Photodynamic therapy on bacterial reduction in dental caries. *In vivo* study

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ABSTRACT

The reduction of pathogenic microorganisms in supragingival plaque is one of the principal factors in caries prevention and control. A large number of microorganisms have been reported to be inactivated *in vitro* by photodynamic therapy (PDT). The purpose of this study was to develop a rat model to investigate the effects of PDT on bacterial reduction in induced dental caries. Twenty four rats were orally inoculated with *Streptococcus mutans* cells (ATCC 25175) for three consecutive days. The animals were fed with a cariogenic diet and water with 10% of sucrose *ad libitum*, during all experimental period. Caries lesion formation was confirmed by Optical Coherence Tomography (OCT) 5 days after the beginning of the experiment. Then, the animals were randomly divided into two groups: Control Group: twelve animals were untreated by either light or photosensitizer; and PDT Group: twelve animals were treated with 100μM of methylene blue for 5min and irradiated by a Light Emitting Diode (LED) at $\lambda = 640\pm30$nm, fluence of 172J/cm^2, output power of 240mW, and exposure time of 3min. Microbiological samples were collected before, immediately after, 3, 7 and 10 days after treatment and the number of total microaerophiles was counted. OCT images showed areas of enamel demineralization on rat molars. Microbiological analysis showed a significant bacterial reduction after PDT. Furthermore, the number of total microaerophiles in PDT group remained lower than control group until 10 days post-treatment. These findings suggest that PDT could be an alternative approach to reduce bacteria in dental caries.

Keywords: photoinactivation, methylene blue, enamel demineralization, optical coherence tomography

1. INTRODUCTION

Dental caries has been indicated as one of the most contagious chronic disease in humans[1], and it presents localized or progressive forms[2]. Furthemore, income populations in underdeveloped countries and immunecompromised patients are described as most susceptible target of this disease, which produce progressive demineralization of dental tissue, tooth cavity, pain and endodontic lesions. Fortunately, lesions evolution can be neutralized at any stage of its development by biofilm control that produces a favorable environment to stop tooth demineralization[3, 4]. The dental biofilm supports a micro-ecosystem of bacteria that exhibit a variety of physiological characteristics. In particular, the production of acid through the metabolism of carboydrates by acidogenic microorganisms within these biofilms, and the subsequent decrease in environmental pH is responsible for demineralization of the tooth surface and formation of dental caries. Once the acidic environment has been established, *Streptococci mutans* and other aciduric bacteria may increase and promote lesion development by sustaining the environment characterized by net mineral loss. Hence, high proportions of aciduric bacteria may be considered biomarkers of sites of particularly rapid caries progression.[5]

Photodynamic therapy (PDT) is a phototherapy based on the utilization of photosensitizers in combination with harmless visible light of the correct wavelength to excite the photosensitizer[6]. The cells that are considered therapeutic targets are stained with the photosensitizing agent and irradiated with light[7]. The photodynamic process rapidly generates reactive oxygen species (ROS) as for instance peroxides, hydroxyl radicals, superoxide ions and singlet oxygen, the last one being implicated as the major causative agent of cellular damage in photodynamic process[8]. Some studies have

Biophotonics: Photonic Solutions for Better Health Care II, edited by Jürgen Popp, Wolfgang Drexler, Valery V. Tuchin, Dennis L. Matthews, Proc. of SPIE Vol. 7715, 77151R

reported that PDT-induced bacterial killing reduced bacterial numbers by more than 10-fold in *Streptococcus mutans, Streptococcus sobrinus* and *Streptococcus sanguinis* biofilms[9-12].

Optical coherence tomography (OCT) is a diagnostic imaging technology in which the coherence features of photons are exploited, leading to an imaging technology that is capable of producing high-resolution cross-sectional images of the internal microstructure of living tissue. This technique is based on the detection of optical properties of tissue, like the backscattering coefficient and refraction index variation. With this information, it is possible to construct an image of internal structures. In the OCT image, we use a false color map that represents the backscattering coefficient. The white color represents a high-scattering coefficient and the black color represents a low-scattering coefficient. OCT in dentistry has been used to *in vitro* studies evaluating enamel interface restoration[13], early caries diagnosis[14, 15] and analysis of the performance of dental materials [16].

The utilization of animal models has substantially improved the knowledge about dental caries. Rats have been used successfully to study anticaries efficacy of chemical agents, immunization, cariogenicity of diet, maturation of enamel and bacterial involvement in caries process[17, 18]. The PDT has previously been used to kill pathogenic microorganisms *in vitro* however, its use to treat caries *in vivo* infected animal models or patients has not still been described in literature[19].

