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Antimicrobial Photodynamic Therapy as a Strategy to Arrest Enamel Demineralization: A Short-Term Study on Incipient Caries in a Rat Model†

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ABSTRACT

In this study we developed a rat model of incipient caries to investigate the short-term effects of antimicrobial photodynamic therapy (aPDT) on oral microbiota regulation and demineralization arrestment. Twenty-nine male rats were submitted to caries induction. Early carious lesion was confirmed by optical coherence tomography (OCT) 5 days after experiment beginning in five animals. The remaining animals ($n = 24$) were randomly divided into two groups: control ($n = 12$), animals were untreated; and aPDT ($n = 12$), animals were treated with 100 μM of methylene blue for 5 min and irradiated by a light emitting diode at $\lambda = 645 \pm 30$ nm, fluence rate of 480 mW cm^{-2} and exposure time of 3 min. Bacterial burden was evaluated before, immediately after, 3, 7 and 10 days following treatment, and total number of microaerophilic bacteria was counted. OCT was also used to quantify teeth demineralization. A significant bacterial decrease of about 1.6 log was observed immediately after aPDT. Besides, bacterial load in aPDT group remained lower than control until 10 days post-treatment ($P < 0.05$) and variation of optical attenuation coefficient before and after aPDT was 15%, corroborating to caries arrestment. Put together, these findings suggest that aPDT was competent to reduce cariogenic bacteria and to avoid further mineral loss.

INTRODUCTION

Caries disease is a worldly spread chronic infection that causes tooth hard-tissue lesions and it can cause cavities, endodontic lesions and severe pain. Caries disease is caused by a consortium of bacteria organized in biofilm that support a microecosystem that exhibits characteristics of acid production as result of carbohydrate fermentation process. Caries lesion development is characterized by a localized imbalance in pH that increases hydroxyapatite solubility and tooth demineralization. Once the acidic environment has been established, *Streptococcus mutans* and other aciduric bacteria sustain the acid environment and promote lesion progression (1).

The use of fluoride-containing products and the establishment of preventive measures in kindergartens and schools have improved the oral health of children and young people in a good number of industrialized regions. However, caries continues to be a public health challenge in several countries, especially in groups with less access to preventive protocols and with caries-tending lifestyle (2,3).

Furthermore, it has been shown that genetic differences in populations exist with respect to sensitivity to caries, and successful preventive strategies for the majority of the population are insufficient for some group of individuals (4), in which the use of antimicrobial approaches, in theory, could control the infection. In fact, the literature does not support this tactic in long-term prevention based on clinical evidences (1,5).

Minimal intervention is a key phrase in today's dental practice and has led to change of paradigms in dentistry. Among the therapies for oral diseases, modern caries management includes noninvasive strategies for demineralized tissue repair based on the current knowledge about caries process. The basic principle is to establish conditions that encourage remineralization of incipient carious lesions through a biological or therapeutic strategy rather than the traditional restorative treatment (6,7). Although a complete reverse of initial demineralized lesions could be desired, repair approaches can effectively arrest caries with no detrimental clinical consequences (8). Antimicrobial photodynamic therapy (aPDT) arises in this scenario as a powerful technique to reduce microbiota in the oral cavity and avoid caries progression, as if untreated caries may evolve throughout the outer enamel layer of a tooth into the dentin.

Antimicrobial PDT combines a nontoxic photoactivatable dye or photosensitizer with harmless visible light of the correct wavelength to excite the dye to its reactive triplet state, which will then generate reactive oxygen species (ROS) such as singlet oxygen and hydroxyl radicals that are toxic to cells (9–12). aPDT is an emerging alternative to treat localized infections (13–15), including oral diseases (16) such as periodontitis (13,17–19), adjuvant approach for endodontic treatment (20,21) and caries (22,23).

Despite the growing use of aPDT in dentistry and the vast literature attesting its remarkable aptitude to inactivate

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microbial cells and biofilms, this study demonstrates a concrete usage with potential to be incorporated in a clinical situation for public health. Preclinical trials constitute an important step to define the impact of new treatment modalities of caries control and to identify effective strategies and therapeutic parameters for clinical studies. Within this context, the aim of this work was to develop an animal model of early carious lesions to investigate the short-term effects of MB-mediated aPDT as a treatment approach to arrest caries progression and avoid further mineral loss. We evaluated the microbial load from affected teeth and we used optical coherent tomography (OCT) to quantify enamel demineralization.

MATERIALS AND METHODS

Bacterial strain and inoculum. *Streptococcus mutans* strain ATCC 25175 (kindly provided by Dr. Márcia P. A. Mayer from ICB/USP) was subcultured from vial stock onto TSA (Tryptic Soy Agar) at 37°C under a 5% CO_2 atmosphere. Bacteria inoculum was prepared from 48 h culture in brain heart infusion. Turbidity of cell suspension was adjusted in an optical spectrophotometer in order to obtain suspensions of 10^8 cells mL^{-1} (optical density of 0.15 at $\lambda = 530$ nm).

