PLOS ONE

RESEARCH ARTICLE

Evaluation of antimicrobial photodynamic therapy with acidic methylene blue for the treatment of experimental periodontitis

Juliano Milanezi de Almeida[1,2]*, Henrique Rinaldi Matheus[1,2], Breno Edson Sendão Alves[1,2], David Jonathan Rodrigues Gusman[1,2], Maria José Hitomi Nagata[1,2], Elisa Mara de Abreu Furquim[1,2], Edilson Ervolino[2,3]

1 Periodontics Division, Department of Diagnosis and Surgery, School of Dentistry, São Paulo State University (Unesp), Araçatuba, Brazil, **2** School of Dentistry, Nucleus of Study and Research in Periodontics and Implantology (NEPPI), São Paulo State University (Unesp), Araçatuba, SP, Brazil, **3** Department of Basic Science, School of Dentistry, São Paulo State University (Unesp), Araçatuba, Brazil

* jumilanezi@hotmail.com

OPEN ACCESS

Citation: de Almeida JM, Matheus HR, Sendão Alves BE, Rodrigues Gusman DJ, Nagata MJH, de Abreu Furquim EM, et al. (2022) Evaluation of antimicrobial photodynamic therapy with acidic methylene blue for the treatment of experimental periodontitis. PLoS ONE 17(2): e0263103. https://doi.org/10.1371/journal.pone.0263103

Editor: Ewa Tomaszewska, University of Life Sciences in Lublin, POLAND

Received: January 27, 2021

Accepted: January 13, 2022

Published: February 10, 2022

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: The author(s) received no specific funding for this work.

Competing interests: The author Juliano Milanezi de Almeida declares that has no conflict of interests related to this study. The author Henrique Rinaldi Matheus declares that has no conflict of interests related to this study. The author Breno Edson

Abstract

Objective

To investigate the security and effectiveness of antimicrobial photodynamic therapy (aPDT) with a citric acid-based methylene blue (MB) on the periodontal repair following the treatment of ligature-induced experimental periodontitis (EP) in rats.

Material and methods

Were used 120 male rats, randomly divided into 4 experimental groups (n = 30): no treatment (NT), SRP alone (SRP), SRP plus aPDT using conventional MB pH 7.0 (aPDT-pH7), SRP plus aPDT using acidic MB pH 1.0 (aPDT-pH1). EP was induced at day 0 by the placement of a ligature around the mandibular left first molars. Ten animals per group/period were euthanized at 14, 22 and 37 days. Histopathological, histometric (percentage of bone in the furcation [PBF]) and immunohistochemical (for tartrate-resistant acid phosphatase [TRAP] and osteocalcin [OCN]) analyses were performed. Data were statistically analyzed.

Results

aPDT-pH1 showed the highest PBF as compared with the other treatments. Collectively, tissues' reaction to both dyes were controlled and healthy for the periodontium. Both aPDT protocols reduced the extent and intensity of the local inflammatory response, reduced the alveolar bone resorption, and promoted a better structural arrangement of the connective tissue as compared with SRP. TRAP expression was downregulated while OCN expression was upregulated by aPDT as compared with SRP alone.

Sendão Alves declares that has no conflict of interests related to this study. The author David Jonathan Rodrigues Gusman declares that has no conflict of interests related to this study. The author Elisa Mara de Abreu Furquim declares that has no conflict of interests related to this study. The author Maria José Hitomi Nagata declares that has no conflict of interests related to this study. The author Edilson Ervolino declares that has no conflict of interests related to this study. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

Conclusion

Our data implicate that the novel MB pH 1.0 is as safe as the conventional MB for use in aPDT and raises its additional benefit of increasing the amount of alveolar bone in the furcation.

Introduction

Periodontitis is a highly prevalent oral disease in the United States, estimated to affect over 42% of dentate US adults 30 years or older [1], and the primary cause for tooth loss in the world [2]. Periodontal breakdown is the consequence of the combination of complex illnesses, interactive amongst inflammatory and immunologic systems of a host, subgingival microbiota, and environmental factors [3]. Even disorders within this microbiota being capable to result in dysbiosis, a specific microenvironment and susceptibility are essential to trigger the disease [4]. Hence, periodontal therapy will be the most effective when capable to encompass the many variables as possible.

The use of antimicrobial photodynamic therapy (aPDT) has been increasingly investigated as an adjunctive approach to non-surgical scaling and root planing (SRP) [5] for the treatment of periodontitis in sites with impaired access, such as deep periodontal pockets and furcation areas [6, 7]. The impact of photobiomodulation therapy ranges from biomolecules (e.g. nucleic acids, amino acids, carbohydrates) to entire organisms [8]. In aPDT, the reactive oxygen species released from the interaction between light at a specific wavelength and photosensitizer (PS) cause irreversible damage to the cytoplasmic membrane and DNA of microorganisms [9, 10].

Despite the benefits of aPDT [8], the positive effects found in animal experiments do not represent potential clinical relevance in human studies [11]. The complexity of periodontitis, limited reachability to biofilms, and the focus of aPDT in one variable (i.e. microbiota) lead to this scenario, and, hence, the proposition of improvements in aPDT may increase the clinical relevance of the therapy. Therefore, aiming to improve the outcomes obtained with aPDT, the literature reports alterations to its initial protocol, such as multiple aPDT sessions [12], distinct light sources [13, 14] and PS [15], and incorporation of nanoparticles [16].

The rate of growth, cell wall composition, and presence of polysaccharide intercellular adhesin (PIA) differ between cells growing in biofilms and their planktonic forms, which may block the penetration of the photosensitizer and light to the deeper layers and thereby reduce the photosensitizing process [17]. This way, alterations in PS in order to target this topic may enhance its antimicrobial properties. Ruggeri Jr et al. [18] supported the hypothesis that manual or ultrasonic instrumentation alone is not able to expose the sound dentin matrix, whereas a subsequent citric acid conditioning exposes collagen fibrils and associated proteoglycans, indicating deeper penetration into mineralized tissues. Also, in addition to the reduced oral biofilm formation achieved with citric acid etching [19], root conditioning with citric acid promotes the adhesion of the fibrin network onto the dentin surface, which may further enhance connective tissue attachment to the root surface [20].

Animal experimentation is known to be the first step for *in vivo* validation [21]. In periodontics, rodent models of experimental periodontitis allow not only the understanding of the pathogenesis [22] but also the validation of hypotheses related to therapies for periodontal treatment [7].

Substantiated by the favorable properties of citric acid on mineralized tissues and reduction of oral microorganisms formation (both related with periodontal treatment and repair), and

based on the increased biocompatibility of methylene blue (MB) acidulated with citric acid pH 1.0 over conventional MB (pH 7.0) [23], this *in vivo* experiment aimed to assess the hypothesis that a novel acidic MB could provide additional benefits over conventional MB, used in aPDT as adjunctive therapy to SRP for the treatment of experimental periodontitis (EP).

Materials and methods

Animals

The study followed a randomized, single-blind, controlled design, and was conducted in accordance with the ARRIVE Guidelines: Animal Research: Reporting In Vivo Experiments [23]. The protocols were approved by the Ethics Committee on Animal Use under protocol the 310–2016 of São Paulo State University, UNESP, School of Dentistry, Araçatuba. Previous experience of our research group [7] determined n = 10 enough to reject the null hypothesis in the histopathological, histometric, and immunohistochemical analyses. One hundred twenty healthy 3-month-old male rats (*Rattus norvegicus*, *albinus*; Wistar) weighing 250–300 g were kept in plastic boxes with wood shavings, under 12 hr/12 hr light/dark cycles, 22 ± 2˚C ambient temperature, 20 air changes per hour, 55 ± 5% humidity, receiving feed and water ad libitum, and monitored daily. A blinded staff external to the study used the Minitab® 17 software (Minitab Inc., State College, PA, USA) to perform simple randomization (1:1 allocation ratio) of the animals to one of the four experimental groups: no treatment (NT), SRP, aPDT-pH7 and aPDT-pH1 (Fig 1). The euthanasia were performed at 14, 22, or 37 days after EP induction in groups SRP, aPDT-pH7, and aPDT-pH1, with an overdose (150 mg/kg) of sodium thiopental (Cristália Ltda., Itapira, SP, Brazil), consisting of ten animals per period.