The purpose of this study was to develop an induced-dental caries rat model to investigate the effects of PDT on bacterial reduction.

2. MATERIALS AND METHODS

2.1 Inoculum Preparation

The microorganism used in this study was *Streptococcus mutans* (ATCC 25175), which was sub-cultured from vial stock onto TSA (Tryptic Soy Agar). The incubation conditions were in 5-10% CO_2 atmosphere for 48h at 37ºC. Inoculum was prepared with 10^8 cells/mL of *S. mutans* on BHI (Brain heart infusion). The *S. mutans* suspensions were measured at 530nm in an optical spectrophotometer in the transmittance of 70±5 %.

2.2 Establishment of rat caries model

Twenty six male Wistar rats with 20 days of age were selected for this study. All animal work was performed under and complied with the local Animals Ethics Committee. In the first day of experiment, twenty four rats were anesthetized by ketamine/xylazine intra-peritoneal injection. Thereafter, they were inoculated with *Streptococcus mutans* in BHI suspension. Two hundred-μL of microorganism suspension was injected directly into the oral cavity.

In the second and third days of experiment, the animals were also infected with *S. mutans*. The suspension was added to the animal's food; thus, all animals received the microorganisms during three consecutive days. The experimental schedule is shown in Table 1.

Table 1- Experimental schedule

Procedure Description	Day
Animal birth	0
Animal weaned, cariogenic diet beginning and first day of bacterial inoculation	20
Second day of inoculation of *S. mutans*	21
Third day of inoculation of *S. mutans*	22
Treatment and e first assessment	25
Second assessment	28
Third assessment	32
Fourth assessment	35

The animals were mainted under standard laboratory conditions receiving 50g/dia of cariogenic diet[20] and to increase the cariogenic challenge, the animals also received water with 10% of sucrose[18] *ad libitum*, during all experiment. In this study, two animals not submitted to caries induction protocol were used as healthy teeth for bacterial comparison.

2.3 Caries lesions diagnosis by OCT

Optical coherence tomography images were produced with a Thorlabs OCT system (OCP930SRS, Thorlabs, Newton,NJ, USA), wich comprised a superluminescent light emitting diode working at $\lambda = 930 \pm 5$nm with 100 ± 5nm FWHM and an optical power of 2mW, resolution of 6.2 μm in air and maximum image depth of 1.6mm in air. This system was used to generate cross-sectional images of rat molars fixed in dental wax, and put on the sample holder capable of maintaining the fiber straight, without natural distortions.

2.4 Photosensitizer and light sources

The solution of MB (methylene blue) was prepared by dissolution of the powder (Sigma Ltd, Poole, UK) in distillated water in a concentration of 10mM, which was filtered through a sterile filter membrane (022μm, Millipore, São Paulo, Brazil). Before the PDT session, the concentrated photosensitizer solution was diluted in distillated water in the rate of 1/100 that resulted in a final concentration of 100μM [21].

The light sources used for the photosensitizer excitation was a light emitting diode (LED) device (MMOPTICS, São Carlos, Brazil) with an emission band centered at $\lambda = 640 \pm 30$nm. The irradiation parameters used were irradiance of 480mW/cm^2, output power of 240mW, spot diameter of 0.8cm (0.5cm^2 area) and exposure time of 180s.

2.5 Experimental procedure

Three days after the last inoculation, the animals were again anesthetized and randomly distributed into two groups (n=12): C (contol group) and PDT (photodynamic therapy group). Animals of the C group received 0.2mL of saline solution and were untreated by either light or photosensitizer. PDT group was treated with 0.2mL of 100μM methylene blue for five minutes (pre-irradiation time) and then irradiated for 3min. The light parameters are shown in Table 2. Irradiation was performed on upper molars of the right side with the probe in contact with the teeth. One irradiation was enough to cover the whole affected area.

Table 2- Light parameters

Parameters	
Wavelength (nm)	$\lambda = 640 \pm 30$
Spot size (cm^2)	0.5
Exposure time (s)	180
Output power (mW)	240
Fluence rate (mW/cm^2)	480
Fluence (J/cm^2)	172

2.6 Microbiological analysis

Both groups were evaluated immediately after the treatment and following 3, 7 and 10 days, as shown in the experimental schedule (Table 1). For each evaluation, we used three animals by group. The animals were euthanized in CO_2 chamber, and then the maxillaries were surgically removed, dissected and the molars were carefully extracted. For microbial recovery, the teeth were stored in individual eppendorfs containing 1mL of sterile 0.85% saline solution. The eppendorfs were agitated in vortex for 30s and maintained in rest for 30 minutes. After this period, the cells suspensions

were serially diluted in PBS to generate dilution of 10^{-1} to 10^{-4} times the original concentration. Ten-µL aliquots of each diluition were streaked onto a TSA plaque in triplicate and incubated for 24h at 37°C to allow colony formation.

