental em ratos

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Tese apresentada à Faculdade de Odontologia de Araçatuba – Universidade Estadual Paulista "Júlio de Mesquita Filho" - UNESP, como parte dos requisitos para a obtenção do título de Doutor em Odontologia, área de concentração em Periodontia

Orientadora: Profa. Associada Letícia Helena Theodoro

Co-orientador: Prof. Titular Valdir Gouveia Garcia

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Dedico esta conquista aos grandes amores da minha vida:

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Eu saí de casa muito cedo e, infelizmente, nunca mais voltei. Mas, se tem algo que eu sinto falta todos os dias é isso. São eles. Eles são minha saudade rotineira. Minha fala emocionada. O número de telefone que eu ligo pra recarregar as forças e nutrir a fé em tudo. Eles são meu abrigo à distância e a certeza de que eu jamais estou sozinha.
Quando decidi pelo curso de doutorado da FOA/UNESP, eu sabia que seriam anos de muitas saudades e distância, e posso afirmar que este foi o maior desafio desta caminhada. Lembro-me perfeitamente de sentar com minha mãe na frente do computador e pesquisar as linhas de ônibus entre a minha cidade e Araçatuba. Naquele dia eu tive a certeza de que grandes desafios me esperavam. Recomeçar, fazer novas amizades, me adaptar com a nova cidade e diminuir a frequência de retorno para a minha casa. Meus pais também estavam cientes disso tudo e jamais me limitaram. Pelo contrário, falamos religiosamente todos os dias pelo telefone e o amor que eu recebo deles me faz seguir em frente e me inspira a dar sempre o melhor de mim. Meus pais são os maiores exemplos de força, generosidade, sabedoria e humildade da minha vida. São minha fonte de amor inesgotável. Eles são a base tudo! Eles são tudo!

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Esta conquista não é minha, esta conquista é nossa!
Amo vocês infinitamente!

"O amor é a força mais sutil do mundo."
Mahatma Gandhi

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Ao meu guia e protetor,

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"I touch the sky when my knees hit the ground"

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"Muitas vezes as pessoas são egocêntricas, ilógicas e insensatas.
Perdoe-as assim mesmo

Se você é gentil, as pessoas podem acusá-lo de interesseiro.
Seja gentil assim mesmo.

Se você é um vencedor, terá alguns falsos amigos e alguns inimigos verdadeiros.
Vença assim mesmo.

Se você é honesto e franco, as pessoas podem enganá-lo.
Seja honesto e franco assim mesmo.

O que você levou anos para construir, alguém pode destruir de uma hora para outra.
Construa assim mesmo.

Se você tem paz e é feliz, as pessoas podem sentir inveja.
Seja feliz assim mesmo.

O bem que você faz hoje, pode ser esquecido amanhã.
Faça o bem assim mesmo.

Dê ao mundo o melhor de você, mas isso pode não ser o bastante.
Dê o melhor de você assim mesmo.

Veja você que, no final das contas, é tudo entre você e Deus.
Nunca foi entre você e os outros."

Madre Tereza de Calcutá

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Luiz Antônio Nuernberg.

"Ter o amor de um irmão na vida é saber que nunca se estará só, pois aconteça o que acontecer esse laço jamais será quebrado" – Autor desconhecido

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"Sejamos atentos uns aos outros para incentivar a caridade e as boas obras porque será usada conosco a mesma medida que usamos com os outros. Que o Senhor nos dê mãos puras e coração inocente" – Pe. Elcio Alberton

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"Há pessoas que marcam a nossa vida, que despertam algo especial em nós, que abrem nossos olhos de modo irreversível e transformam a nossa maneira de ver o mundo." – Autor desconhecido

Professora Letícia Helena Theodoro,

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"Não existe o esquecimento total: as pegadas impressas na alma são indestrutíveis"
Thomas De Quincey
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O Alto Preço de Viver Longe de Casa

(...)

A vida de quem inventa de voar é paradoxal, todo dia. É o peito eternamente divido. É chorar porque queria estar lá, sem deixar de querer estar aqui. É ver o céu e o inferno na partida, o pesadelo e o sonho na permanência. É se orgulhar da escolha que te ofereceu mil tesouros e se odiar pela mesma escolha que te subtraiu outras mil pedras preciosas.

O preço é alto. A gente se questiona, a gente se culpa, a gente se angustia. Mas o destino, a vida e o peito às vezes pedem que a gente embarque. Alguns não vão. Mas nós, que fomos, viemos e iremos, não estamos livres do medo e de tantas fraquezas. Mas estamos para sempre livres do medo de nunca termos tentado.

(...)

Ruth Manus

NUERNBERG, MAA. Efeito do fotossensibilizador butyl azul de toluidina na terapia fotodinâmica antimicrobiana para o tratamento da periodontite experimental em ratos. [Tese de Doutorado]. Araçatuba: Faculdade de Odontologia da Universidade Estadual Paulista; 2019.

O presente estudo avaliou pela primeira vez "in vivo" os efeitos de três concentrações do butyl azul de toluidina (BuTB) como agente fotossensibilizador na terapia fotodinâmica antimicrobiana (aPDT), como terapia coadjuvante a raspagem e alisamento radicular (RAR), para o tratamento de periodontite experimental (PE) em ratos. A PE foi induzida por meio da instalação de um fio de algodão ao redor do primeiro molar inferior esquerdo. Posteriormente os animais foram aleatoriamente distribuídos em 7 grupos com 15 animais cada, através de uma tabela gerada por computador, de acordo com os seguintes tratamentos: RAR (n=15) - RAR seguido de irrigação local de solução salina fisiológica; BuTB-0,1 (n=15) - RAR seguido de aplicação local de BuTB na concentração de 0,1 mg/mL; aPDT-0,1 (n=15) - RAR seguido da aplicação local de BuTB na concentração de 0,1 mg/mL e irradiação com laser de diodo (LD) de InGaAlP (660 nm, 40 mW, 60 s, 2,4 J); BuTB-0,5 (n=15) – RAR seguido de aplicação local de BuTB na concentração de 0,5 mg/mL; aPDT-0,5 (n=15) – RAR seguido da aplicação local de BuTB na concentração de 0,5 mg/mL e irradiação com LD; BuTB-2,0 (n=15) - RAR seguido de aplicação local de BuTB na concentração de 2 mg/mL; aPDT-2,0 (n=15) - RAR seguido da aplicação local de BuTB na concentração de 2 mg/mL e irradiação com LD. Decorridos 7, 15 e 30 dias pós-tratamento, 5 animais de cada grupo foram submetidos à eutanásia. A área de furca dos molares foi submetida às análises histológica, histométrica e dos padrões de imunomarcação para TGF-ß1, OCN e TRAP. Os dados foram submetidos à análise estatística ($\alpha = 5\%$). De acordo com a análise histométrica na região de furca, todos os grupos experimentais apresentaram menor perda óssea comparado ao grupo controle. Histologicamente, os espécimes do aPDT-0,5 apresentaram uma resposta inflamatória local

mais branda e menos extensa, com melhor reestruturação tecidual em todos os períodos. Aos 30 dias observou-se resolução total da resposta inflamatória local, com presença de tecido conjuntivo denso. Alguns espécimes apresentavam trabéculas ósseas com contorno regular revestido com osteoblastos ativos, incluindo áreas de neoformação óssea. O tratamento com aPDT na concentração de 0,5 mg/mL resultou em padrões mais altos de imunomarcação de TGF-ß1 em todos os períodos e de OCN aos 30 dias. Diante dos resultados obtidos, todas as concentrações do novo fotossensibilizador BuTB trouxeram resultados adicionais ao tratamento da PE em relação a RAR. No entanto, a aPDT realizada com a concentração de 0,5 mg/mL resultou em benefícios adicionais na resposta inflamatória local e melhor reestruturação tecidual.

Palavras-chave

Periodontite; Raspagem dentária; Fotoquimioterapia; Modelos animais de doenças.

NUERNBERG, MAA. Effects of butyl toluidine blue photosensitizer on antimicrobial photodynamic therapy for experimental periodontitis treatment in rats. [Tese de Doutorado]. Araçatuba: Faculdade de Odontologia da Universidade Estadual Paulista; 2019.