- Group NT (n = 30): EP induction and no treatment performed (ligatures remained around the mandibular left first molars during the entire experiment);
- Group SRP (n = 30): EP induction and treatment with SRP and subgingival irrigation with physiological saline solution (PSS), 7 days after EP induction;
- Group aPDT-pH7 (n = 30): EP induction and treatment with SRP plus aPDT using conventional MB pH 7.0 (100μg/ml)), 7 days after EP induction;
- Group aPDT-pH1 (n = 30) EP induction and treatment with SRP plus aPDT using a novel citric acid-based MB pH 1.0 (100μg/ml), 7 days after EP induction.

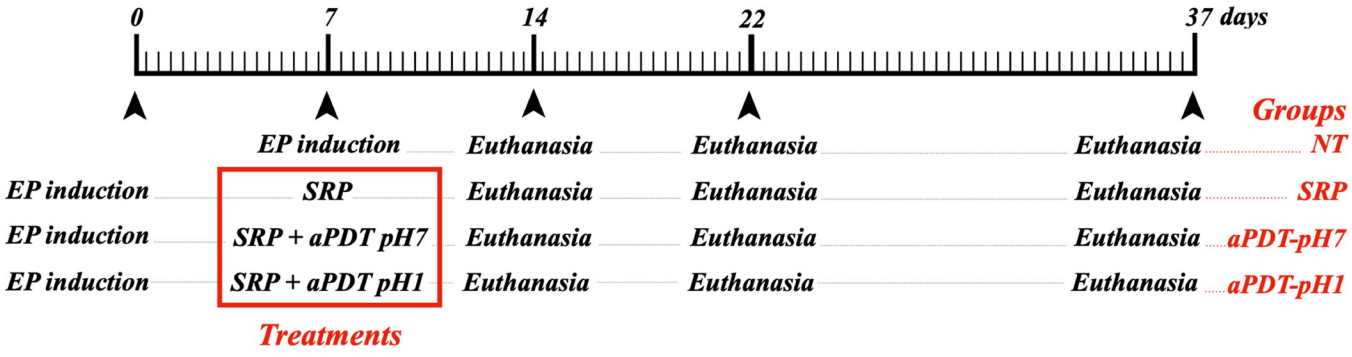

Fig 1. Scheme illustrating the experimental procedures performed during the study for each group.

https://doi.org/10.1371/journal.pone.0263103.g001

Anaesthesia

For all experimental procedures, the animals were anesthetized via intramuscular injection with xylazine hydrochloride (6 mg/kg of body weight) and ketamine hydrochloride (70 mg/kg of body weight).

Experimental periodontitis induction

At day 0, a #24 cotton thread (Cotton Chain N˚. 24, Coats Corrente, São Paulo, SP, Brazil) was placed around the mandibular left first molars to induce EP [7, 24] in groups SRP, aPDT-pH7, and aPDT-pH1. In group NT, EP was induced at day 7 by using the same method.

Preparation of the dyes

The conventional PS was prepared (MB 100 μg/ml) in distilled water at native (not adjusted) pH 7.0. Initially, for the novel PS, was prepared an unsaturated solution of citric acid adjusted to pH 1.0. After preparation, this solution was used as a solvent for the MB in order to achieve a concentration of 100 μg/ml. At the moment of the experiment, the pH of the dye was rechecked and kept stable.

Treatments

Were performed by the same calibrated operator [JMA], masked to the experimental groups, 7 days after EP induction. SRP was conducted in accordance with the protocol described by Almeida et al. (2008) [7]. In groups treated with aPDT, either the MB pH 7.0 or pH 1.0 were carefully inserted and kept within the periodontal pocket for 60 s and then irradiated with a red (AlGaInP) diode laser (TheraLase—DMC, Sao Carlos, SP, Brazil) in one point at the buccal and one point at the lingual surfaces of the mandibular left first molars. Laser parameters for each point were 660 nm, 35mW, 74.2 J/cm^2, 2.10 J, spot 0.0283 cm^2, contact, continuous mode.

Tissue processing

The left hemimandibles were fixed in buffered 4% formaldehyde for 48 hr and demineralized in 10% ethylenediaminetetraacetic acid (EDTA) solution. Then, were submitted to conventional histological processing and paraffin embedding. Semi-serial 4 μm thick sections were obtained from buccal to lingual progression. Five equidistant sections from each specimen were stained with haematoxylin and eosin (H&E) for histopathological and histometric analyses [7]. Four additional sections from each specimen were subjected to the indirect immunoperoxidase method, using the following primary anti-bodies: goat anti-osteocalcin (OCN) (Santa Cruz Biotechnology®) and goat anti-tartrate-acid phosphatase (TRAP) (Santa Cruz Biotechnology®). The indirect immunoperoxidase technique was conducted in accordance with Matheus et al. (2018) [25].

Microscopy analysis procedure

Analyses were performed by a calibrated and masked staff using image analysis software (AxioVision 4.8.2; Carl Zeiss MicroImaging GmbH, Jena, TH, Germany). Histopathological analysis was conducted by a certified histologist [EE] based on the criteria described by Gusman et al. (2019) [26]. Each parameter was scored from 0 to 3 and is shown in Table 1. For the histometric analysis, the entire furcation (FA) was delineated and considered 100% of the area to be analyzed. The entire bone area (BA) was delineated within the limits of FA. The ratio of BA to FA was calculated and the results were expressed as the percentage of bone in the

Table 1. Scores and specimens' distribution according to the parameters of the histologic analysis in groups NT, SRP, aPDT-pH7 and aPDT-pH1, with intragroup and intergroup comparisons.

HISTOLOGICAL ANALYSIS												
SCORES FOR EACH PARAMETER	% ANIMALS PER SCORE											
	EXPERIMENTAL GROUPS/TIMES											
	NT			SRP			aPDT-pH7			aPDT-pH1		
	14d	22d	37d	14d	22d	37d	14d	22d	37d	14d	22d	37d
INTENSITY OF LOCAL INFLAMMATORY RESPONSE												
absence of inflammation	-	-	-	-	-	-	-	-	80	-	-	60
small amount of inflammatory cells	-	-	-	-	40	100	-	100	20	-	100	40
moderate amount of inflammatory cells	-	40	40	20	60	-	100	-	-	100	-	-
large amount of inflammatory cells	100	60	60	80	-	-	-	-	-	-	-	-
MEDIAN	**3**	**3**	**3**	**3†**	**2*‡**	**1*‡**	**2†‡§**	**1*‡§**	**0*‡§**	**2†‡§**	**1*‡§**	**0*‡§**
EXTENSION OF THE INFLAMMATORY PROCESS												
0) absence of inflammation	-	-	-	-	-	-	-	-	80	-	-	60
1) extending to part of the connective tissue	-	-	-	-	40	60	100	100	20	100	100	40
2) extending to the whole connective tissue	60	60	80	100	60	40	-	-	-	-	-	-
3) extending to the whole connective tissue and to the alveolar bone	40	40	20	-	-	-	-	-	-	-	-	-
MEDIAN	**2**	**2**	**2**	**2**	**2*‡**	**1‡**	**1‡§**	**1*‡§**	**0*‡§**	**1‡§**	**1*‡§**	**1*‡§**
EXTERNAL ROOT RESORPTION (CEMENTUM AND DENTIN)												
0) absence of external root resorption	-	-	-	40	20	-	40	-	-	40	-	-
1) only inactive resorption areas	-	-	20	-	20	40	-	20	60	-	40	40
2) few active resorption areas	60	60	60	60	60	60	60	80	40	60	60	60
3) many active resorption areas	40	40	20	-	-	-	-	-	-	-	-	-
MEDIAN	**2**	**2**	**2**	**2‡**	**2‡**	**2**	**2‡**	**2‡**	**1**	**2‡**	**2‡**	**2**
ALVEOLAR BONE RESORPTION												
0) within normality patterns	-	-	-	-	-	-	-	20	20	-	-	-
1) small amount of alveolar bone resorption	-	-	-	-	20	40	20	40	60	40	40	80
2) moderate amount of alveolar bone resorption	20	100	100	60	60	60	80	40	20	60	60	20
3) large amount of alveolar bone resorption	80	-	-	40	20	-	-	-	-	-	-	-
MEDIAN	**3**	**2**	**2**	**2**	**2***	**2**	**2‡§**	**1*‡§**	**1‡**	**2‡§**	**2**	**1‡**
PATTERN OF THE CONNECTIVE TISSUE STRUCTURE												
0) moderate amount of fibroblasts and large amount of collagen fibers (dense connective tissue)	-	-	-	-	-	-	-	80	80	-	40	60
1) moderate amount of fibroblasts and collagen fibers	-	-	-	-	60	60	-	20	20	-	60	40
2) small amount of fibroblasts and collagen fibers	80	80	80	100	40	40	100	-	-	100	-	-
3) severe tissue breakdown and areas with necrosis	20	20	20									
MEDIAN	**2**	**2**	**2**	**2**	**1*‡**	**1*‡**	**2**	**0*‡§**	**0*‡§**	**2**	**1*‡§**	**0*‡§**
PATTERN OF THE ALVEOLAR BONE STRUCTURE												
0) bone trabeculae with regular contour, surrounded by many active osteoblasts, including areas of new bone formation	-	-	-	-	-	-	-	-	20	-	20	40
1) bone trabeculae with irregular contour, surrounded by many active osteoblasts and osteoclasts	20	20	20	20	100	100	20	100	80	20	80	60
2) bone trabeculae with irregular contour, surrounded by active osteoclasts	80	80	80	80	-	-	80	-	-	80	-	-
3) areas of necrotic bone and bone trabeculae with irregular contour, surrounded by many active osteoclasts	-	-	-	-	-	-	-	-	-	-	-	-
MEDIAN	**2**	**2**	**2**	**2**	**1*‡**	**1*‡**	**2**	**1*‡**	**1*‡**	**2**	**1*‡**	**1*‡**