2.7 Statistics

The yeast colonies were counted and converted into colony-forming units (cfu) for analysis. All samples were submitted to this process and statistical analysis of the experimental data was perfomed using one-way analysis of variance (ANOVA). Mean comparisons were carried out with the Tukey's test, which retains the overall significance level at 5% ($p \leq 0.05$).

3. RESULTS AND DISCUSSION

3.1 OCT

Figure 1 displays clinical and OCT images of rat molar 5 days after beginning of the experiment and before the treatment. We can observe a fissure on the occlusal surface on figure 1A indicating caries formation. Figure 1B shows a longitudinal section by OCT. The depression observed correspond to the sulcus of figure1A. The intense bright characterizes a higher scattering of this area suggesting the enamel demineralization[14].

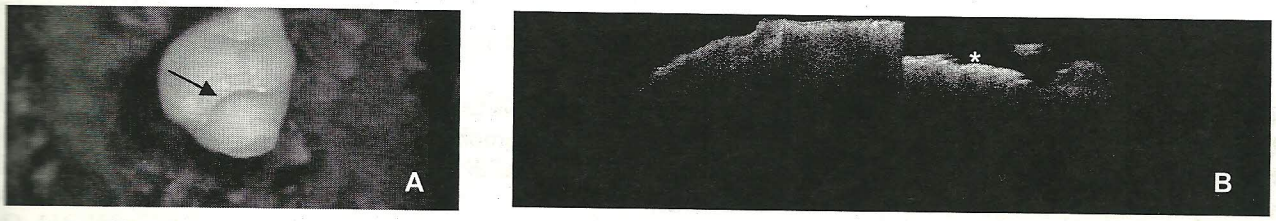

Figure 1. A- Clinical image of the rat molar occlusal surface. The arrow points to the sulcular area. B- OCT image of a longitudinal section corresponding to the sulcus of image A. Note the enamel demineralization represented by the bright area (*).

3.2 Microbiological analysis

The number of microaerophilic bacteria recovered from infected rats (C group, day 25) was similar to that from healthy teeth. This result suggests that the oral rat microbiota was only qualitatively altered after *S. mutans* inoculation. Our study showed that microaerophilic organisms can be photoinactivated *in vivo* by light emiting diode ($\lambda = 640 \pm 30$nm) after have been sensitized with methylene blue. A significant bacterial reduction was observed after PDT for all experimental moments. In addition, the number of viable bacteria in PDT group remained significantly lower than in control group. Besides bacterial reduction, these results suggest that recolonization of dental sites were not observed after PDT 10 days post-treatment (Figure 2).

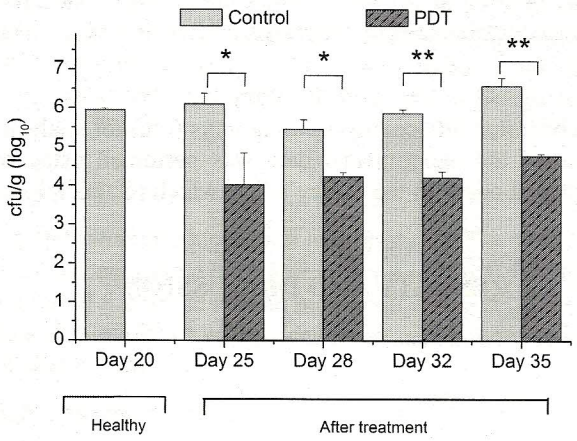

Figure 2. Microaerophilic bacteria recovered during the experiment. Column represents means values and bars are SD of the number of recovered cells from experimental animals (n=3). Symbols represent statistical significant differences between groups at each experimental moment (* $p<0.05$ and ** $p<0.01$).

4. CONCLUSION

In this study, rat caries model was used for studying microaerophilic bacteria reduction with PDT. Caries lesions could be successfully developed in rat molars, as confirmed by OCT. Microaerophilic organisms were photoinactivated *in vivo* by light emiting diode (λ= 640 ± 30nm) after have been sensitized with methylene blue. The reduction of bacterial viability was kept by 2 logs even 10 days after the treatment.

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