The present study evaluated for the first time the effects of three concentrations of butyl toluidine blue (BuTB) as a photosensitizing agent on antimicrobial photodynamic therapy (aPDT), as adjuvant therapy to scaling and root planing (SRP), for the treatment of experimental periodontitis (EP) in rats. EP was induced by placing a cotton thread around the lower left first molar. Subsequently, the animals were randomly distributed into seven groups with 15 animals each, through a computer generated table, according to the following treatments: SRP (n = 15), SRP followed by local irrigation of physiological saline solution; BuTB-0.1 (n = 15), SRP followed by local application of 0.1 mg/mL BuTB; aPDT-0.1 (n = 15), SRP followed by local application of BuTB at 0.1 mg/mL concentration and irradiation with InGaAlP diode laser (DL) (660 nm, 40 mW, 60 s, 4 J); BuTB-0.5 (n = 15), SRP followed by local application of BuTB at 0.5 mg/mL concentration; aPDT-0.5 (n = 15), SRP followed by local application of BuTB at 0.5 mg/mL concentration and DL irradiation; BuTB-2.0 (n = 15), SRP followed by local application of BuTB at 2 mg/mL concentration; aPDT-2.0 (n = 15), SRP followed by local application of BuTB at 2 mg/mL concentration and DL irradiation. The animals (n=5) from each group were submitted to euthanasia at 7, 15 and 30 days post-treatment. The furcation area of the first lower molar was submitted to histological, histometric and immunohistochemical analyses to identify TGF-ß1, OCN and TRAP. The data were submitted to statistical analysis ($\alpha = 5\%$). According to the histometric analysis in the furcation region, all experimental groups presented lower bone loss compared to the control group. Histologically, the aPDT -0.5 specimens presented a milder and less extensive local inflammatory response, with better tissue remodeling in all periods. Total resolution of the local inflammatory response was observed at 30 days with presence of mature connective tissue. Some specimens presented bone trabeculae

with a regular contour and active osteoblasts, including areas of bone neoformation. Treatment with aPDT-0.5 also resulted in higher immunolabelling patterns of TGFβ1 at all periods and of OCN at 30 days. All concentrations of the new photosensitizer BuTB resulted in significant improvement for EP treatment in relation to SRP. However, aPDT combined with BuTB at 0.5 mg / mL showed the best benefits for inflammatory response and periodontal repair process.

Keywords

Periodontitis; Dental Scaling; Photochemotherapy; Animal disease model.

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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, 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.

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, 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.

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 point. (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, 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.

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LISTA DE ABREVIATURAS E SIGLAS

SRP - Scaling and root planing

aPDT - Antimicrobial photodynamic therapy

PS - Photosensitizer

$_1O^2$ - Singlet oxygen

BuTB - Butyl toluidine blue

TBO – Toluidine blue O

ROS – Reactive oxygen species

EP - Experimental periodontitis

TGF-ß1 - Transforming growth factor-β1

OCN - Osteocalcin

TRAP - Tartrate-resistant acid phosphatase

CEUA - Ethics Committee in the Use of Animal

InGaAlP - Indio-Gallium-Aluminum-Phosphorus

DL – Diodo laser

nm - Nanometer

mW - Milliwatts

J/cm2 - Joules per square centimetre

J - Joules

W/cm² - Watts per square centimetre

EDTA - Ethylenediamine tetraacetic acid

M - molar mass

H&E - Hematoxylin and eosin

MB – Methylene blue

µM - Micrometer

PBS - Phosphate-buffered saline

λ - Wavelength

RUNX2 - Runt-related transcription factor 2

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SUMÁRIO

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

Short running title: **Effects of a new photosensitizer on aPDT**

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This research study followed the guidelines of the Journal of Clinical Periodontology.

ABSTRACT

Background: The present study evaluated for the first time the effects of three concentrations of butyl toluidine blue (BuTB) as a photosensitizing agent on antimicrobial photodynamic therapy (aPDT), as adjuvant therapy to scaling and root planing (SRP), for the treatment of experimental periodontitis (EP) in rats.

Material and Methods: EP was induced by placing a cotton thread around the mandibular left first molar. Subsequently, the animals were randomly distributed into seven groups with 15 animals each, according to the following treatments: SRP, SRP followed by local irrigation of physiological saline solution; BuTB-0.1, SRP followed by local application of BuTB at 0.1 mg/mL; aPDT-0.1, SRP followed by local application of BuTB at 0.1 mg/mL and irradiation with InGaAlP diode laser (DL) (660 nm, 40 mW, 60 s, 4 J); BuTB-0.5, SRP followed by local application of BuTB at 0.5 mg/mL; aPDT-0.5, SRP followed by local application of BuTB at 0.5 mg/mL and DL irradiation; BuTB-2.0, SRP followed by local application of BuTB at 2 mg/mL; aPDT-2.0, SRP followed by local application of 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 of the mandibular molar was submitted to histological, histometric and immunohistochemical analyses to identify TGF-ß1, OCN and TRAP. The data were submitted to statistical analysis ($\alpha = 5\%$).

Results: All groups treated with BuTB presented lower bone loss compared to the SRP group. aPDT-0.5 specimens presented a less extensive local inflammatory response, with better tissue remodeling in all periods. Total resolution of the local inflammatory response was observed at 30 days with presence of mature connective tissue and areas of bone neoformation. aPDT-0.5 also resulted in higher immunolabelling patterns of TGFβ1 at all periods and of OCN at 30 days.

Conclusion: All concentrations of the new photosensitizer BuTB resulted in significant improvement for EP treatment. However, aPDT combined with BuTB at 0.5 mg / mL showed the best benefits for inflammatory response and periodontal repair.

Keywords

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

Clinical Relevance

Scientific rationale for the study: In face of the need to develop alternative therapies to antibiotics, the study of aPDT raises interest in many areas of health. In the scope of periodontics, the different results achieved with aPDT as an adjunct to SRP, encourages the search for new parameters and photosensitizers to improve this therapy.

Major Findings: The adjuvant use of aPDT with BuTB at the concentration of 0.5 mg/ mL modulated the inflammatory response and obtained better periodontal repair.

Practical implications: The definition of the most effective BuTB concentration will serve as a starting point for further investigations in animals and humans, contributing to the delineation of effective protocols for clinical practice.

Introduction

Periodontitis is a highly prevalent disease associated with bone loss among adults in different populations (Dye, 2012, Eke et al., 2015, Oppermann et al., 2015). The pathological process of the disease is marked by the action of different microbial species and modulation of local and systemic factors that alter host response (Roberts and Darveau, 2015, Tonetti et al., 2018). Non-surgical treatment of scaling and root planing (SRP) is the initial recommended therapy for infection control (Smiley et al., 2015, Fang et al., 2016). However, some limitations of this mechanical therapy and the better understanding of periodontal disease pathogenesis have led many researchers to seek adjunctive methods for SRP in order to obtain clinical benefits with a low risk of side effects (Fang et al., 2016).

Antimicrobial photodynamic therapy (aPDT) has been studied as a promising adjuvant therapy (Meisel and Kocher, 2005, Kikuchi et al., 2015). The aPDT involves the combination of a photoactive agent, called photosensitizer (PS), associated with light energy and presence of oxygen (Wainwright, 1998). The therapeutic technique consists of the administration of a PS in the area to be treated, which requires a short pre-irradiation time for suitable

biodistribution of the drug within or on the microorganisms, followed by irradiation with light at the wavelength compatible with the PS absorption spectrum (Maisch, 2007b). 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. Essentially, the therapy is based on the generation of free radicals and singlet oxygen ($^1O_2$), which are cytotoxic to cells (Wainwright, 1998). The development of microbial resistance for this cytotoxic action is unlikely as $^1O_2$ is a primitive molecule and it acts in different molecular sites of the pathogen (Hamblin and Hasan, 2004, Maisch, 2015, Wainwright et al., 2017, Hu et al., 2018).

aPDT allows better access to areas not reached by SRP as well as prevents damage to host tissues in the infected area due to its bacterial selectivity (Hamblin and Hasan, 2004). In relation to its antimicrobial effectiveness, some *in vitro* studies have demonstrated a bactericidal effect of aPDT against some bacterial species associated with the etiology of periodontitis, particularly *Porphyromonas gingivalis* (Street et al., 2010, Oruba et al., 2015, Oruba et al., 2017). In addition to the antimicrobial effects, low-level laser irradiation seems to positively influence the response of periodontal tissues affected by the disease (Woodruff et al., 2004, Pejcic et al., 2010, Makhlouf et al., 2012, Aykol et al., 2011, Calderín et al., 2013) .

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 (Atieh, 2010, Azaripour et al., 2018, Chambrone et al., 2018). However, the extent of this statistical clinical attachment gain obtained with the combination of aPDT and SRP do not represent significant clinical relevance (Chambrone et al., 2018), stimulating further research to improve the parameters and elements involved in aPDT.

The aPDT has a set of differentials and peculiarities, with emphasis on the possibility of prospect different compositions of photosensitizing molecules with selectivity characteristics

(Maisch, 2007a). The search for a PS that is effective in photodynamic inactivation of bacteria and fungi started in 1903 and the selection of an ideal drug is still a challenge (Wainwright and McLean, 2017).

Given the need for further research to improve photosensitizers, this study demonstrates for the first time the *in vivo* effects of three concentrations of a new PS, butyl toluidine blue (BuTB). BuTB was developed by physicochemical modifications of the molecular structure of the toluidine blue O (TBO), a phenothiazine dye. Compared with the original compound, BuTB exhibited an increase in lipophilicity and decreased molecular aggregation behavior, characteristics that positively interfere both in the production of reactive oxygen species (ROS) and in the efficiency of cellular interaction (Wainwright et al., 2016).

In the present study, BuTB was evaluated as a photosensitizing agent in aPDT, as adjuvant to SRP, in the treatment of experimental periodontitis (EP) in rats. The effectiveness of each BuTB concentration was evaluated in the furcation region of the mandibular first molar using histological and histometric analysis of alveolar bone loss. Additionally, immunohistochemical analysis was performed to search for the transforming growth factor-β1 (TGF-β1), one of the main cytokines involved in the periodontal repair processes, as well as for osteocalcin (OCN), an important marker of the bone mineralization process and for tartrate-resistant acid phosphatase (TRAP), an osteoclast biomarker.