Intragroup comparisons

*, statistically significant difference with 14 days at the same group ($p \leq 0.05$), †, statistically significant difference with 22 and 37 days at the same group ($p \leq 0.05$). Intergroup comparisons: ‡, statistically significant difference with NT at the same time points ($p \leq 0.05$); §, statistically significant difference with SRP at the same time points ($p \leq 0.05$). Statistical tests: Kruskal-Wallis and Dunn.

https://doi.org/10.1371/journal.pone.0263103.t001

furcation (PBF). Remeasurement of the 5 sections stained with H&E was performed 1 week after the first measurement in order to assess the intra-examiner reliability and reproducibility by Kappa statistic.

TRAP immunolabeling was evaluated by counting TRAP-positive cells at × 200 magnification within TA. A semiquantitative analysis of the immunolabelling of OCN was performed at × 400 magnification [26]. The immunolabelling was characterized as follows:

- Score 0: no immunolabelling (total absence of immunoreactive [IR] cells);
- Score 1: low immunolabelling (IR in ∼1/4 of cells per area);
- Score 2: moderate immunolabelling (IR in ∼1/2 of cells per area);
- Score 3: high immunolabelling (IR in ∼3/4 of cells per area).

Primary and secondary outcomes

The primary outcome was defined as the bone formation within the furcation. Hence, PBF was considered the primary outcome variable. The secondary outcome was to define the tissues' response and safety of the novel MB when compared with MB pH 7.0, through histopathological and immunohistochemical analyses.

Statistical analysis

Data were analyzed using BioStat software (BioStat version 5.0, Belém, PA, Brazil). Cohen's kappa coefficient was used to calculate the agreement between the measurements of PBF. The normality of distribution of the collect data was accessed by Shapiro–Wilk test. The scores evaluated in the histopathological and immunohistochemical analysis of OCN were submitted to analysis of variance with Kruskal–Wallis test and post-test of multiple comparisons of Dunn ($p \leq 0.05$). Parametric data (PBF and TRAP) were analyzed with analysis of variance (One-way ANOVA) and post-test of multiple comparisons of Tukey ($p \leq 0.05$).

Results

Histometric analysis of PBF

Cohen's Kappa coefficient showed 96% agreement between measurements. The results of PBF are shown in Fig 2. In intragroup analysis, aPDT-pH7 showed lower PBF at 14 days (64.97% ±5.38) compared with 37 days (72.96%±3.46) ($p \leq 0.05$). aPDT-pH1 showed lower PBF at 14 days (66.33%±7.35) compared with 22 (75.05%±2.66) and 37 days (78.4%±3.65) ($p \leq 0.05$). In intergroup analysis, group NT showed lower PBF at 14, 22 and 37 days (27.09%±6.85; 31.33% ±7.41; 29.67%±5.68) when compared with groups SRP, aPDT-pH7 and aPDT-pH1 at the same time points ($p \leq 0.05$). Group SRP showed lower PBF at 22 (57.03%±10.46) and 37 days (63.24%±5.58) when compared with aPDT-pH7 and aPDT-pH1 at the same time points ($p \leq 0.05$). aPDT-pH7 showed lower PBF at 22 (68.48%±3.32) and 37 days (72.96%±3.46) when compared with aPDT-pH1 at the same time points ($p \leq 0.05$).

Histopathological analysis of periodontal tissues

Experimental periodontitis severe in group NT, as confirmed by the higher extent of the local inflammatory response and progressive damage to periodontium when compared with the other groups. In group SRP was noticed decreased extent of local inflammatory response, as well as more favorable repair process over the experimental periods when compared with group NT. Groups aPDT-pH7 and aPDT-pH1 showed very similar histopathologic features,

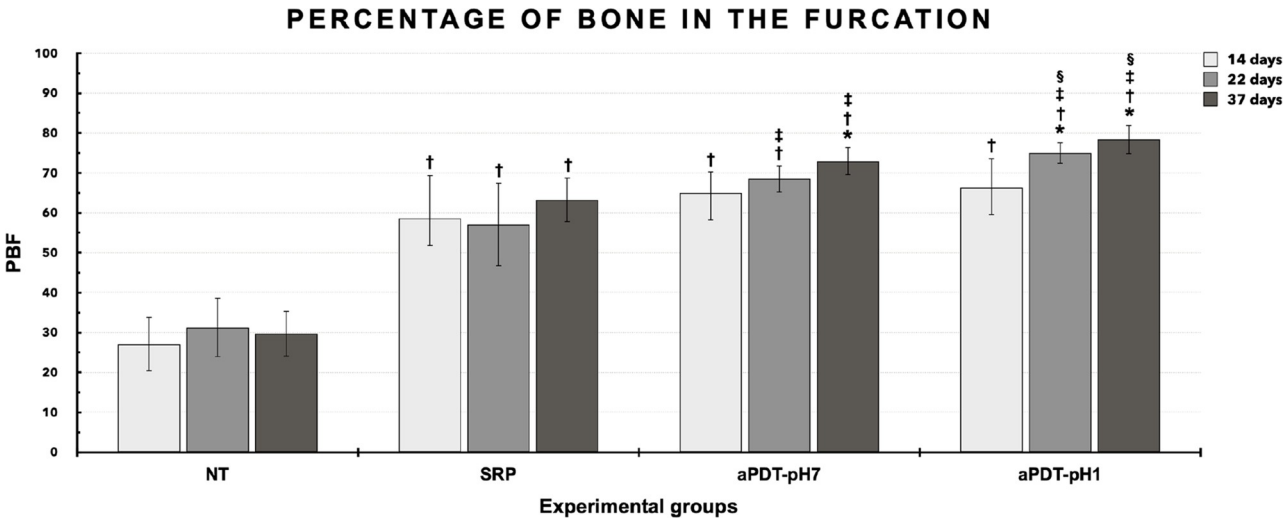

Fig 2. Graph showing the quantification (M±SD) of the percentage of bone in the furcation (PBF) of the mandibular left first molars for each group and period. Statistical tests: ANOVA and Tukey. Symbols: *, statistically significant difference with 14 days at the same group ($p \leq 0.05$); †, statistically significant difference with NT at the same time points ($p \leq 0.05$); ‡, statistically significant difference with SRP at the same time points ($p \leq 0.05$); §, statistically significant difference with aPDT-pH7 at the same time points ($p \leq 0.05$).

https://doi.org/10.1371/journal.pone.0263103.g002

and when compared with group SRP, both presented reduced extent of local inflammatory response and greater tissue repair process after treatment (Fig 3). The parameters, scores, specimens' distributions, and statistical analysis of periodontal tissues in NT, SRP, aPDT-pH7 and aPDT-pH1 are shown in Table 1.