Rat caries model. Thirty-two male Wistar rats with 20 days of age were selected for this study (Fig. 1). The experiments were conducted in compliance with the local Animal Ethics Committee (28/CEPA-IPEN/SP). Twenty-nine animals were housed under standard laboratory conditions and fed 50 g day^{-1} of cariogenic diet (24) during the whole experiment. To increase the cariogenic challenge, rats also received water with 10% of sucrose (25) *ad libitum*. During three consecutive days, animals were orally infected with *S. mutans* (24). In the first inoculation, rats were anesthetized with ketamine (80 mg kg^{-1}) and xylazine (10 mg kg^{-1}) by intraperitoneal injection, and orally inoculated with 200 μL of *S. mutans* suspension. In the next 2 days, bacteria suspension was added to the food. The remaining three animals (not submitted to caries protocol) were used for OCT analysis of sound enamel. The experimental schedule is shown in Fig. 2.

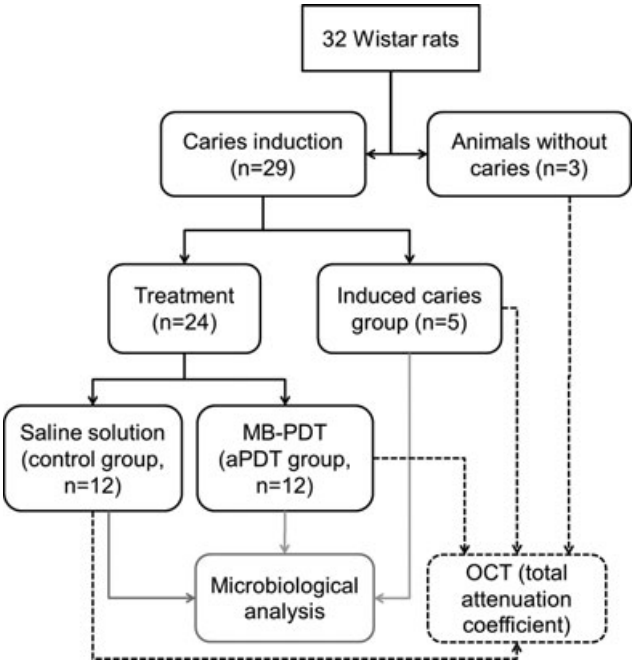

Figure 1. Flowchart diagram of the study design.

Photosensitizer and light source. Methylene blue (MB) (Sigma-Aldrich, Poole, UK) stock solution was prepared in distilled water at a concentration of 10 mM, filtered through a sterile 0.22 μm filter membrane and stored in the dark. Before the experiments, 100 μM MB solution was prepared by 100-fold dilution of the stock solution in distilled water (13,26).

The light source used for the photosensitizer excitation was a light emitting diode (LED) with an emission at $\lambda = 645 \pm 30$ nm (MMOPTICS, São Carlos, Brazil). The total output power provided by the device was 240 mW, and the spot area was 0.5 cm^2 (Fig. 3).

Antimicrobial photodynamic therapy. Three days after the last bacterial inoculation (Fig. 1), 24 animals were randomly distributed into control and aPDT group (12 animals per group). Animals from control group received 200 μL of saline solution and were not treated by either light or photosensitizer. In aPDT group, 200 μL of 100 μM MB (18) was applied in the upper molars. After 5 min (preirradiation time) the teeth were irradiated for 3 min at a fluence rate of 480 mW cm^{-2}. Irradiation was performed with the probe in contact with the teeth (Fig. 4).

Microbiological analysis. Five animals submitted to caries induction protocol (induced-caries group) were euthanized before the treatment (at day 0) and used for microbiological comparison and OCT (Fig. 1). Thereafter, animals from control and aPDT groups were evaluated immediately after the treatment and following 3, 7 and 10 days (three animals per evaluation). Rats were euthanized, the maxillae were surgically removed and dissected, and the molars were carefully extracted and weighed. For microbial recovery, three molars from each animal were stored in eppendorfs containing 1 mL of sterile 0.85% saline solution (one sample per animal). The eppendorfs were agitated in vortex for 30 s. Thereafter, cell suspensions were serially diluted 10-fold in PBS on 96-well plates to generate dilution of 10^{-1} to 10^{-4} times the original concentrations. Ten microliter aliquots of each dilution were streaked horizontally (27) onto a TSA plate in triplicate. Plates were incubated for 24 h at 37°C in 5% CO_2 atmosphere to allow colony formation from all microaerophilic bacteria. Bacterial colonies were counted and converted into colony-forming units per gram (cfu g^{-1}). The survival fraction values were obtained by dividing the data for the mean of bacterial load from animals submitted to caries induction (before treatment).