M**aterial and Methods**

Animals

This study was conducted on 105 male, 3-month old, adult rats (*Rattus novergicus albinus*, Wistar) weighing approximately 180 to 250 g. The animals were kept in plastic cages with access to feed and water *ad libitum*. The animals were kept in an environment with controlled temperature (22 ± 2º C) and light cycle (12 hours clear and 12 hours dark) one week before the experimental procedures. For all experimental procedures, the animals received

general anesthesia with the combination of 70 mg/kg ketamine hydrochloride (Dopalen, Industry and Commerce Ltda., Paulínia, SP, Brazil) and 6 mg/kg xylazine hydrochloride (Xilazin, Rhobifarma Indústria Farmacêutica Ltda, Hortolândia, SP, Brazil) applied intramuscularly in the biceps femoris of the right leg. All protocols were approved by the Ethics Committee on Animal Use (CEUA) of the School of Dentistry of Araçatuba (Process number 2015-00586, Sao Paulo State University, UNESP, SP, Brazil), and followed the principles of the "ARRIVE Guidelines".

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 (Garcia et al., 2014). The ligature was removed seven days after installation, and the animals were randomly assigned by a computer-generated table distributed into 7 groups with 15 animals each, according to the following treatments: SRP (n = 15), animals treated with SRP followed by local irrigation of physiological saline solution; BuTB-0.1 (n = 15), SRP treated animals followed by local application of BuTB at 0.1 mg/mL concentration; aPDT-0.1 (n = 15) , SRP-treated animals followed by local application of 0.1 mg/mL BuTB and irradiation with InGaAlP diodo laser (DL) (660 nm, 40 mW , 60 s, 2.4 J); BuTB-0.5 (n = 15), SRP-treated animals followed by local application of BuTB at a concentration of 0.5 mg/mL; aPDT-0.5 (n = 15), animals treated with SRP followed by local application of BuTB at 0.5 mg/mL concentration and DL irradiation; BuTB-2.0 (n = 15), animals treated with SRP followed by local application of BuTB at 2 mg/mL concentration; aPDT-2.0 (n = 15), SRP treated animals followed by local application of BuTB at 2 mg/ mL concentration and DL irradiation.

Scaling and root planing treatment

All animals received SRP treatment with mini-five 1-2-hand manual curettes (Hu-Fridey 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 (Garcia et al., 2014). The SRP procedures were performed by the same experienced operator, who was trained and blinded to the experimental groups (MAAN).

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 (Wainwright et al., 2016). Irrigation was done with the aid of an insulin syringe, carefully directing the tip of the needle into the tooth / gingival tissue following homeostasis of the area. DL irradiation was performed one minute after the drug remained in the tissue.

The laser used in the present study was the Indio-Gallium-Aluminum-Phosphorus (InGaAlP) DL with 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.

Experimental periods

After 7, 15 and 30 days post-treatment, five animals from each group were submitted to euthanasia by the injection of a lethal dose of thiopental 150mg/ kg (Cristália, Produtos Químicos Farmacêuticos Ltda., Itapira, SP, Brazil) associated with lidocaine hydrochloride 2%

10mg/kg (Novafarma Indústria Farmacêutica Ltda, Anápolis, GO, Brazil). The left hemimandibules were dissected and fixed in 4 % formaldehyde in 0.1 M buffered solution for 48 hours for histological, histometric and immunohistochemical analyzes.

Histological procedures

The left hemimandibules were washed in running water and decalcified in 10 % ethylenediamine tetraacetic acid (EDTA) solution. Following demineralization, the samples were washed in running water for 24 hours, dehydrated in ethanol, cleared in xylenes, embedded and blocked in paraffin. Semi-serial (4 μm) sections were obtained in the mesio-distal direction. Some sections were stained with hematoxylin and eosin (H&E) for histologic and histometric analysis and others were submitted to immunohistochemical method.

For the immunohistochemical reaction, the histological sections were deparaffinized in xylenes and hydrated in a decreasing series of ethanol. Antigen retrieval was performed by immersing the histological slides in 0.1 M citrate buffer, pH 7.4 (DIVA DECLOAKER®, Biocare Medical, Concord, CA, USA), in a pressurized chamber (DECLOAKING CHAMBER®, Biocare Medical, Concord, CA, USA) at 95° C for 20 minutes. At the end of each step of the immunohistochemical reaction, the histological slides were washed in 0.1 M PBS, pH 7.4. Subsequently, the slides were immersed in 3 % hydrogen peroxide for 1 hour and 1 % bovine serum albumin for 12 hours to block the endogenous peroxidase and block the non-specific sites, respectively. The slides containing samples from each experimental group were divided into three batches, and each batch was incubated with one of the following primary antibodies: goat anti-rat OCN (Osteocalcin, SC-18319, Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-rat TRAP (Tartrate-resistant acid phosphatase, SC-30833, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-rat TGF-β1 (Transforming growth factor beta 1, SC-146, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Next, the sections were incubated with biotinylated secondary antibody for 2 hours and with streptavidin- horseradish

peroxidase (HRP) conjugate for 1 hour (Universal Dako Labeled HRP Streptavidin-Biotin Kit®, Dako Laboratories, CA, USA). The immunoreaction was developed using diaminobenzidine 3, 3'-tetrahydrochloride (DAB chromogen Kit®, Dako Laboratories, CA, USA) as a chromogen and H_2O_2 as a substrate. The sections were then counterstained with Harris Hematoxylin, dehydrated in ethanol, cleared in xylene, mounted with Permount (Fisher Scientific, San Diego, CA, USA) and glass coverslips. As a negative control, the histological sections were submitted to the previously described procedures, omitting the primary antibodies.

Histological analysis

A certified histologist who was blinded to the treatments (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.

Histometric analysis

Bone loss in the furcation region 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). After exclusion of the first and last histological section, in which the furcation region is evident, three equidistant sections of each specimen were selected for histomorphometric analysis. A trained and blinded examiner (EE) performed this selection. Another calibrated and blinded examiner performed the histomorphometric analysis (MAAN). The same examiner measured the bone loss of each specimen two times, on different days, for reducing data variation.

Immunohistochemical analysis

A trained and blinded examiner selected the histological sections to be analyzed (MAAN). A certified and blinded histologist (EE) performed the immunohistochemical analyzes. Immunolabeling was defined as a brownish color present in the cytosolic compartment of the cells. In each animal, three histological sections were analyzed equidistant from the furcation region of the mandibular first molar with induced periodontitis. In these sections, 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 (Garcia et al., 2014). The coronal limit of this area was the alveolar ridge crest, from which it extends apically by a distance of 1200 µm (Garcia et al., 2014). For OCN and TGF-β1, a semi quantitative analysis of the immunolabeling was performed throughout the furcation area as follows: Score 0 - absence of immunolabeling (total absence of immunoreactive cells); Score 1 - Low immunolabeling pattern (25 % of positive cells per area); Score 2 - moderate immunolabeling pattern (50 % of positive cells per area) and; Score 3 - high immunolabeling pattern (75 % of positive cells per area) (Garcia et al., 2014).

Examiner calibration

Before the histometric and immunohistochemical analysis, an examiner was trained through two analyzes of bone loss and TRAP count of thirty samples, 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 (0.97).

Statistical analysis

The sample calculation was performed considering each animal as a unit and 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. The results demonstrated that with a sample size of 5 ($p<0.05$) the power of the study is 95%.

Statistical analyzes of all data were 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 analysis of variance (One-Factor ANOVA), 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.

Results

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.

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-0.1 and

aPDT-0.1 ($p < 0.05$) and there was no statistically significant difference in relation to the BuTB-0.5 group ($p > 0.05$).

Immunohistochemical analysis

The immunolabeling for TGF-ß1, OCN and TRAP demonstrated high specificity in the detection of such proteins, which was confirmed by the total absence of labeling in the negative control of the immunohistochemical reaction.

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 aDT-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, there was a higher immunolabeling pattern in BuTB-0.5 compared to SRP ($p < 0.05$). In addition, 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).

Immunohistochemical analysis to identify the number of positive TRAP cells corroborated the results of the histometric analysis. 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 lower number of TRAT-positive cells only at 15 days ($p < 0.05$) (Figure 6).

Discussion

The present study evaluated for the first time the *in vivo* effect of BuTB, as a photosensitizer agent in aPDT used as an adjunctive therapy to SRP, during EP treatment in rats. Three different concentrations of BuTB were evaluated by histometric, histological and immunohistochemical analyzes. All the evaluated concentrations showed favorable results for the control of alveolar bone loss. However, animals receiving treatment with aPDT using BuTB at the concentration of 0.5 mg/mL (aPDT-0.5 group) showed greater control of the local inflammatory response with better periodontal tissue repair than animals treated with the other concentrations, in all experimental periods. Corroborating this data, the aPDT-0.5 group presented higher immunolabeling pattern of TGFβ1 at all periods and for OCN at 30 days.