Immunohistochemical analysis

The immunoperoxidase technique for detection of TRAP and OCN showed high specificity for these antigens, as confirmed by the absence of immunolabeling in negative control. In intragroup analysis, group NT showed higher number of TRAP-positive cells at 37 (63.5±10.5 cells) days when compared with 14 (41.8±5.80 cells) and 22 days (47.7±5.3 cells) ($p \leq 0.05$). In intergroup analysis, group NT (41.8±5.80; 47.7±5.3; 63.5±10.5 cells) showed higher number of TRAP-positive cells at 37 days when compared with group SRP (49.2±7.3 cells), at 22 and 37 days when compared with aPDT-pH7 (30.2±12.6; 26.4±7.9 cells), and in all experimental periods when compared with aPDT-pH1 (24.6±9.2; 22.4±10.6; 34±7.9 cells) ($p \leq 0.05$). Group SRP (45.2±4.4; 52.2±9; 49.2±7.3 cells) showed higher number of TRAP-positive cells at 14, 22 and 37 days when compared with aPDT-pH7 and aPDT-pH1 at the same time points ($p \leq 0.05$) (Fig 4).

In intragroup analysis for OCN, aPDT-pH7 and aPDT-pH1 showed lower immunolabeling pattern at 14 days when compared with 22 and 37 days ($p \leq 0.05$). In intergroup analysis, group NT showed no statistically significant difference with group SRP and lower immunolabeling pattern when compared with groups aPDT-pH7 and aPDT-pH1 in all experimental periods ($p \leq 0.05$). Group SRP showed lower immunolabeling pattern when compared with groups aPDT-pH7 and aPDT-pH1 at 22 and 37 days ($p \leq 0.05$) (Fig 5).

Discussion

To target more than one system and/or tissue enrolled with periodontal treatment and repair (i.e. microbiota, specific microenvironment, host immune and inflammatory systems, bone, root surface, and collagen fibers) may be the key for adjunctive therapies to provide standout

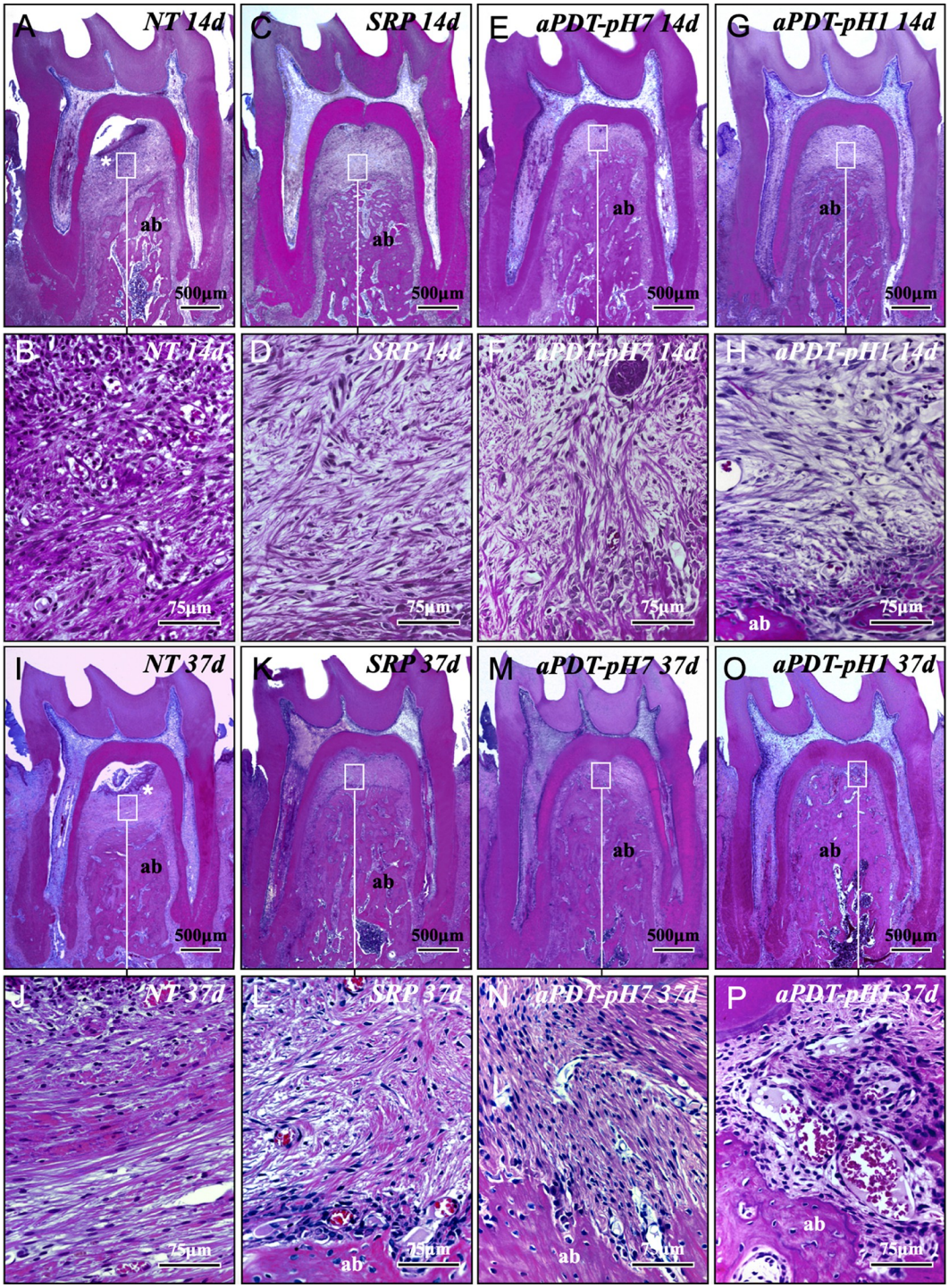

Fig 3. Histopathologic features of the periodontal tissues in the furcation region of the mandibular left first molars. Abbreviations and symbols: ab, alveolar bone; *, inflammatory infiltrate. a- h: photomicrographs showing the features of the periodontal tissues at 14 in NT (A, B), SRP (C, D), aPDT-pH7 (E, F) and aPDT-pH1 (G, H). I—P: photomicrographs showing the features of the periodontal tissues at 37 in NT (I, J), SRP (K, L), aPDT-pH7 (M, N) and aPDT-pH1 (O, P). A, C, E, G, I, K, M, and O allow an overview of the furcation, while B, D, F, H, J, L, N, and P provide a closer view of the inflammatory infiltrate, fibroblasts, arrangement of collagens fibers and alveolar bone in the furcation. Staining: Haematoxylin & Eosin. Scale Bars: A, C, E, G, I, K, M, and O: 500 μm; B, D, F, H, J, L, N, and P: 75 μm.