Caries lesion diagnosis and quantification by OCT. Optical coherence tomography (OCT) is a noninvasive diagnostic imaging technology that has been used in dentistry to evaluate enamel interface restoration, analysis of the performance of dental materials, and early caries detection (28–30). The technique is derived of quantitative measurements of the backscattered light intensity as a function of depth into the region of interest (ROI) so that it is appropriate for detecting changes in optical scattering due to morphological alterations within teeth (31).

The OCT analysis was used to confirm the presence of incipient caries lesions before aPDT by determining the degree of enamel demineralization. It was also used to analyze the effects of aPDT

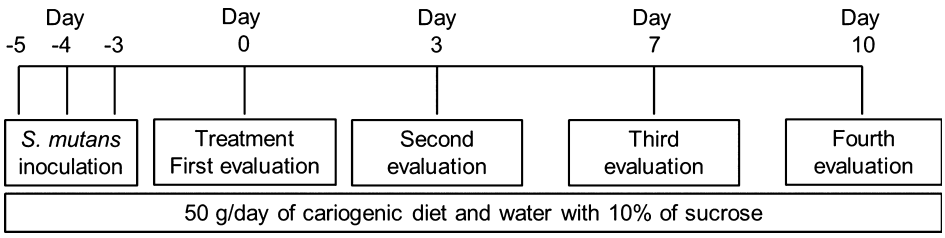

Figure 2. Experimental schedule of caries induced in rats' molars. *Streptococcus mutans* was inoculated on day −5, −4 and −3. Early carious lesions were confirmed before treatment (day 0). Bacterial load and OCT were accomplished immediately after aPDT and on days 3, 7, 10 post-treatment.

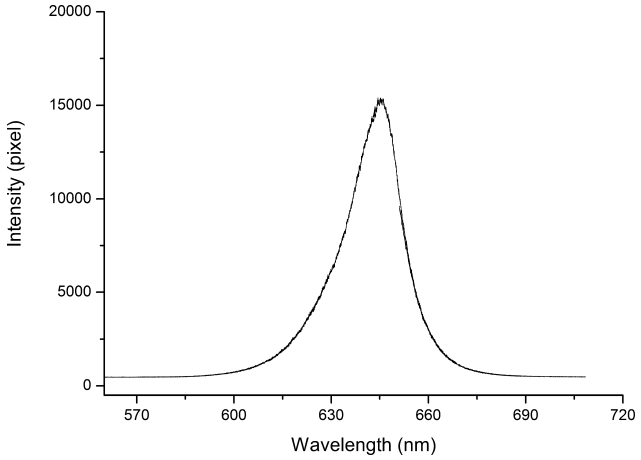

Figure 3. Emission spectrum of the LED used in this study.

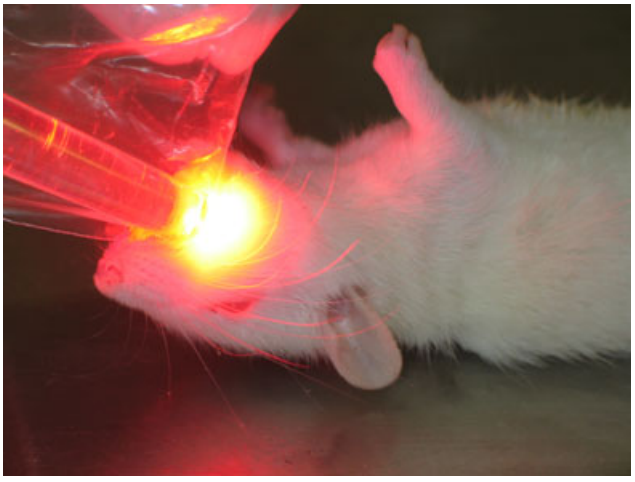

Figure 4. Clinical photograph of aPDT in caries. Methylene blue (100 μM) was applied in the upper molars and maintained for 5 min, and irradiation using a LED at $\lambda = 640 \pm 30$ nm, fluence rate of 480 mW cm^{-2} and exposure time of 3 min.

10 days after treatment and both analyses (control and aPDT) were compared. OCT images were produced with a Thorlabs OCT system (OCP930SRS, Thorlabs, Newton, NJ) that consists of a superluminescent LED emitting at $\lambda = 930 \pm 5$ nm with 100 ± 5 nm FWHM and an optical power of 2 mW. Resolution for this system is of 6.2 μm and maximum image depth of 1.6 mm in air. We used OCT to create cross-sectional images of rat molars fixed in dental wax. The molars were placed in a standard way on the sample holder able to keep the fiber straight, without usual warps.