Periodontitis is clinically characterized by an exacerbated, ineffective and non-resolving inflammation of the tissues supporting the teeth, with consequent teeth loss (Meyle and Chapple, 2015). In the experimental model used in this study, as in humans, the development of the disease and alveolar bone loss is dependent on bacteria. Ligature installation leads to plaque accumulation, which acts as a key factor for the development of a dysbiotic microbiota (Graves et al., 2008). In good periodontal health status there is a homeostatic balance between the number of microorganisms and the composition of the microbiota with the host response. In dysbiosis, there is an increase in the number and proportion of bacteria associated with periodontal disease. The dysbiotic microbiota induces periodontal tissue destruction by means of a dysregulated inflammatory immune response of the host (Kilian et al., 2016). In this experimental model, bone loss occurs predictably over a period of 7 days (Graves et al., 2008).

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 application of 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 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 phenothiazine, methylene blue (MB) and TBO photosensitizers, at the concentration of 0.1 mg/mL (Alberton Nuernberg et al., 2019).

The bone loss results in the furcation region obtained with the aPDT treatment with BuTB are comparatively better than results obtained in previous studies with similar methodology that used MB and TBO (Garcia et al., 2013b, Garcia et al., 2014) In relation to TBO, the BuTB presents an increase in λmax values, an increase in $_1O^2$ quantum yield, a decrease in aggregation behavior and an increase in lipophilicity (Wainwright et al., 2016). These characteristics positively influence PS uptake and subcellular distribution (Bacellar et al., 2014, Benov, 2015). Besides the potential for ROS production, the efficacy of a PS agent is determined by the degree of its interaction with the target (Wainwright, 2018, Bacellar et al., 2018). 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 (Wainwright and McLean, 2017). 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 (George et al., 2009, Hu et al., 2018). This hypothesis can explain both the increased photodynamic efficacy and increased dark toxicity against microbial cells. 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 in our study obtained positive results in relation to the extent and intensity of the inflammatory process and cellularity pattern of the connective and bone tissues. Among the three PS concentrations tested, the 0.5 mg/mL (aPDT-0.5 group) concentration followed by DL irradiation showed a local inflammatory response of less intensity and extension, associated

with better restructuring of the connective and bone tissues. 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 concentration in relation to the 2 mg/mL concentration may be related to the aggregation behavior. Although BuTB shows lower aggregation than the original compound, the increase of the PS concentration favors stacking interactions (Wainwright and McLean, 2017). 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, with adjuvant use of aPDT, in which the smallest concentrations of both PS were the most effective ones (Garcia et al., 2014). 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 concentration. Further studies with microbiological analysis will provide important elucidations regarding the antimicrobial effect on periodontopathogens.

A previous study analyzed the *in vitro* photo-antimicrobial efficiency of BuTB, demonstrating a significantly increased activity against Gram-negative bacteria, such as *Pseudomonas aeruginosa* (Wainwright et al., 2016). The best bone loss control observed in this 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. A clinical study evaluating the adjuvant use of aPDT to SRP, followed by surgery for periodontal treatment in furcation class III, evidenced an additional effect of aPDT on increasing TGFβ1 concentration in the crevicular fluid at 45

postoperative days (Souza et al., 2013). Increased TGFβ1 levels in the crevicular fluid have been pointed out as a marker of prognosis for the progress of tissue repair (Kuru et al., 2004). TGFβ1 is a cytokine with pleiotropic properties, with a wide variety of effects on cell migration, differentiation and proliferation. TGFβ1 is involved in the regulation of inflammation and immune response in wound healing (Dereka et al., 2006, Barrientos et al., 2008, Koivisto et al., 2014) and in bone resorption control (Fox and Lovibond, 2005, Tang et al., 2017, Kasagi and Chen, 2013). Among the three treatment groups with aPDT, the 0.5 mg/mL BuTB concentration was once again highlighted in all periods. 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, without the presence of light, 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 (Sodek and McKee, 2000).

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. Light with wavelength in the red (600 to 700 nm) or near infrared (780 to 1200 nm) spectral regions interacts with the cells, leading to photomodulations at the molecular, cellular and tissue level, resulting in anti-inflammatory, analgesic and biostimulation effects (de Freitas and Hamblin, 2016). A previous study that evaluated the effect of photobiomodulation on EP in rats evidenced an accelerated periodontal repair process in animals treated with DL, associated or not to SRP, and an increase in the expression of RUNX2 and OCN (Theodoro et al., 2015). Another study, with in vivo analyzes of human osteoblasts cultured in hypoxia, demonstrated that photobiomodulation stimulates osteoblast differentiation and proliferation and increases BMP-2, OCN and TGFβ1

expression (Pyo et al., 2013). 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 PS concentration 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 (Hayman, 2008). The TRAP immunolabeling pattern is related to the data obtained in the bone loss histometric analysis in the furcation region. 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 (Garcia et al., 2011, Garcia et al., 2013a, Garcia et al., 2014, Gualberto et al., 2016, de Oliveira et al., 2016, Garcia et al., 2018).

The results of the present preclinical study make an important contribution in the evaluation of BuTB. 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. An additional *in vivo* analysis of the antimicrobial action of BuTB may add important evidence and will contribute to explain the benefits in the inflammatory response and tissue repair observed in the present study.

Conclusion

The use of BuTB as a photosensitizer agent, associated or not to DL, as adjuvant therapy for treatment of EP showed promising results at all concentrations employed. Among the concentrations evaluated, the adjuvant treatment with aPDT using BuTB at the concentration

of 0.5 mg/mL showed better control of the local inflammatory response and better tissue repair process.

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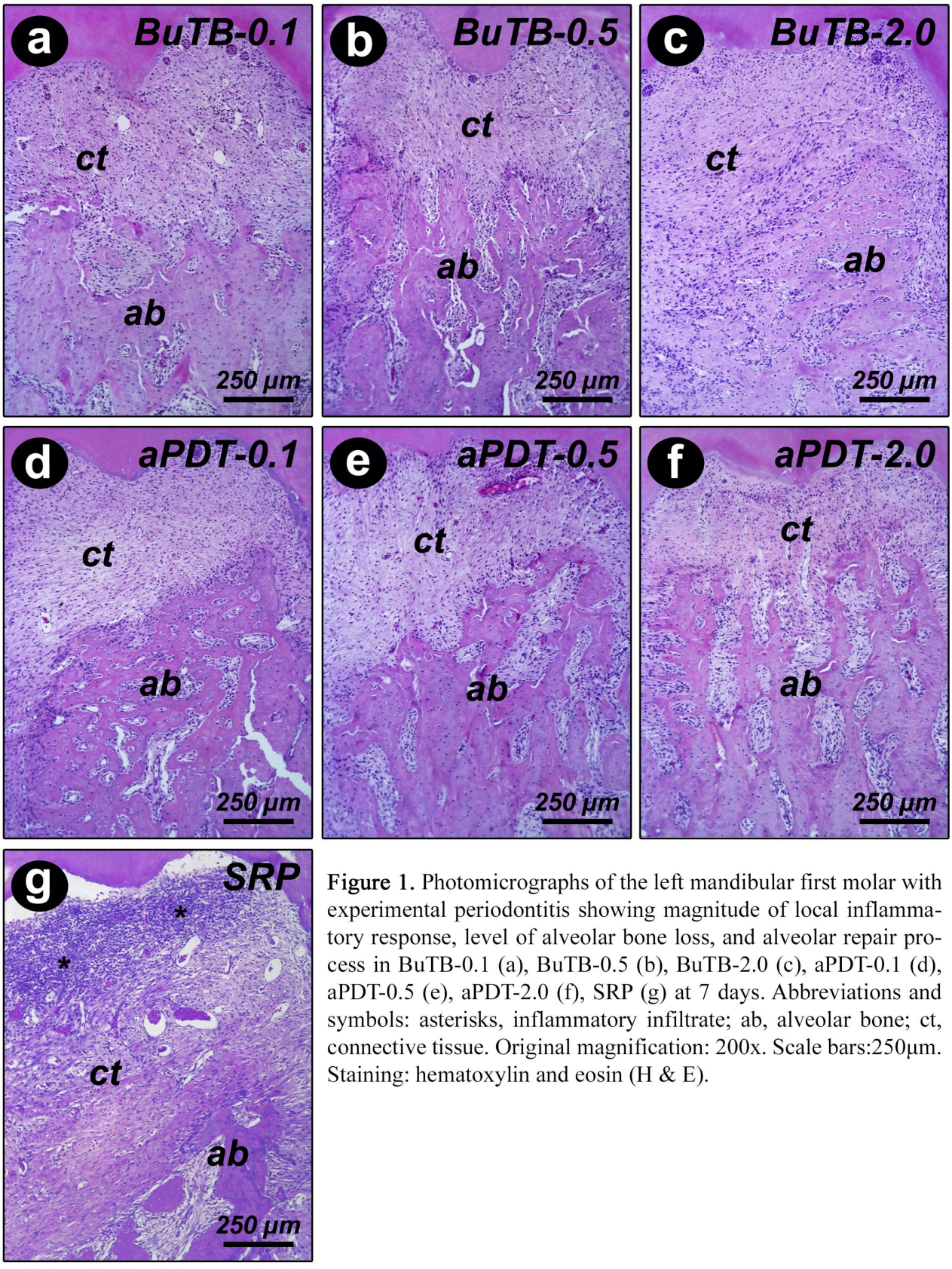

Figure 1. Photomicrographs of the left mandibular first molar with experimental periodontitis showing magnitude of local inflammatory response, level of alveolar bone loss, and alveolar repair process in BuTB-0.1 (a), BuTB-0.5 (b), BuTB-2.0 (c), aPDT-0.1 (d), aPDT-0.5 (e), aPDT-2.0 (f), SRP (g) at 7 days. Abbreviations and symbols: asterisks, inflammatory infiltrate; ab, alveolar bone; ct, connective tissue. Original magnification: 200x. Scale bars:250µm. Staining: hematoxylin and eosin (H & E).