https://doi.org/10.1371/journal.pone.0263103.g003

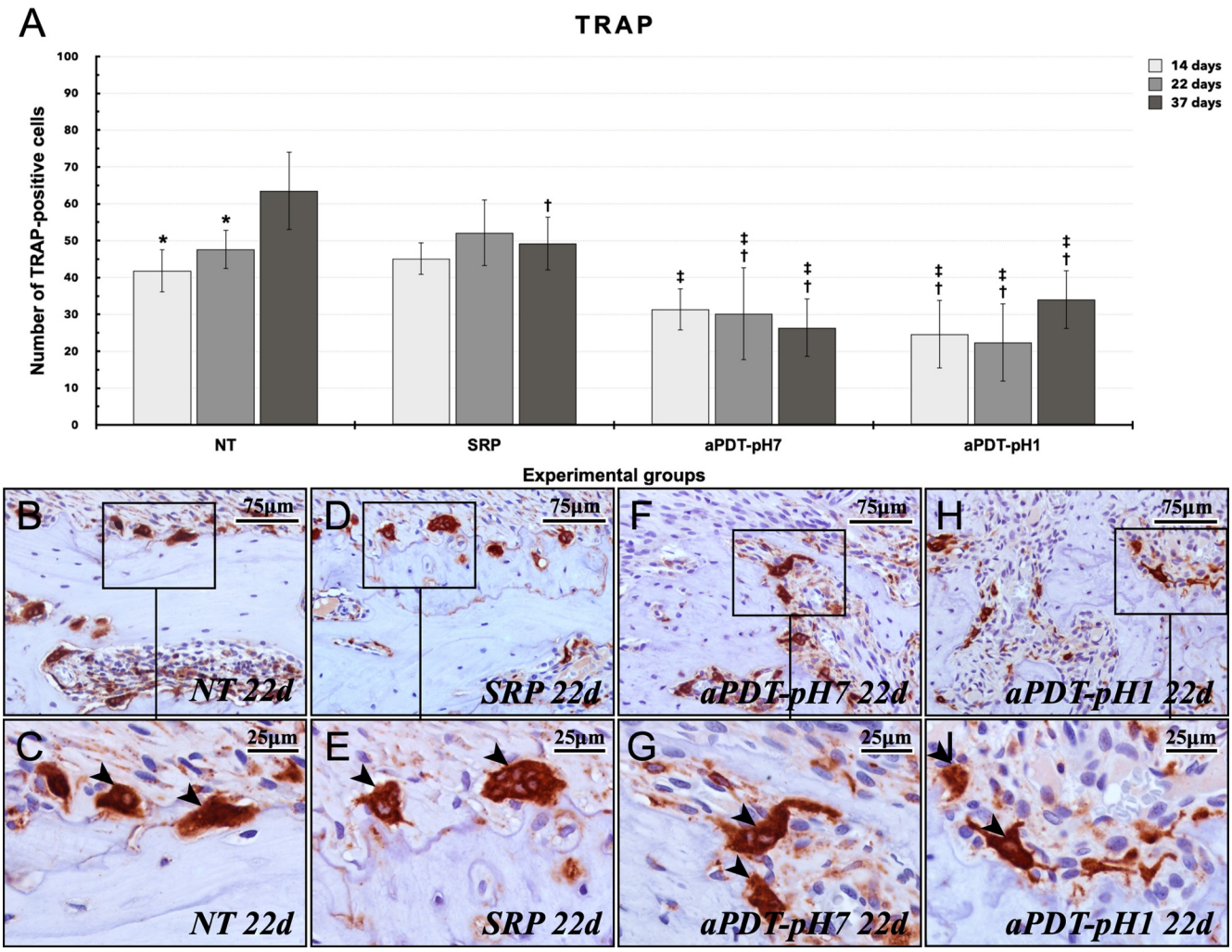

Fig 4. TRAP immunobelling in the furcation region of the mandibular left first molars at 22 days. (A) Means and standard deviations (M±SD) of the number of TRAP-positive cells in the furcation for each group and period. Statistical tests: ANOVA and Tukey. Symbols: *, statistically significant difference with 37 days at the same group ($p \leq 0.05$); †, statistically significant difference with NT at the same time points ($p \leq 0.05$); ‡, statistically significant difference with SRP at the same time points ($p \leq 0.05$). (B—I) Photomicrographs showing the TRAP-positive cells (black arrowheads) at 22 days in groups NT (B, C), SRP (D, E), aPDT-pH7 (F, G) and aPDT-pH1 (H, I). Counter-staining: Harris' haematoxylin. Scale bars: B, D, F, and H: 75µm; C, E, G, and I: 25 µm.

https://doi.org/10.1371/journal.pone.0263103.g004

additional benefits over traditional forms of treating periodontitis. The present study evaluated the effectiveness and safety of a citric acid-based MB pH 1.0 to be used with aPDT as adjunctive therapy to SRP for the treatment of experimental periodontitis. The results support that the acidic MB is as safe as the conventional MB (pH 7.0) used with aPDT so far (i.e. similar histopathologic features, tissues' reaction, and expression of bone-related biomarkers). Additionally, our hypothesis that the acidic MB pH 1.0 could provide additional benefits over conventional MB was confirmed by the higher PBF in group aPDT-pH1 at 22 and 37 days, when compared with aPDT-pH7.

The methodology of EP induction by ligature is an experimental model widely used in researches evaluating periodontal diseases [7, 22]. The histopathologic features obtained 7 days after ligature placement are very similar [22] to those observed in humans, and, also, the equivalent complexity of the microbiota is confirmed by the absence of substantial periodontal breakdown in *"germ-free"* animals [27]. Furthermore, animal studies might mimic the closest

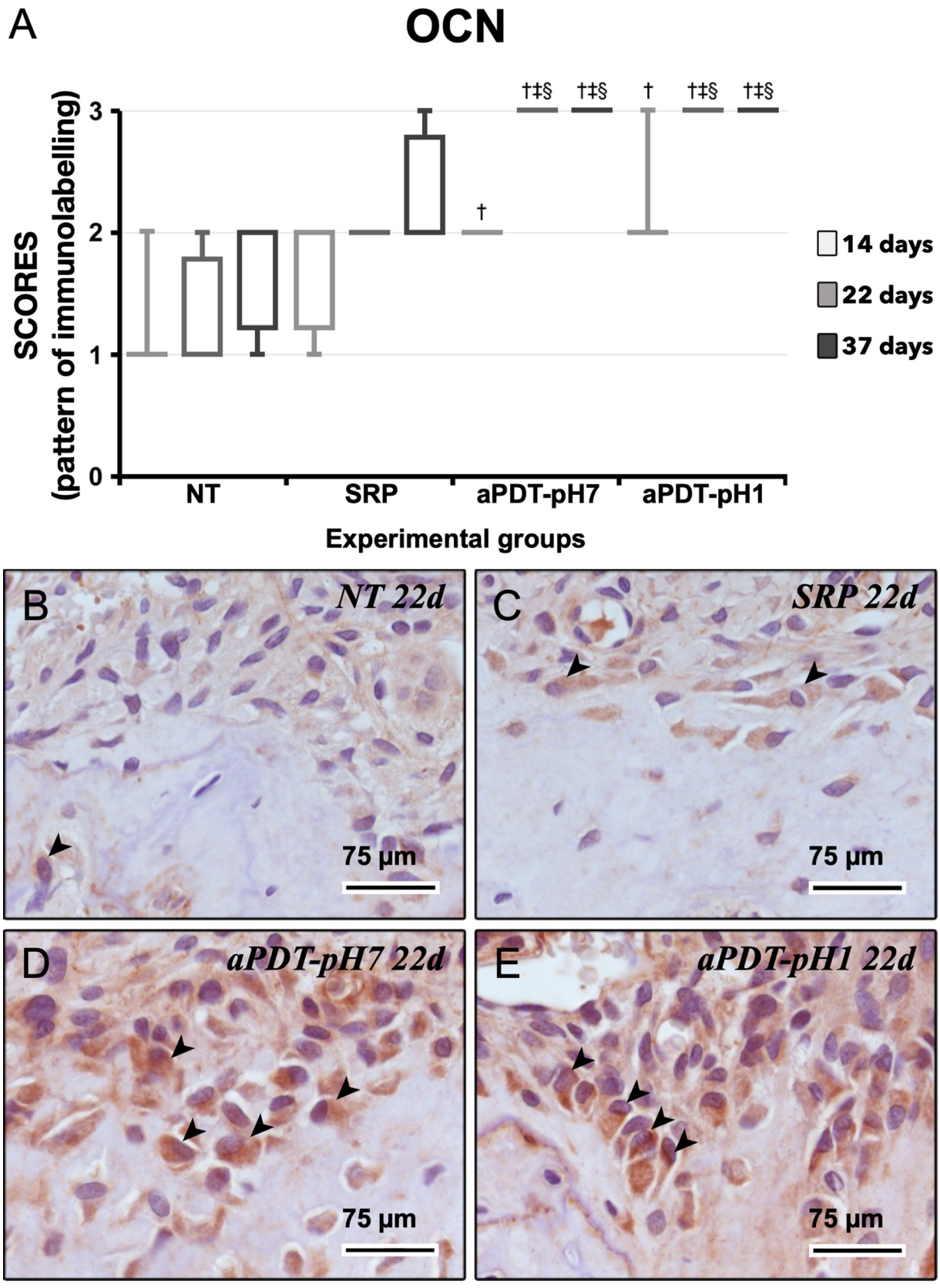
A
OCN
SCORES (pattern of immunolabelling)
0
1
2
3
†‡§
†‡§
†
†‡§
†‡§
†
14 days
22 days
37 days
NT
SRP
aPDT-pH7
aPDT-pH1
Experimental groups
B
NT 22d
75 µm
C
SRP 22d
75 µm
D
aPDT-pH7 22d
75 µm
E
aPDT-pH1 22d
75 µm

Fig 5. OCN immunobelling in the furcation region of the mandibular left first molars at 22 days. (A) Graph showing the median and interquartile range of the scores for OCN in the furcation for each group and period. Statistical tests: Kuskal-Wallis and Dunn. Symbols: ‡, statistically significant difference with 14 days at the same group ($p \leq 0.05$); †, statistically significant difference with NT at the same time points ($p \leq 0.05$); §, statistically significant difference with SRP at the same time points ($p \leq 0.05$). B–E: photomicrographs showing the immunolabelling pattern for OCN and OCN-positive cells (black arrowheads) at 22 days in groups NT (B), SRP (C), aPDT-pH7 (D) and aPDT-pH1 (E). Counter-staining: Harris' haematoxylin. Scale bars: 75 μm.

https://doi.org/10.1371/journal.pone.0263103.g005

situation to the clinical scenario in order to establish valuable cause and effect relationships. On this topic, the reduced dimensions and the impaired access to the furcation of rats' molars validate the application of adjunctive therapies for the treatment of EP in these areas [6]. Also, neither maintaining (present experiment) nor removing the ligature 7 days after induction would compromise the comparisons in this model, since the removal of the ligature didn't prevent the occurrence of alveolar bone loss in the furcation [28]. Hence, this experimental model is capable to validate the hypothesis and to confirm the security and effectiveness of novel therapies for the treatment of periodontal disease in humans [22].