To verify optical attenuation in dental tissue from the OCT signal, specific software was developed. Firstly, all the OCT scans in the ROI that we selected were aligned to monitor the surface curvature, and then, an OCT signal mean value was generated by computing the arithmetic median. In this work, we choose three ROIs per animal ($n = 3$ per animal) in the OCT averaged signal from occlusal surface of the dental enamel initiating some microns below the sample surface until 200 μm of depth. Secondly, we picked ROIs of 100 μm, and the program performed an exponential fit using the function:

$$f(x) = a \cdot \exp(-b \cdot x) + c$$

where a is the amplitude of backscattering signal in the surface, b is the total optical attenuation coefficient (TOAC, in μm^{-1}) inside the sample and c is the background level due to signal noise.

The TOAC values were obtained from control and aPDT groups, animals without caries (sound enamel) and submitted to caries induction (three animals per group). OCT data were presented as the TOAC variation (TOACV). The coefficients were normalized by the mean value of sound enamel and the percentage of variation was obtained.

Statistical analysis. Values are given as means, and error bars are standard deviations. Shapiro–Wilk W-test showed normal distribution on microbiological samples that were then submitted to statistical comparisons using one-way analysis of variance (ANOVA). Mean comparisons were carried out with the Tukey's test. Significance differences were established on $P < 0.05$.

Statistical analysis from OCT data was accomplished using Kruskal–Wallis ANOVA test and comparisons between groups were analyzed *via* the Mann–Whitney test at 5% significance level.

RESULTS

During all experimental period, the rats gained body mass and did not show any signs of complication.

Our methodology of induced caries model promoted the development of incipient caries lesions that were confirmed by OCT analysis. Figure 5 displays typical OCT images of sound and induced-caries on rat molars. Significant differences could be observed between the samples. Figure 5A shows a caries-free tooth. The brightness observed in Fig. 5B characterized by a higher scattering of this region evidences enamel demineralization.

The initial mean value of infectious load accessed from five animals with induced caries lesions was 5.6 $\log_{10}$. Survival fraction of microaerophilic burden of overall experimental period is presented in Fig. 6. Our data demonstrate that aPDT significantly reduced bacterium viability. Immediately after therapy, the mean infectious burden was reduced to 4.02 $\log_{10}$ resulting in a mean log reduction of 1.58% or 97.4% ($P < 0.05$).

Ten days after intervention, bacterial load of control group showed a significantly greater average of bacterial counts compared to its initial value (1 log increase, $P < 0.05$) indicating that caries continues to progress. On the other hand, statistically significant reduction ($P < 0.05$) of bacterial burden in aPDT group persisted until the end of experiment. This result demonstrates that microbial load was stable and did not return to initial values (Fig. 6). In fact, 3, 7 and 10 days post-treatment, a mean infectious burden of 5.39, 5.87 and 6.56 was achieved in the control group, while a significantly higher diminution ($P < 0.05$) was observed following aPDT (4.24, 4.2 and 4.78, respectively).

Figure 7 exhibits the TOACV for control and aPDT groups. Molars from animals submitted to caries induction were also evaluated at day 0 (induced-caries group). All data were normalized by the mean value of sound enamel, and signal change was expressed as percentage of variation. The TOACV was significantly higher for all groups compared with sound molar indicating that there was enamel demineralization. Besides, observe that median optical attenuation variation of decayed teeth before treatment was about 55%. Ten days post-treatment, aPDT group showed a median attenuation variation of 40%. No statistically significant differences were observed between induced-caries teeth before treatment and aPDT group ($P = 0.09$). On the other hand, optical attenuation for control group was 62%. This value was significantly higher compared with decayed molars before

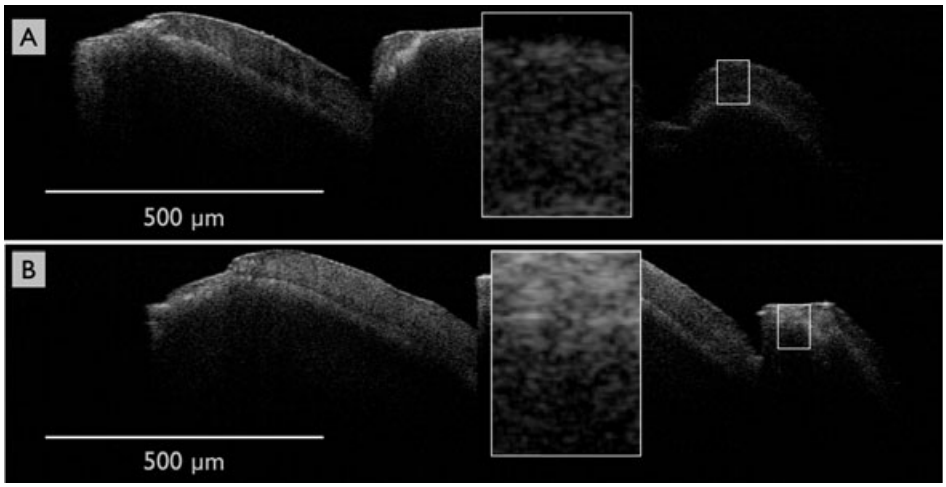

Figure 5. OCT images of rat molars. (A) Sound and (B) carious teeth evaluated at day 0 before treatment. Observe the increase of backscattered OCT signal in demineralized tissue of caries lesion. Inserts show a higher magnification of the region of interest analyzed.