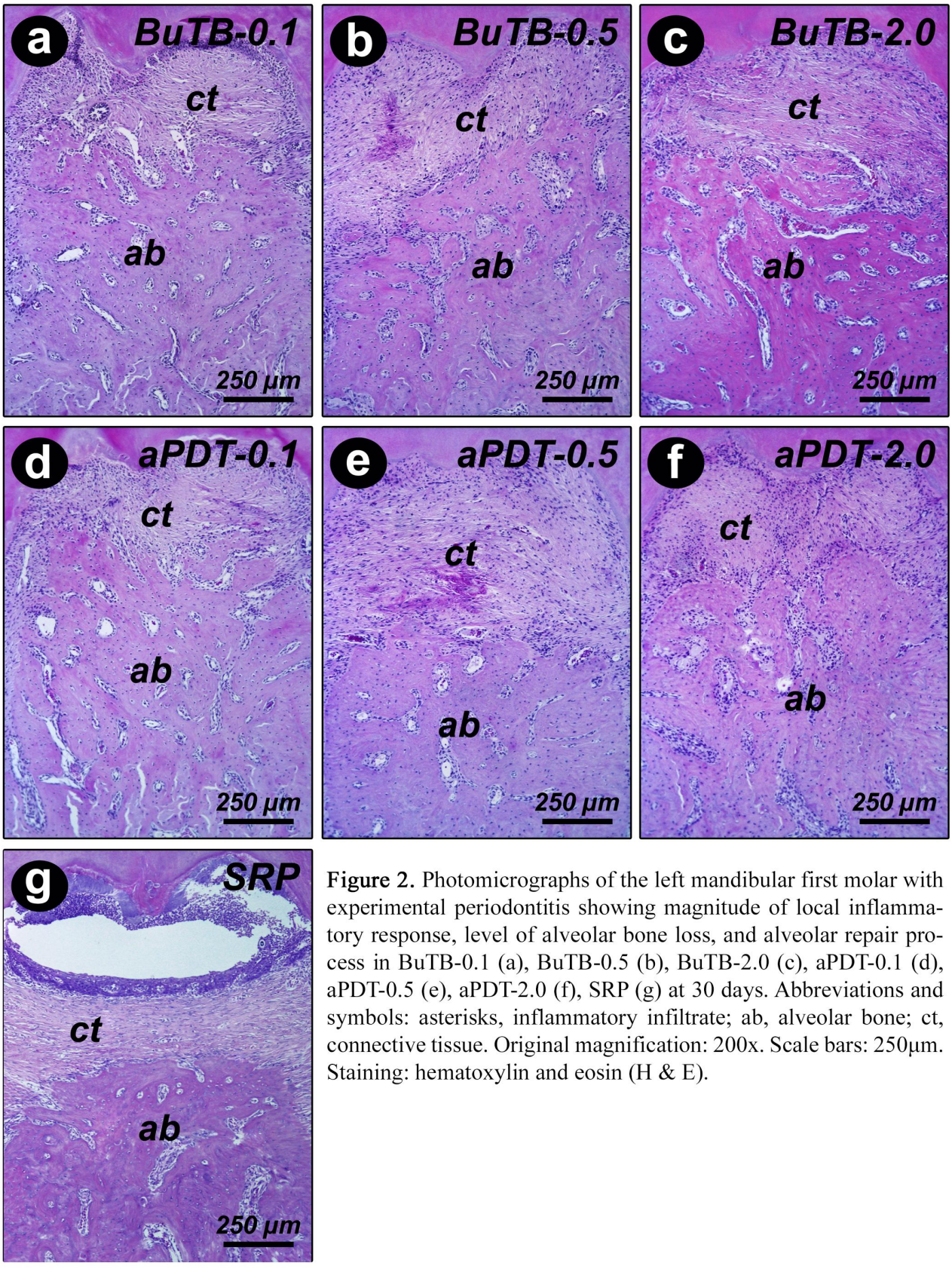

Figure 2. Photomicrographs of the left mandibular first molar with experimental periodontitis showing magnitude of local inflammatory response, level of alveolar bone loss, and alveolar repair process in BuTB-0.1 (a), BuTB-0.5 (b), BuTB-2.0 (c), aPDT-0.1 (d), aPDT-0.5 (e), aPDT-2.0 (f), SRP (g) at 30 days. Abbreviations and symbols: asterisks, inflammatory infiltrate; ab, alveolar bone; ct, connective tissue. Original magnification: 200x. Scale bars: 250µm. Staining: hematoxylin and eosin (H & E).

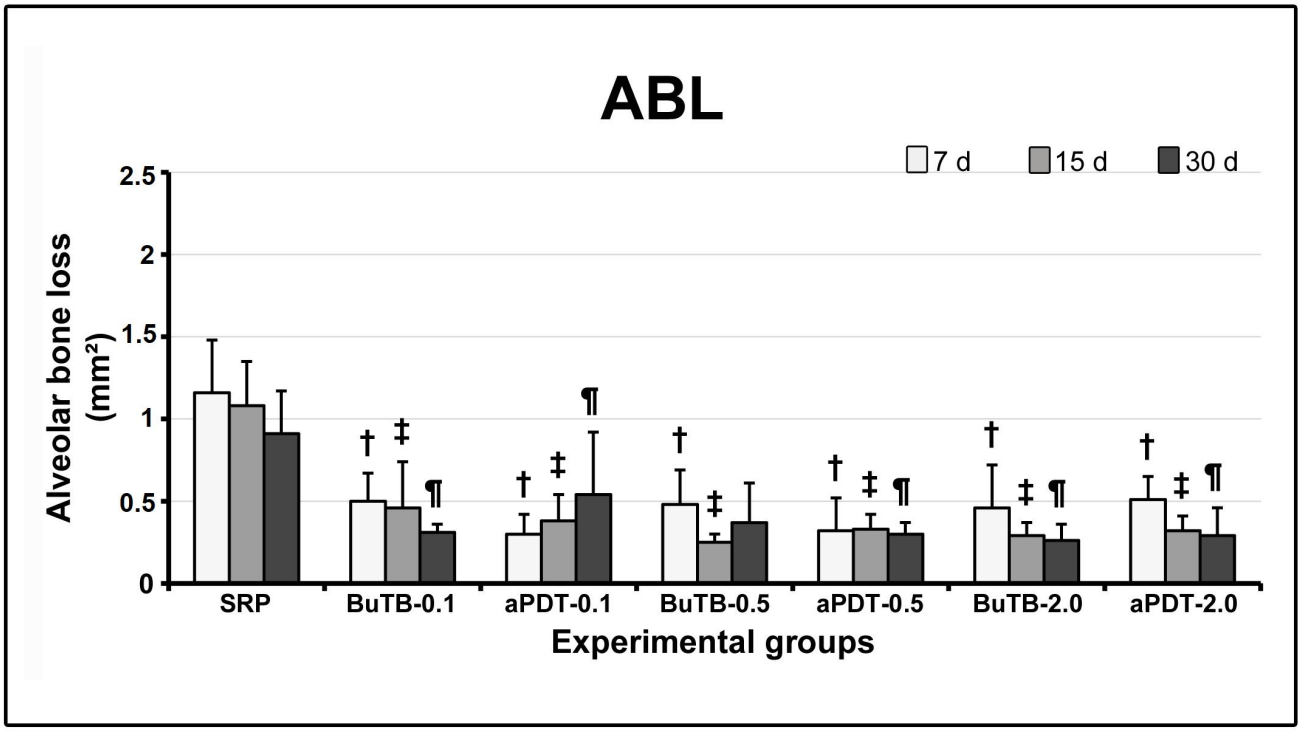

Figure 3. Mean and standard deviation of the area of alveolar bone loss (mm²) in the furcation region of the left mandibular first molar, in the different experimental groups and evaluation periods. Abbreviations and symbols: ABL, alveolar bone loss; †, Statistically significant difference in relation to the SPR group at 7 days; ‡, Statistically significant difference in relation to the SPR group at 15 days; ¶, Statistically significant difference in relation to the SPR group at 30 days.