The innate host molecules and inflammatory cells are significantly different when healthy and diseased sites are compared. Although bacteria are present in healthy sites, they coexist in a homeostatic relationship with periodontal tissues, in which an orchestrated expression of select innate host-defense mediators are observed [29, 30]. Conversely, periodontitis sites disorderly express more and different mediators compared with healthy sites [3, 31]. aPDT is known to target microorganisms related to the pathogenesis of periodontitis, however, it was hypothesized by Braham et al. [32] that aPDT may promote periodontal healing not only by killing bacteria but also by inhibiting destructive host responses. Hence, the higher biocompatibility of MB pH 1.0 found by Gusman et al. [33] is likely to support some of the better results found in group aPDT-pH1.

Persistence on the expression of prostaglandin-E_2 harms bone formation through upregulation of RANKL and inhibition of osteoprotegerin [34]. Osteoblasts and osteoclast are two of the main cells responsible for bone metabolism and maintenance of the hierarchical structure of bone [35]. Osteocalcin regulates bone mineralization and turnover [36], while TRAP is expressed from differentiation until apoptosis of osteoclasts [37], therefore, both proteins are important regulators of the bone tissue. With regard to the expression of both biomarkers, this experiment showed that both aPDT protocols (aPDT-pH7 and aPDT-pH1) reduced the number of TRAP-positive cells and increased the immunolabelling pattern of OCN in the furcation when compared with only SRP.

Noteworthy, there is a strict relationship between alveolar bone loss and the success of periodontal therapies [38]. In spite of a possible positive effect of aPDT with MB acidulated with citric acid at pH 1.0 for the treatment of periodontitis, as evidenced by the increased PBF at 22 and 37 days, when compared with 14 days in group aPDT-pH1, further experimental research pursuing deep knowledge on this topic, as well as researches evaluating other parameters shall be conducted in order to eventually translated their results to the clinical scenario.

Even though the mechanisms by which the acidic MB increased the amount of alveolar bone when compared to neutral MB are not completely elucidated, it is known that the interaction of citric acid with mineralized surfaces leads to ionic-exchange processes, increasing the release of Ca^{++} [39] and precipitation of $Ca_3(citrate)_2.4H_20$ onto apatite surface [40], plausible triggers for bone formation. Furthermore, this beneficial property over molecules that positively affect bone might not be understood as limited to bone tissue, since calcium and calcium citrate regulate fundamental cellular events, such as mitochondrial activity [41]. Also, despite the distinct purpose and methods from this experiment, de Rezende et al. [42] observed that demineralization of bone surfaces with citric acid pH 1.0 provided an appropriate substrate for pre-osteoblast proliferation and bone mineralization.

Innumerous PS have been used with aPDT for the treatment of periodontitis [43]. Curcumin [44], toluidine blue [45], chlorin [46], and MB [47] are able to promote a significant antifungal and antibacterial effect against microorganisms. However, the impaired penetration of PS deep in the biofilm matrix decreases the effectiveness of the few PS molecules that are able to penetrate the biofilm matrix [48]. Such drawbacks can be overcome by the development of modified PS [49]. Most experiments confirmed the efficacy of the optimization of MB derivatives to kill bacteria [50, 51] but usually fail to assess their effects on periodontal tissues.

Negatively charged citric acid is believed to inactivate bacteria by either destabilizing the outer membrane or by sequestering essential metals from the growth environment [52, 53]. Recently, Burel et al. [54] evaluated the impact of pH on citric acid antimicrobial activity against Gram-negative bacteria. Their [54] results indicate that tribasic citric acid was the most effective in damaging the bacterial membrane. However, a reduction in the absolute number of bacteria and damages to cell walls were found regardless of the tested pH. No similar experiment has been carried in order to evaluate the effectiveness of citric acid on targeting the membrane or killing periodontal pathogens. Hence, the promising findings of Burel et al. [54] encourage further research on the plausible efficacy of using citric acid-based therapies for reducing bacterial colonization related to the pathogenesis of periodontitis.

The harm to tissues is reasoned by the term "acid strength", described as the potential of acid to lose protons H+. For instance, this property can be adjusted by topology structure and morphology, and crystallinity in addition to the chemical composition [55]. Although in a contaminated environment no statistical significance in inflammatory parameters has been observed between acidic and neutral MB, a subcutaneous test showed the best biocompatibility for MB pH 1.0 [22] as compared with MB pH 7.0.

The use of citric acid for decontamination and repair has being the focus of *in vitro* and *in vivo* experiments in implantology. Souza et al. [19] demonstrated that citric acid significantly reduced the biofilm related with peri-implantitis, as well as enhanced the electrochemical stability of titanium. Htet et al. [56] observed increased bone formation following the combination of mechanical and chemical treatment with citric acid for disinfection of anodized implant surface, which may encourage further research using the acidic MB for the treatment of peri-implantitis as well.

To the best of our knowledge, this is the first experiment to investigate the influence of an acidic dye used in aPDT for the treatment of EP in an *in vivo* model. As with any animal model, the data presented by this experiment shall be interpreted with caution, since the contact of acidic substances with denuded root surface is frequently related with dentin hypersensitivity in humans.

Collectively, our data indicate that aPDT using the citric acid-based MB pH 1.0 is as secure for periodontal tissues as the conventional MB (pH 7.0) used in aPDT so far. Our findings suggest an additional benefit of stimulating bone formation in the furcation following aPDT with the acidic MB.

Supporting information

S1 Dataset. POF dataset.
(PDF)

S2 Dataset. TRAP dataset.
(PDF)

Acknowledgments

It was conducted at the Department of Diagnosis and Surgery—Division of Periodontics, São Paulo State University (UNESP), School of Dentistry, Araçatuba, São Paulo, Brazil. The authors thank the laboratory of Osteobiology Applied to Dentistry for assistance in the process of histological and immuhistochemical samples.

Author Contributions

Conceptualization: Juliano Milanezi de Almeida, Henrique Rinaldi Matheus, David Jonathan Rodrigues Gusman, Maria José Hitomi Nagata.

Data curation: Juliano Milanezi de Almeida, Breno Edson Sendão Alves, David Jonathan Rodrigues Gusman, Maria José Hitomi Nagata, Elisa Mara de Abreu Furquim, Edilson Ervolino.

Formal analysis: Henrique Rinaldi Matheus, Breno Edson Sendão Alves, Elisa Mara de Abreu Furquim, Edilson Ervolino.

Investigation: Juliano Milanezi de Almeida, Henrique Rinaldi Matheus, Breno Edson Sendão Alves, David Jonathan Rodrigues Gusman, Maria José Hitomi Nagata, Elisa Mara de Abreu Furquim, Edilson Ervolino.

Methodology: Juliano Milanezi de Almeida, Henrique Rinaldi Matheus, Elisa Mara de Abreu Furquim.

Project administration: Juliano Milanezi de Almeida, Henrique Rinaldi Matheus, David Jonathan Rodrigues Gusman, Maria José Hitomi Nagata, Edilson Ervolino.

Software: Juliano Milanezi de Almeida, Henrique Rinaldi Matheus, Breno Edson Sendão Alves, Elisa Mara de Abreu Furquim.

Supervision: Juliano Milanezi de Almeida, David Jonathan Rodrigues Gusman, Maria José Hitomi Nagata, Edilson Ervolino.

Validation: Juliano Milanezi de Almeida, Breno Edson Sendão Alves, Maria José Hitomi Nagata, Edilson Ervolino.