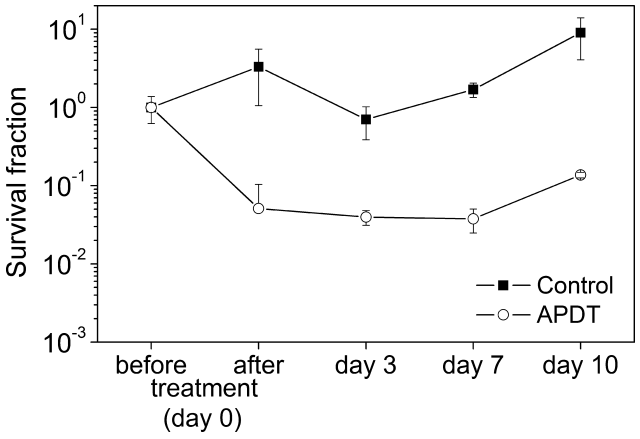

Figure 6. Effect of aPDT on oral microaerophilic bacteria. Data are means values. Bars are standard deviations. Three animals per group were used in each moment. On day 0, five animals were used.

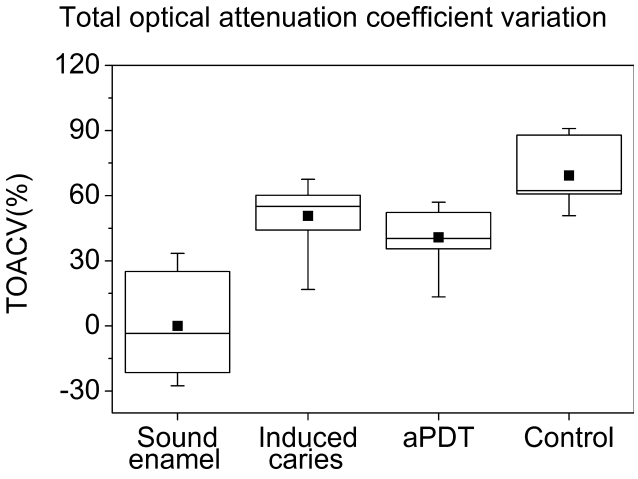

Figure 7. Evaluation of dental enamel demineralization by OCT represented by the total optical attenuation coefficient variation (TOACV). Sound enamel group depicts data obtained from samples caries free. Induced-caries group was evaluated before treatment. Samples from control and aPDT groups were evaluated 10 days after treatment. Attenuation coefficients from OCT signals were normalized by sound enamel mean value. Box data are lower quartile, median and upper quartile; ■ is mean and bars maximum and minimum values. $N = 9$ per group.

treatment ($P = 0.04$) and aPDT group ($P = 0.001$). Put together, our results demonstrate that aPDT was able to avoid further mineral loss while control group showed disease progression.

DISCUSSION

Dental caries is a common form of infectious disease that causes the demineralization of dental hard tissue leading to cavity formation, pain, endodontic infection and possible tooth loss if left untreated. The factors that determine if there will be or will not be progression of the disease are susceptible host, cariogenic microorganisms, diet and time (6).

Key elements of a biological approach dedicated mainly to prevention and early intervention for caries are the usage of remineralizing agents to tooth structure or regulation of cariogenic biofilms. Nowadays, the standard method to prevent dental caries is through constant exposure of the teeth to low concentrations of fluoride. This approach would turn the host less susceptible to acid demineralization and enhance teeth remineralization. Dietary as well as biofilm control would be another possibility but it relies on patient cooperation, which is especially difficult in children and teenagers. Furthermore, in the past 50 years there was increased prevalence of fluorosis in optimally fluoridated communities, as well as in fluoride-deficient areas, probably due to dietary fluoride supplements (32). The ingestion of fluoride-containing toothpastes used by toddlers and young children without supervision can provide another source of fluoride exposure (33).

In this work, we investigated the use of aPDT as a strategy to avoid enamel demineralization and arrest caries progression by controlling the oral microbiota. The proposition to intervene *via* aPDT on the microorganims would render the individual less susceptible to caries. Our results demonstrate that aPDT is a suitable method to control tooth demineralization through bacterial reduction.