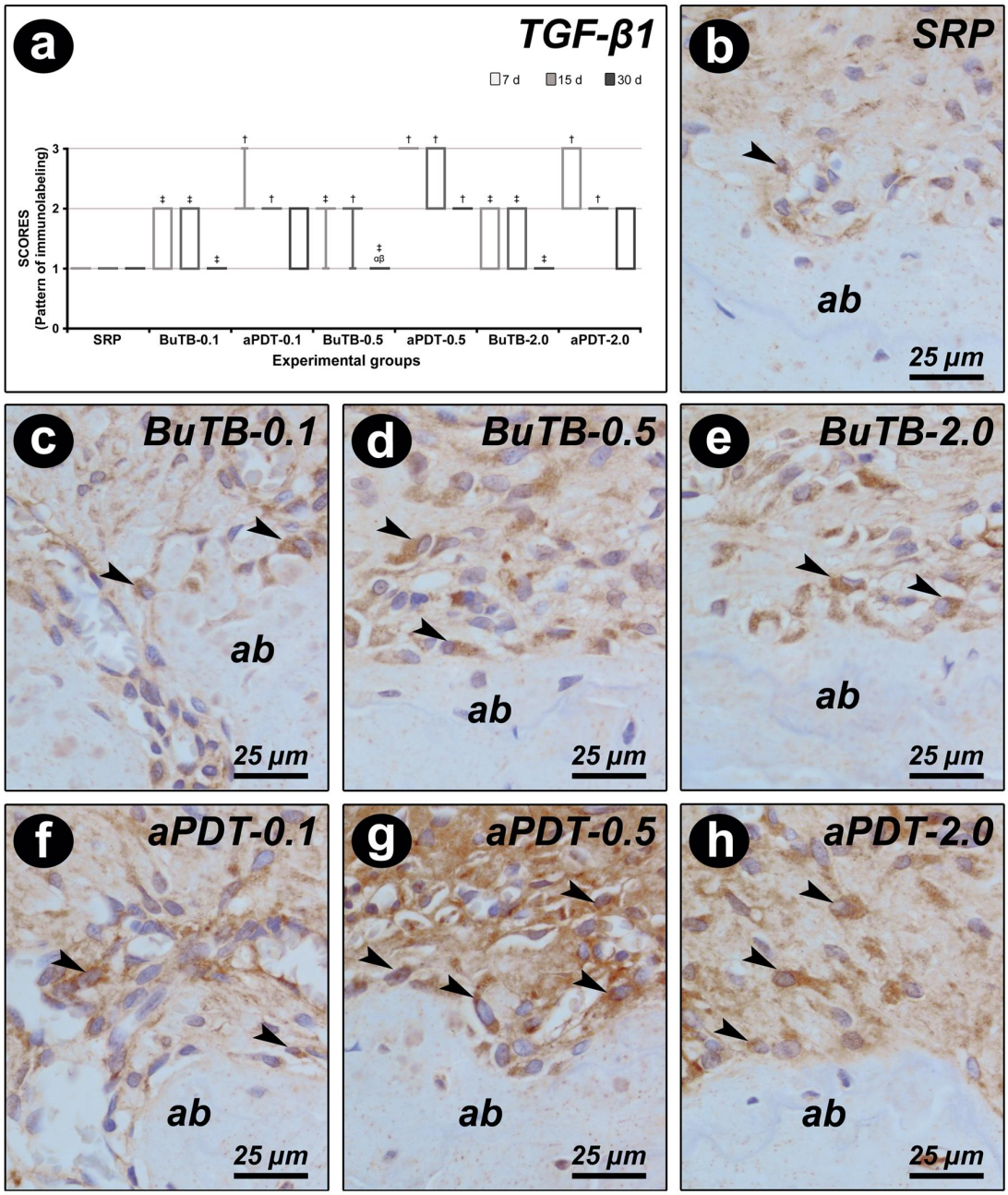

Figure 4. Immunolabeling pattern for TGF-β1 in the furcation region of the left mandibular first molar. (a) Median and interquartile deviation of the scores attributed to the immunolabeling pattern for TGF-β1. (b-h) Photomicrographs showing immunolabeling pattern for TGF-β1 in SRP (b), BuTB-0.1 (c), 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, 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.

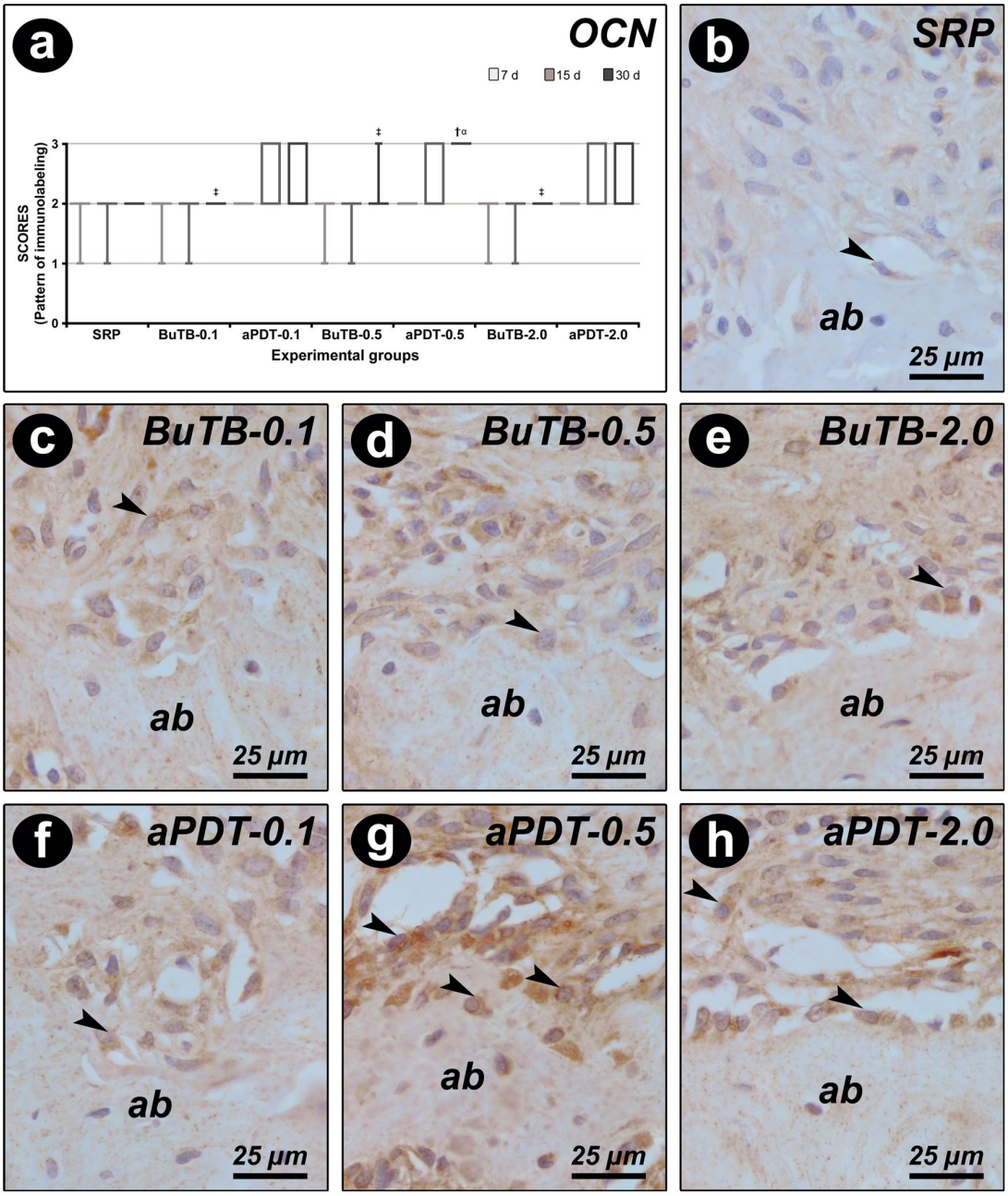

Figure 5. Immunolabeling pattern for OCN in the furcation region of the left mandibular first molar. (a) Median and interquartile deviation of the scores attributed to the immunolabeling pattern for OCN. (b-h) Photomicrographs showing immunolabeling pattern for OCN in SRP (b), BuTB-0.1 (c), BuTB-0.5 (d), BuTB-2.0 (e), aPDT-0.1 (f), aPDT-0.5 (g), aPDT-2.0 (h), at 30 days. Abbreviations and symbols: arrows, 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.