Visualization: Henrique Rinaldi Matheus.

Writing – original draft: Henrique Rinaldi Matheus, Breno Edson Sendão Alves, David Jonathan Rodrigues Gusman, Elisa Mara de Abreu Furquim.

Writing – review & editing: Juliano Milanezi de Almeida, Maria José Hitomi Nagata, Edilson Ervolino.

References

1. Eke PI, Thornton-Evans GO, Wei L, Borgnakke WS, Dye BA, Genco RJ (2018) Periodontitis in US Adults: National Health and Nutrition Examination Survey 2009–2014. J Am Dent Assoc 149:576–588. https://doi.org/10.1016/j.adaj.2018.04.023 PMID: 29957185
2. Kassebaum NJ, Bernabé E, Dahiya M, Bhandari B, Murray CJ, Marcenes W (2014) Global burden of severe periodontitis in 1990–2010: a systematic review and meta-regression. J Dent Res 93:1045–1053. https://doi.org/10.1177/0022034514552491 PMID: 25261053
3. Bartold PM, Van Dyke TE (2013) Periodontitis: a host-mediated disruption of microbial homeostasis. Unlearning learned concepts. Periodontol 2000 62:203–217. https://doi.org/10.1111/j.1600-0757.2012.00450.x PMID: 23574467

4. Darveau RP, Hajishengallis G, Curtis MA (2012) Porphyromonas gingivalis as a potential community activist for disease. J Dent Res 91:816–820. https://doi.org/10.1177/0022034512453589 PMID: 22772362

5. Mizutani K, Aoki A, Coluzzi D, Yukna R, Wang CY, Pavlic V, et al (2016) Lasers in minimally invasive periodontal and peri-implant therapy. Periodontol 2000 71:185–212. https://doi.org/10.1111/prd.12123 PMID: 27045437

6. Renvert S, Persson GR (2002) A systematic review on the use of residual probing depth, bleeding on probing and furcation status following initial periodontal therapy to predict further attachment and tooth loss. J Clin Periodontol 29:82–89. https://doi.org/10.1034/j.1600-051x.29.s-3.2.x PMID: 12787209

7. de Almeida JM, Theodoro LH, Bosco AF, Nagata MJ, Bonfante S, Garcia VG (2008) Treatment of experimental periodontal disease by photodynamic therapy in rats with diabetes. J Periodontol 79:2156–65. https://doi.org/10.1902/jop.2008.080103 PMID: 18980525

8. Arany PR (2016) Craniofacial Wound Healing with Photobiomodulation Therapy: New Insights and Current Challenges. J Dent Res 95:977–984. https://doi.org/10.1177/0022034516648939 PMID: 27161014

9. Romanova NA, Brovko LY, Moore L, Pometun E, Savitsky AP, Ugarova NN, et al (2003) Assessment of photodynamic destruction of Escherichia coli O157:H7 and Listeria monocytogenes by using ATP bioluminescence. Appl Environ Microbiol 69:6393–6398. https://doi.org/10.1128/AEM.69.11.6393-6398.2003 PMID: 14602591

10. Jori G, Fabris C, Soncin M, Ferro S, Coppellotti O, Dei D, et al (2006) Photodynamic therapy in the treatment of microbial infections: basic principles and perspective applications. Lasers Surg Med 38:468–81. https://doi.org/10.1002/lsm.20361 PMID: 16788934

11. Chambrone L, Wang HL, Romanos GE (2018) Antimicrobial photodynamic therapy for the treatment of periodontitis and peri-implantitis: An American Academy of Periodontology best evidence review. J Periodontol 89:783–803. https://doi.org/10.1902/jop.2017.170172 PMID: 30133749

12. Cadore UB, Reis MBL, Martins SHL, Invernici MM, Novaes AB Jr, Taba M Jr, et al (2019) Multiple sessions of antimicrobial photodynamic therapy associated with surgical periodontal treatment in patients with chronic periodontitis. J Periodontol 90:339–349. https://doi.org/10.1002/JPER.18-0373 PMID: 30383298

13. Nikinmaa S, Alapulli H, Auvinen P, Vaara M, Rantala J, Kankuri E, et al (2020) Dual-light photodynamic therapy administered daily provides a sustained antibacterial effect on biofilm and prevents Streptococcus mutans adaptation. PLoS One 15:e0232775. https://doi.org/10.1371/journal.pone.0232775 PMID: 32374766

14. Allaker RP, Douglas CW (2009) Novel anti-microbial therapies for dental plaque-related diseases. Int J Antimicrob Agents 33:8–13. https://doi.org/10.1016/j.ijantimicag.2008.07.014 PMID: 18804350

15. Ghorbani J, Rahban D, Aghamiri S, Teymouri A, Bahador A (2018) Photosensitizers in antibacterial photodynamic therapy: an overview. Laser Ther 27:293–302. https://doi.org/10.5978/islsm.27_18-RA-01 PMID: 31182904

16. Qi M, Chi M, Sun X, Xie X, Weir MD, Oates TW, et al (2019) Novel nanomaterial-based antibacterial photodynamic therapies to combat oral bacterial biofilms and infectious diseases. Int J Nanomedicine 14:6937–6956. https://doi.org/10.2147/IJN.S212807 PMID: 31695368

17. Sharma M, Visai L, Bragheri F, Cristiani I, Gupta PK, Speziale P (2008) Toluidine blue-mediated photodynamic effects on staphylococcal biofilms. Antimicrob Agents Chemother 52:299–305. https://doi.org/10.1128/AAC.00988-07 PMID: 17967908

18. Ruggeri A Jr, Prati C, Mazzoni A, Nucci C, Di Lenarda R, Mazzotti G, et al (2007) Effects of citric acid and EDTA conditioning on exposed root dentin: An immunohistochemical analysis of collagen and proteoglycans. Arch Oral Biol 52:1–8. https://doi.org/10.1016/j.archoralbio.2006.07.001 PMID: 17098210

19. Souza JGS, Cordeiro JM, Lima CV, Barão VAR (2019) Citric acid reduces oral biofilm and influences the electrochemical behavior of titanium: An in situ and in vitro study. J Periodontol 90:149–158. https://doi.org/10.1002/JPER.18-0178 PMID: 30088827

20. Subramanian S, Appukuttan D, Tadepalli A, Gnana PPS, Athmarao RT (2017) Root conditioning with citric acid and ethylenediaminetetraacetic acid and their effect on fibrin clot adhesion to dentin-a scanning electron microscopic study. J Clin Diagn Res 11:ZC82–ZC85. https://doi.org/10.7860/JCDR/2017/27768.10443 PMID: 28969280

21. Hollinger JO, Kleinschmidt JC (1990) The critical size defect as an experimental model to test bone repair materials. J Craniofac Surg 1:60–68. https://doi.org/10.1097/00001665-199001000-00011 PMID: 1965154

22. de Molon RS, de Avila ED, Boas Nogueira AV, Chaves de Souza JA, Avila-Campos MJ, de Andrade CR, et al (2014) Evaluation of the host response in various models of induced periodontal disease in mice. J Periodontol 85:465–477. https://doi.org/10.1902/jop.2013.130225 PMID: 23805811

23. Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, et al (2020) The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol 18:e3000410. https://doi.org/10.1371/journal.pbio.3000410 PMID: 32663219

24. Johnson IH (1975) Effects of local irritation and dextran sulphate administration on the periodontium of the rat. J Periodontal Res 10:332–345. https://doi.org/10.1111/j.1600-0765.1975.tb00042.x PMID: 129558

25. Matheus HR, Ervolino E, Faleiros PL, Novaes VCN, Theodoro LH, Garcia VG, et al (2018) Cisplatin chemotherapy impairs the peri-implant bone repair around titanium implants: An in vivo study in rats. J Clin Periodontol 45:241–252. https://doi.org/10.1111/jcpe.12824 PMID: 28965362

26. Gusman DJR, Ervolino E, Theodoro LH, Garcia VG, Nagata MJH, Alves BES, et al (2019) Antineoplastic agents exacerbate periodontal inflammation and aggravate experimental periodontitis. J Clin Periodontol 46:457–469. https://doi.org/10.1111/jcpe.13101 PMID: 30854670

27. Rovin S, Costich ER, Gordon HA (1966) The influence of bacteria and irritation in the initiation of periodontal disease in germfree and conventional rats. J Periodontal Res 1:193–204. https://doi.org/10.1111/j.1600-0765.1966.tb01860.x PMID: 4225530