Initially we evaluated if our experimental model was adequate to induce early caries. As rat molars have tiny dimensions and white spots could not be observed by visual inspection, we used OCT to confirm enamel demineralization before aPDT. A brighter area was noted in caries-induced group corroborating mineral loss, which is intimately related to caries diagnosis (29). After that, we chose to apply aPDT using methylene blue and a red LED.

It has been reported that cariogenic microorganisms organized in biofilm and even in a clinical situation can be sensitive to aPDT (16,22,34). However, for a successful aPDT some factors must be considered, *e.g.* photosensitizer and light source. MB is a phenotiazinic dye that has been used to kill microbial cells due to its physico-chemical properties, such as a large long-wavelength absorption band and a high quantum yield for the generation of both long-lived triplet excited state and cytotoxic ROS (35–37). Besides, it has been shown that MB dimer species as well as the monomer species are responsible for photodisruption of bacteria (36,37). For this reason we choose to use a LED as it presents a large emission band centered on $\lambda = 640 \pm 30$ nm capable to excite dimers and monomers of MB.

Our study showed that MB in combination with a red LED significantly reduced bacterial load in carious sites. A bacterial decrease of about 1.6 $\log_{10}$ was observed, and interestingly a significant reduction was maintained for 10 days after therapy. Bacterial killing and disorganization of biofilm could explain the reduction of microbial load following aPDT (11,34,38,39). These effects have been reported in oral biofilms and biofilm related oral diseases, such as periodontitis, in which use of aPDT as an adjuvant treatment has demonstrated the improvement of important clinical aspects (17–19). Two points should be highlighted from our results; first, no recolonization with microaerophilic bacteria was observed and the reasons for that are not completely clear so far, since cariogenic diet was maintained until the end of the study. Second, the importance of 1.6 logs of killing relies on clinical significance. In fact, in a clinical situation the purpose of the treatment is not to eradicate total microorganisms in oral cavity but to control microbiota and bring it to a standstill situation compatible to health. Our findings from OCT corroborate that after aPDT the demineralization process was arrested.

The total attenuation coefficient of OCT signal within the tooth is a parameter that measures the light-tooth interaction and it is sensitive to tooth demineralization occurring under enamel surface (31). In this work, we calculated the TOACV related to healthy teeth. The greater TOACV, higher demineralization occurs in dental structure. All groups showed higher TOACV values than sound molars, indicating that some degree of demineralization affected the teeth submitted to caries induction. Moreover, TOACV from 930 nm OCT signal of aPDT group was significantly smaller than control group 10 days post-treatment but similar to decayed teeth (before treatment). These findings reveal that aPDT was competent to avoid further mineral loss, whereas in the absence of an intervention carious lesions continued to progress.

Besides a significant caries reduction resulted from prevention strategies (1,3,7,38), there is still a need for the development of antimicrobial agents that are safe for use and their mode of action should be synergistic or complementary with that of fluoride (5,40). The correlation between bacterial reduction and arrested enamel demineralization in our work provides real evidence that aPDT could avoid caries progression. Besides, aPDT shows harmless side-effects on biologic tissues and also it does not promote structural changes on dental hard tissue (13,38).

It is noteworthy that in our model the cariogenic diet was provided to the animals during the experimental period, and as no external source of fluoride was given to the animals besides that supplied in the drinking water, the lower demineralization observed on aPDT group can be directly linked with the microbial reduction.

The original finding that aPDT regulates oral microaerophilic bacteria *in vivo* and arrests tooth demineralization has to be confirmed by long-term and clinical studies. This unique approach also represents a new possibility in caries prevention that can be extremely useful to be implemented in public health mostly in high-risk population. Besides, interventions during childhood could improve long-term health.