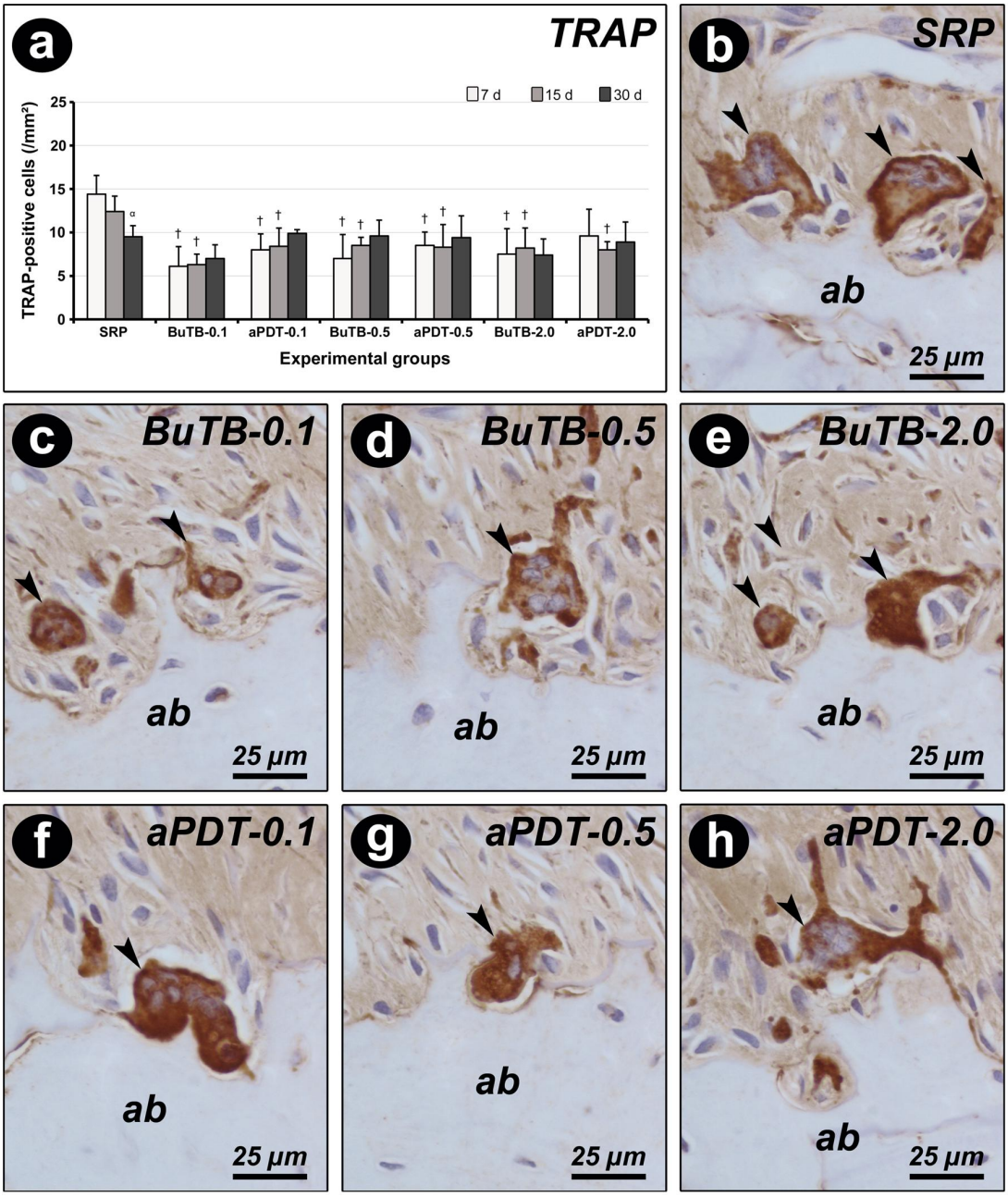

Figure 6. Immunolabeling pattern for TRAP in the furcation region of the left mandibular first molar. (a) Mean and standard deviation of the number of TRAP-positive cells per mm² according to treatments and time point. (b-h) Photomicrographs showing immunolabeling pattern for TRAP in SRP (b), BuTB-0.1 (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, 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.

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%																				

Anexo A – Certificado da Comissão de Ética no Uso de Animais (CEUA)

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Relatório Final do trabalho intitulado **"Efeito de um novo fotossensibilizador na PDT para o tratamento da periodontite experimental em ratos"**, Processo FOA nº 2015-00586, sob responsabilidade de Letícia Helena Theodoro e colaboração de Valdir Gouveia Garcia, Edilson Ervolino, Mark Wainwright e Marta Aparecida Alberton Nuernberg foi aprovado pela CEUA em 10 de Maio de 2019.

CERTIFICATE

We certify that the study entitled **"Effect of new photosensitizer on PDT for treatment of experimental periodontitis in rats"**, Process FOA nº 2015-00586, under the supervision of Letícia Helena Theodoro and collaboration of Valdir Gouveia Garcia, Edilson Ervolino, Mark Wainwright and Marta Aparecida Alberton Nuernberg had its the Final Report approved by the CEUA on May 10, 2019.

Prof. Dr. Leonardo Perez Faverani
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
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Anexo B – Normas para publicação segundo o Periódico “Journal of Clinical Periodontology”

Author Guidelines

Sections

1. SUBMISSION

Authors should kindly note that submission implies that the content has not been published or submitted for publication elsewhere except as a brief abstract in the proceedings of a scientific meeting or symposium.

Once the submission materials have been prepared in accordance with the Author Guidelines, manuscripts should be submitted online at https://mc.manuscriptcentral.com/jcpe

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Preprint policy
This journal will consider for review articles previously available as preprints on non-commercial servers such as ArXiv, bioRxiv, psyArXiv, SocArXiv, engrXiv, etc. Authors may also post the submitted version of a manuscript to non-commercial servers at any time. Authors are requested to update any pre-publication versions with a link to the final published article.

For help with submissions, please contact: cpeedoffice@wiley.com

2. AIMS AND SCOPE

The aim of the Journal of Clinical Periodontology is to provide the platform for exchange of scientific and clinical progress in the field of Periodontology and allied disciplines, and to do so at the highest possible level. The Journal also aims to facilitate the application of new scientific knowledge to the daily practice of the concerned disciplines and addresses both practicing clinicians and academics. The Journal is the official publication of the European Federation of Periodontology but wishes to retain its international scope.

Journal of Clinical Periodontology publishes original contributions of high scientific merit in the fields of periodontology and implant dentistry. Its scope encompasses the physiology and pathology of the periodontium, the tissue integration of dental implants, the biology and the modulation of periodontal and alveolar bone healing and regeneration, diagnosis, epidemiology, prevention and therapy of periodontal disease, the clinical aspects of tooth replacement with dental implants, and the comprehensive rehabilitation of the periodontal patient. Review articles by experts on new developments in basic and applied periodontal science and associated dental disciplines, advances in periodontal or implant techniques and procedures, and case reports which illustrate important new information are also welcome.

3. MANUSCRIPT CATEGORIES AND REQUIREMENTS

Journal of Clinical Periodontology publishes original research articles, reviews, clinical innovation reports and case reports. The latter will be published only if they provide new fundamental knowledge and if they use language understandable to the clinician. It is expected that any manuscript submitted represents unpublished original research.

i. Original Research Articles

Original Research articles must describe significant and original experimental observations and provide sufficient detail so that the observations can be critically evaluated and, if necessary, repeated. Original articles will be published under the heading of clinical periodontology, implant dentistry or pre-clinical sciences and must conform to the highest international standards in the field.

Word limit: 3,500 words maximum, excluding references.
Abstract: 200 words maximum; must be structured, under the sub-headings: Aim(s), Materials and methods, Results, Conclusion(s).
Figures/Tables: Total of no more than 7 figures and tables.

Introduction: should be focused, outlining the historical or logical origins of the study and not summarize the results; exhaustive literature reviews are not appropriate. It should close with the explicit statement of the specific aims of the investigation.

Material and Methods: must contain sufficient detail such that, in combination with the references cited, all clinical trials and experiments reported can be fully reproduced. As a condition of publication, authors are required to make materials and methods used freely available to academic researchers for their own use. This includes antibodies and the constructs used to make transgenic animals, although not the animals themselves.

Results: should present the observations with minimal reference to earlier literature or to possible interpretations.

Discussion: may usefully start with a brief summary of the major findings, but repetition of parts of the abstract or of the results section should be avoided. The discussion section should end with a brief conclusion and a comment on the potential clinical relevance of the findings. Statements and interpretation of the data should be appropriately supported by original references.

The discussion may usefully be structured with the following points in mind (modified from the proposal by Richard Horton (2002), The Hidden Research Paper, The Journal of the American Medical Association, 287, 2775-2778). Not all points will apply to all studies and its use is optional, but we believe it will improve the discussion section to keep these points in mind.

Summary of key finding
Primary outcome measure(s)
Secondary outcome measure(s)
Results as they relate to a prior hypothesis
Strengths and Limitations of the Study

Study Question
Study Design
Data Collection
Analysis
Interpretation
Possible effects of bias on outcomes
Interpretation and Implications in the Context of the Totality of Evidence

Is there a systematic review to refer to?
If not, could one be reasonably done here and now?
What this study adds to the available evidence
Effects on patient care and health policy
Possible mechanisms
Controversies Raised by This Study Future Research Directions

For this particular research collaboration
Underlying mechanisms
Clinical research

ii. Clinical Innovation Reports
Clinical Innovation Reports are suited to describe significant improvements in clinical practice such as the report of a novel surgical technique, a breakthrough in technology or practical approaches to recognized clinical challenges. They should conform to the highest scientific and clinical practice standards.