28. De Almeida J, Ervolino E, Bonfietti LH, et al (2015) Adjuvant Therapy With Sodium Alendronate for the Treatment of Experimental Periodontitis in Rats. J Periodontol 86:1166–1175. https://doi.org/10.1902/jop.2015.150166 PMID: 26062841

29. Moughal NA, Adonogianaki E, Thornhill MH, Kinane DF (1992) Endothelial cell leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression in gingival tissue during health and experimentally-induced gingivitis. J Periodontal Res 27:623–630. https://doi.org/10.1111/j.1600-0765.1992.tb01746.x PMID: 1281230

30. Tonetti MS (1997) Molecular factors associated with compartmentalization of gingival immune responses and transepithelial neutrophil migration. J Periodontal Res 32:104–109. https://doi.org/10.1111/j.1600-0765.1997.tb01389.x PMID: 9085218

31. Ren L, Leung WK, Darveau RP, Jin L (2005) The expression profile of lipopolysaccharide-binding protein, membrane-bound CD14, and toll-like receptors 2 and 4 in chronic periodontitis. J Periodontol 76:1950–1959. https://doi.org/10.1902/jop.2005.76.11.1950 PMID: 16274315

32. Braham P, Herron C, Street C, Darveau R (2009) Antimicrobial photodynamic therapy may promote periodontal healing through multiple mechanisms. J Periodontol 80:1790–1798. https://doi.org/10.1902/jop.2009.090214 PMID: 19905948

33. Gusman DJR, Cintra LTA, Novaes VCN, Matheus HR, de Araujo NJ, de Almeida JM (2018) pH influences the biocompatibility of methylene blue solutions. Clin Oral Investig 22:361–367. https://doi.org/10.1007/s00784-017-2120-4 PMID: 28536783

34. Horowitz M, Kacena M, Lorenzo J. Genetics and mutations affecting osteoclast development and function. In Bronner F., Farach-Carson M. & Rubin J. (Eds.), Bone resorption. London: Springer-Verlag; 2006:91–107.

35. Adamopoulos IE (2018) Inflammation in bone physiology and pathology. Curr Opin Rheumatol 30:59–64. https://doi.org/10.1097/BOR.0000000000000449 PMID: 29016371

36. Ivaska KK, Hentunen TA, Vääräniemi J, Ylipahkala H, Pettersson K, Väänänen HK (2004) Release of intact and fragmented osteocalcin molecules from bone matrix during bone resorption in vitro. J Biol Chem 279:18361–18369. https://doi.org/10.1074/jbc.M314324200 PMID: 14970229

37. Minkin C (1982) Bone acid phosphatase: tartrate-resistant acid phosphatase as a marker of osteoclast function. Calcif Tissue Int 34:285–290. https://doi.org/10.1007/BF02411252 PMID: 6809291

38. Polimeni G, Xiropaidis AV, Wikesjö UM (2006) Biology and principles of periodontal wound healing/regeneration. Periodontol 2000 41:30–47. https://doi.org/10.1111/j.1600-0757.2006.00157.x PMID: 16686925

39. Sterrett JD, Bankey T, Murphy HJ (1993) Dentin demineralization. The effects of citric acid concentration and application time. J Clin Periodontol 20:366–370. https://doi.org/10.1111/j.1600-051x.1993.tb00374.x PMID: 8501277

40. Misra DN (1996) Interaction of citric acid with hydroxyapatite: surface exchange of ions and precipitation of calcium citrate. J Dent Res 75:1418–1425. https://doi.org/10.1177/00220345960750061401 PMID: 8831638

41. Bertero E, Maack C (2018) Calcium Signaling and Reactive Oxygen Species in Mitochondria. Circ Res 122:1460–1478. https://doi.org/10.1161/CIRCRESAHA.118.310082 PMID: 29748369

42. de Rezende ML, Coesta PT, de Oliveira RC, Salmeron S, Sant'Ana AC, Damante CA, et al (2015) Bone demineralization with citric acid enhances adhesion and spreading of preosteoblasts. J Periodontol 86:146–154. https://doi.org/10.1902/jop.2014.130657 PMID: 25272980

43. Carrera ET, Dias HB, Corbi SCT, Marcantonio RAC, Bernardi ACA, Bagnato VS, et al (2016) The application of antimicrobial photodynamic therapy (aPDT) in dentistry: a critical review. Laser Phys 26:123001. https://doi.org/10.1088/1054-660X/26/12/123001 PMID: 29151775

44. Araújo NC, Fontana CR, Bagnato VS, Gerbi ME (2014) Photodynamic antimicrobial therapy of curcumin in biofilms and carious dentine. Lasers Med Sci 29:629–635. https://doi.org/10.1007/s10103-013-1369-3 PMID: 23793414

45. Qin YL, Luan XL, Bi LJ, Sheng YQ, Zhou CN, Zhang ZG (2008) Comparison of toluidine blue-mediated photodynamic therapy and conventional scaling treatment for periodontitis in rats. J Periodontal Res 43:162–167. https://doi.org/10.1111/j.1600-0765.2007.01007.x PMID: 18302617

46. Pfitzner A, Sigusch BW, Albrecht V, Glockmann E (2004) Killing of periodontopathogenic bacteria by photodynamic therapy. J Periodontol 75:1343–1349. https://doi.org/10.1902/jop.2004.75.10.1343 PMID: 15562911

47. de Oliveira RR, Schwartz-Filho HO, Novaes AB Jr, Taba M Jr (2007) Antimicrobial photodynamic therapy in the non-surgical treatment of aggressive periodontitis: a preliminary randomized controlled clinical study. J Periodontol 78:965–973. https://doi.org/10.1902/jop.2007.060494 PMID: 17539707

48. Shih MH, Huang FC (2013) Repetitive methylene blue-mediated photoantimicrobial chemotherapy changes the susceptibility and expression of the outer membrane proteins of Pseudomonas aeruginosa. Photodiagnosis Photodyn Ther 10:664–671. https://doi.org/10.1016/j.pdpdt.2013.07.003 PMID: 24284125

49. Dascalu Rusu LM, Moldovan M, Prodan D, Ciotlaus I, Popescu V, Baldea I, et al (2020) Assessment and Characterization of Some New Photosensitizers for Antimicrobial Photodynamic Therapy (aPDT). Materials (Basel) 13:3012. https://doi.org/10.3390/ma13133012 PMID: 32640635

50. Klepac-Ceraj V, Patel N, Song X, Holewa C, Patel C, Kent R, et al (2011) Photodynamic effects of methylene blue-loaded polymeric nanoparticles on dental plaque bacteria. Lasers Surg Med 43:600–606. https://doi.org/10.1002/lsm.21069 PMID: 22057487

51. de Freitas LM, Calixto GM, Chorilli M, Giusti JS, Bagnato VS, Soukos NS, et al (2016) Polymeric Nanoparticle-Based Photodynamic Therapy for Chronic Periodontitis in Vivo. Int J Mol Sci 17:769. https://doi.org/10.3390/ijms17050769 PMID: 27213356

52. Beuchat LR, Golden DA (1989) Antimicrobials occurring naturally in foods. Food Technol 43, 134–142.

53. Buchanan RL, Golden MH (1994) Interaction of Citric Acid Concentration and pH on the Kinetics of Listeria monocytogenes Inactivation. J Food Prot 57:567–570. https://doi.org/10.4315/0362-028X-57.7.567 PMID: 31121707

54. Burel C, Kala A, Purevdorj-Gage L (2021) Impact of pH on citric acid antimicrobial activity against Gram-negative bacteria. Lett Appl Microbiol 72:332–340. https://doi.org/10.1111/lam.13420 PMID: 33099798

55. Zhang X, Zhao Y, Xu S, Yang Y, Liu J, Wei Y, et al (2014) Polystyrene sulphonic acid resins with enhanced acid strength via macromolecular self-assembly within confined nanospace. Nat Commun 5:3170. https://doi.org/10.1038/ncomms4170 PMID: 24463793

56. Htet M, Madi M, Zakaria O, Miyahara T, Xin W, Lin Z, et al (2016) Decontamination of anodized implant surface with different modalities for peri-implantitis treatment: lasers and mechanical debridement with citric acid. J Periodontol 87:953–961. https://doi.org/10.1902/jop.2016.150615 PMID: 26966876