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REFERENCES

1. Sullivan, R., P. Santarpia, S. Lavender, E. Gittins, Z. Liu, M. H. Anderson, J. He, W. Shi and R. Eckert (2011) Clinical efficacy of a specifically targeted antimicrobial peptide mouth rinse: Targeted elimination of *Streptococcus mutans* and prevention of demineralization. *Caries Res.* **45**, 415–428.
2. Declerck, D., R. Leroy, L. Martens, E. Lesaffre, M. J. Garcia-Zattera, S. Vanden Broucke, M. Debyser and K. Hoppenbrouwers (2008) Factors associated with prevalence and severity of caries experience in preschool children. *Community Dent. Oral Epidemiol.* **36**, 168–178.
3. Pieper, K., S. Dressler, M. Heinzel-Gutenbrunner, A. Neuhauser, M. Krecker, K. Wunderlich and A. Jablonski-Momeni (2011) The influence of social status on pre-school children's eating habits, caries experience and caries prevention behavior. *Int. J. Public Health*, DOI: 10.1007/s00038-011-0291-3.
4. Werneck, R. I., M. T. Mira and P. C. Trevilatto (2010) A critical review: An overview of genetic influence on dental caries. *Oral Dis.* **16**, 613–623.
5. ten Cate, J. M. (2009) The need for antibacterial approaches to improve caries control. *Adv. Dent. Res.* **21**, 8–12.
6. Peters, M. C. (2010) Strategies for noninvasive demineralized tissue repair. *Dent. Clin. North Am.* **54**, 507–525.
7. Rao, A. and N. Malhotra (2011) The role of remineralizing agents in dentistry: A review. *Compend. Contin. Educ. Dent.* **32**, 26–33 quiz 34, 36.
8. Gonzalez-Cabezas, C. (2010) The chemistry of caries: Remineralization and demineralization events with direct clinical relevance. *Dent. Clin. North Am.* **54**, 469–478.
9. Wainwright, M., T. Dai and M. R. Hamblin (2011) Antimicrobial photodynamic therapy in the colon: Delivering a light punch to the guts? *Photochem. Photobiol.* **87**, 754–756.
10. Sharma, S. K., T. Dai, G. B. Kharkwal, Y. Y. Huang, L. Huang, V. J. De Arce, G. P. Tegos and M. R. Hamblin (2011) Drug discovery of antimicrobial photosensitizers using animal models. *Curr. Pharm. Des.* **17**, 1303–1319.
11. Huang, L., T. Zhiyentayev, Y. Xuan, D. Azhibek, G. B. Kharkwal and M. R. Hamblin (2011) Photodynamic inactivation of bacteria using polyethylenimine-chlorin(e6) conjugates: Effect of polymer molecular weight, substitution ratio of chlorin(e6) and pH. *Lasers Surg. Med.* **43**, 313–323.