Word limit: 3,000 words maximum, excluding references.
Main text: should be organized with Introduction; Clinical Innovation Report; Discussion and Conclusion.
Figures/Tables: Total of no more than 12 figures and tables.

iii. Case Reports

Case Reports illustrating unusual and clinically relevant observations are acceptable, but their merit needs to provide high priority for publication in the Journal. On rare occasions, completed cases displaying non-obvious solutions to significant clinical challenges will be considered.

Main text: should be organised with Introduction; Case report; Discussion and Conclusion.

iv. Reviews
Reviews are selected for their broad general interest; all are refereed by experts in the field who are asked to comment on issues such as timeliness, general interest and balanced treatment of controversies, as well as on scientific accuracy. Reviews should take a broad view of the field rather than merely summarizing the authors´ own previous work, so extensive citation of the authors´ own publications is discouraged. The use of state-of-the-art evidence-based systematic approaches is expected. Reviews are frequently commissioned by the editors and, as such, authors are encouraged to submit a proposal to the Journal. Review proposals should include a full-page summary of the proposed contents with key references.

Word limit: 4,000 words maximum, excluding references.
Main text: should be organized with Introduction; Review of current literature; Discussion and Conclusion.

Revisions and Resubmissions
Please note that all revisions and resubmissions of papers should also include a separate rebuttal and a tracked changes document to assist in peer review.

4. PREPARING THE SUBMISSION

Cover Letters
Cover letters are not mandatory; however, they may be supplied at the author’s discretion.

Parts of the Manuscript
The manuscript should be submitted in separate files: main text file; figures.

Main Text File
The text file should be presented in the following order:

i. A short informative title containing the major key words. The title should not contain abbreviations (see Wiley's best practice SEO tips);
ii. A short running title of less than 40 characters;
iii. The full names of the authors;
iv. The author's institutional affiliations where the work was conducted, with a footnote for the author’s present address if different from where the work was conducted;
v. Acknowledgments;
vi. Abstract and keywords;
vii. Clinical Relevance
viii. Main text;
ix. References;
x. Tables (each table complete with title and footnotes);
xi. Figure legends;

xiii. Appendices (if relevant).

Figures and supporting information should be supplied as separate files.

Authorship
Please refer to the journal's authorship policy the Editorial Policies and Ethical Considerations section for details on eligibility for author listing.

Acknowledgments
Contributions from anyone who does not meet the criteria for authorship should be listed, with permission from the contributor, in an Acknowledgments section. Financial and material support should also be mentioned. Thanks to anonymous reviewers are not appropriate.

Conflict of Interest Statement
Authors will be asked to provide a conflict of interest statement during the submission process. For details on what to include in this section, see the section 'Conflict of Interest' in the Editorial Policies and Ethical Considerations section below. Submitting authors should ensure they liaise with all co-authors to confirm agreement with the final statement.

Abstract
The abstract is limited to 200 words in length and should not contain abbreviations or references. The abstract should be organized according to the content of the paper.

For Original Research Articles the abstract should be organized with aim, materials and methods, results and conclusions.

For clinical trials, it is encouraged that the abstract finish with the clinical trial registration number on a free public database such as clinicaltrials.gov.

Keywords
Please provide 1-5 keywords. When appropriate keywords are available, they should be taken from those recommended by the US National Library of Medicine's Medical Subject Headings (MeSH) browser list at www.nlm.nih.gov/mesh. Authors may add specific keywords.

Main Text
All manuscripts should emphasize clarity and brevity. Authors should pay special attention to the presentation of their findings so that they may be communicated clearly. Technical jargon should be avoided as much as possible and be clearly explained where its use is unavoidable.
Clinical Relevance
This section is aimed at giving clinicians a reading light to put the present research in perspective. It should be no more than 100 words and should not be a repetition of the abstract. It should provide a clear and concise explanation of the rationale for the study, of what was known before and of how the present results advance knowledge of this field. If appropriate, it may also contain suggestions for clinical practice.

It should be structured with the following headings: Scientific rationale for study; Principal findings; Practical implications.

Authors should pay particular attention to this text as it will be published in a highlighted box within their manuscript; ideally, reading this section should leave clinicians wishing to learn more about the topic and encourage them to read the full article.

References
It is the policy of the Journal to encourage reference to the original papers rather than to literature reviews. Authors should therefore keep citations of reviews to the absolute minimum.

References should be prepared according to the Publication Manual of the American Psychological Association (6th edition). This means in text citations should follow the author-date method whereby the author's last name and the year of publication for the source should appear in the text, for example, (Jones, 1998). The complete reference list should appear alphabetically by name at the end of the paper.

A sample of the most common entries in reference lists appears below. Please note that a DOI should be provided for all references where available. For more information about APA referencing style, please refer to the APA FAQ. Please note that for journal articles, issue numbers are not included unless each issue in the volume begins with page one.

Journal article
Beers, S. R. , & De Bellis, M. D. (2002). Neuropsychological function in children with maltreatment-related posttraumatic stress disorder. The American Journal of Psychiatry, 159, 483–486. doi:10.1176/appi.ajp.159.3.483

Book

Bradley-Johnson, S. (1994). Psychoeducational assessment of students who are visually impaired or blind: Infancy through high school (2nd ed.). Austin, TX: Pro-ed.

Chapter in an Edited Book

Borstrøm, I., & Elbro, C. (1997). Prevention of dyslexia in kindergarten: Effects of phoneme awareness training with children of dyslexic parents. In C. Hulme & M. Snowling (Eds.), Dyslexia: Biology, cognition and intervention (pp. 235–253). London: Whurr.

Internet Document
Norton, R. (2006, November 4). How to train a cat to operate a light switch [Video file]. Retrieved from http://www.youtube.com/watch?v=Vja83KLQXZs

Please note that all unpublished papers (submitted or in press) included in the reference list should be provided in a digital version at submission. The unpublished paper should be uploaded as a supplementary file for review.

Tables
Tables should be self-contained and complement, not duplicate, information contained in the text. They should be supplied as editable files, not pasted as images. Legends should be concise but comprehensive – the table, legend, and footnotes must be understandable without reference to the text. All abbreviations must be defined in footnotes. Footnote symbols: †, ‡,

§, ¶, should be used (in that order) and *, **, *** should be reserved for P-values. Statistical measures such as SD or SEM should be identified in the headings.

Figure Legends
Legends should be concise but comprehensive – the figure and its legend must be understandable without reference to the text. Include definitions of any symbols used and define/explain all abbreviations and units of measurement.

Figures
Although authors are encouraged to send the highest-quality figures possible, for peer-review purposes, a wide variety of formats, sizes, and resolutions are accepted.

Click here for the basic figure requirements for figures submitted with manuscripts for initial peer review, as well as the more detailed post-acceptance figure requirements.

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When citing or making claims based on data, authors must refer to the data at the relevant place in the manuscript text and in addition provide a formal citation in the reference list. We recommend the format proposed by the Joint Declaration of Data Citation Principles:

[dataset] Authors; Year; Dataset title; Data repository or archive; Version (if any); Persistent identifier (e.g. DOI)

Additional Files
Appendices
Appendices will be published after the references. For submission they should be supplied as separate files but referred to in the text.

Supporting Information

Supporting information is information that is not essential to the article, but provides greater depth and background. It is hosted online and appears without editing or typesetting. It may include tables, figures, videos, datasets, etc.

Click here for Wiley's FAQs on supporting information.
Note: if data, scripts, or other artefacts used to generate the analyses presented in the paper are available via a publicly available data repository, authors should include a reference to the location of the material within their paper.

General Style Points
The following points provide general advice on formatting and style.

Abbreviations, Symbols and Nomenclature: Journal of Clinical Periodontology adheres to the conventions outlined in Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors. Abbreviations should be kept to a minimum, particularly those that are not standard. Non-standard abbreviations must be used three or more times and written out completely in the text when first used.

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Author Guidelines Updated 15 January 2019

Anexo C - Lista de verificação de diretrizes para experimentação animal “ARRIVE Guidelines”

ARRIVE

The ARRIVE Guidelines Checklist

Animal Research: Reporting In Vivo Experiments

Carol Kilkenny[1], William J Browne[2], Innes C Cuthill[3], Michael Emerson[4] and Douglas G Altman[5]

[1]The National Centre for the Replacement, Refinement and Reduction of Animals in Research, London, UK, [2]School of Veterinary Science, University of Bristol, Bristol, UK, [3]School of Biological Sciences, University of Bristol, Bristol, UK, [4]National Heart and Lung Institute, Imperial College London, UK, [5]Centre for Statistics in Medicine, University of Oxford, Oxford, UK.

	ITEM	RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	31
INTRODUCTION			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	33
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	35
METHODS			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	36
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	36
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	36
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	35-36

The ARRIVE guidelines. Originally published in *PLoS Biology*, June 2010[1]

Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	35-36
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	36
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	36
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	38-40
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	41
RESULTS			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	41
Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%[2]). b. If any animals or data were not included in the analysis, explain why.	41
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	41-43
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	41
DISCUSSION			
Interpretation/ scientific implications	18	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results[2]. c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.	43-47
Generalisability/ translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	47
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	48

References:
1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
2. Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.