12. Bourre, L., F. Giuntini, I. M. Eggleston, C. A. Mosse, A. J. Macrobert and M. Wilson (2010) Effective photoinactivation of Gram-positive and Gram-negative bacterial strains using an HIV-1 Tat peptide-porphyrin conjugate. *Photochem. Photobiol. Sci.* **9**, 1613–1620.
13. Prates, R. A., A. M. Yamada, L. C. Suzuki, C. M. Franca, S. Cai, M. P. Mayer, A. C. Ribeiro and M. S. Ribeiro (2011) Histomorphometric and microbiological assessment of photodynamic therapy as an adjuvant treatment for periodontitis: A short-term evaluation of inflammatory periodontal conditions and bacterial reduction in a rat model. *Photomed. Laser Surg.* **29**, 835–844.
14. Marotti, J., A. C. Aranha, P. Eduardo Cde and M. S. Ribeiro (2009) Photodynamic therapy can be effective as a treatment for herpes simplex labialis. *Photomed. Laser Surg.* **27**, 357–363.
15. Hayek, R. R., N. S. Araujo, M. A. Gioso, J. Ferreira, C. A. Baptista-Sobrinho, A. M. Yamada and M. S. Ribeiro (2005) Comparative study between the effects of photodynamic therapy and conventional therapy on microbial reduction in ligature-induced peri-implantitis in dogs. *J. Periodontol.* **76**, 1275–1281.
16. Konopka, K. and T. Goslinski (2007) Photodynamic therapy in dentistry. *J. Dent. Res.* **86**, 694–707.
17. Lulic, M., I. Leiggener Gorog, G. E. Salvi, C. A. Ramseier, N. Mattheos and N. P. Lang (2009) One-year outcomes of repeated adjunctive photodynamic therapy during periodontal maintenance: A proof-of-principle randomized-controlled clinical trial. *J. Clin. Periodontol.* **36**, 661–666.
18. Braun, A., C. Dehn, F. Krause and S. Jepsen (2008) Short-term clinical effects of adjunctive antimicrobial photodynamic therapy in periodontal treatment: A randomized clinical trial. *J. Clin. Periodontol.* **35**, 877–884.
19. de Oliveira, R. R., H. O. Schwartz-Filho, A. B. Novaes Jr. and M. Taba Jr. (2007) Antimicrobial photodynamic therapy in the non-surgical treatment of aggressive periodontitis: A preliminary randomized controlled clinical study. *J. Periodontol.* **78**, 965–973.
20. Garcez, A. S., S. C. Nunez, M. R. Hamblin and M. S. Ribeiro (2008) Antimicrobial effects of photodynamic therapy on patients with necrotic pulps and periapical lesion. *J. Endod.* **34**, 138–142.
21. Garcez, A. S., S. C. Nunez, M. R. Hamblim, H. Suzuki and M. S. Ribeiro (2010) Photodynamic therapy associated with conventional endodontic treatment in patients with antibiotic-resistant microflora: A preliminary report. *J. Endod.* **36**, 1463–1466.
22. Guglielmi, C. D., M. R. Simionato, K. M. Ramalho, J. C. Imparato, S. L. Pinheiro and M. A. Luz (2011) Clinical use of photodynamic antimicrobial chemotherapy for the treatment of deep carious lesions. *J. Biomed. Opt.* **16**, 088003.
23. Ciancaglini, P., T. P. Paulino, P. P. Magalhaes, G. Thedei and A. C. Tedesco (2005) Use of visible light-based photodynamic therapy to bacterial photoinactivation. *Biochem. Mol. Biol. Edu.* **33**, 46–49.
24. Muller, K. P., C. R. Rodrigues, S. C. Nunez, R. Rocha, A. O. Jorge and M. S. Ribeiro (2007) Effects of low power red laser on induced-dental caries in rats. *Arch. Oral Biol.* **52**, 648–654.
25. Bowen, W. H. and R. A. Lawrence (2005) Comparison of the cariogenicity of cola, honey, cow milk, human milk, and sucrose. *Pediatrics* **116**, 921–926.
26. Prates, R. A., I. T. Kato, M. S. Ribeiro, G. P. Tegos and M. R. Hamblin (2011) Influence of multidrug efflux systems on methylene blue-mediated photodynamic inactivation of Candida albicans. *J. Antimicrob. Chemother.* **66**, 1525–1532.
27. Jett, B. D., K. L. Hatter, M. M. Huycke and M. S. Gilmore (1997) Simplified agar plate method for quantifying viable bacteria. *BioTechniques* **23**, 648–650.
28. Freitas, A. Z., D. M. Zezell, M. P. A. Mayer, A. C. Ribeiro, A. S. L. Gomes and N. D. Vieira (2009) Determination of dental decay rates with optical coherence tomography. *Laser Phys. Lett.* **6**, 896–900.
29. Freitas, A. Z., D. M. Zezell, N. D. Vieira, A. C. Ribeiro and A. S. L. Gomes (2006) Imaging carious human dental tissue with optical coherence tomography. *J. Appl. Phys.* **99**, 024906.
30. de Melo, L. S., R. E. de Araujo, A. Z. Freitas, D. Zezell, N. D. Vieira, J. Girkin, A. Hall, M. T. Carvalho and A. S. Gomes (2005) Evaluation of enamel dental restoration interface by optical coherence tomography. *J. Biomed. Opt.* **10**, 064027.
31. Popescu, D. P., M. G. Sowa, M. D. Hewko and L. P. Choo-Smith (2008) Assessment of early demineralization in teeth using the signal attenuation in optical coherence tomography images. *J. Biomed. Opt.* **13**, 054053.
32. Billings, R. J., R. J. Berkowitz and G. Watson (2004) Teeth. *Pediatrics* **113**, 1120–1127.
33. Whitford, G. M., S. M. Adair, C. M. Hanes, E. C. Perdue and C. M. Russell (1995) Enamel uptake and patient exposure to fluoride: Comparison of APF gel and foam. *Pediatr. Dent.* **17**, 199–203.
34. Zanin, I. C., R. B. Goncalves, A. B. Junior, C. K. Hope and J. Pratten (2005) Susceptibility of *Streptococcus mutans* biofilms to photodynamic therapy: An in vitro study. *J. Antimicrob. Chemother.* **56**, 324–330.
35. Wainwright, M., D. A. Phoenix, S. L. Laycock, D. R. Wareing and P. A. Wright (1998) Photobactericidal activity of phenothiazinium dyes against methicillin-resistant strains of *Staphylococcus aureus*. *FEMS Microbiol. Lett.* **160**, 177–181.
36. Usacheva, M. N., M. C. Teichert and M. A. Biel (2003) The interaction of lipopolysaccharides with phenothiazine dyes. *Lasers Surg. Med.* **33**, 311–319.
37. Usacheva, M. N., M. C. Teichert and M. A. Biel (2003) The role of the methylene blue and toluidine blue monomers and dimers in the photoinactivation of bacteria. *J. Photochem. Photobiol. B* **71**, 87–98.
38. Soukos, N. S. and J. M. Goodson (2011) Photodynamic therapy in the control of oral biofilms. *Periodontol. 2000* **55**, 143–166.
39. Garcez, A. S., S. C. Nunez, M. S. Baptista, N. A. Daghastanli, R. Itri, M. R. Hamblin and M. S. Ribeiro (2011) Antimicrobial mechanisms behind photodynamic effect in the presence of hydrogen peroxide. *Photochem. Photobiol. Sci.* **10**, 483–490.
40. Twetman, S. (2010) Treatment protocols: Nonfluoride management of the caries disease process and available diagnostics. *Dent. Clin. North Am.* **54**, 527–540.