Antimicrobial photodynamic therapy mediated by methylene blue in surfactant vehicle on periodontopathogens

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Running Title: APDT on periodontopathogens

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ABSTRACT

Background: Periodontal disease (PD) is a chronic inflammatory disease caused by the presence of microbial biofilm. The aim of this study was to evaluate antimicrobial effect of antimicrobial photodynamic therapy (APDT) mediated by methylene blue (MB) in monomer form on *A. actinomycetemcomitans* and *P. gingivalis*.

Methods*: A. actinomycetemcomitans* ATCC 29523 and *P. gingivalis* ATCC 33577 were cultured on anaerobic jars at 37°C for 48 h, and we tested APDT in the presence of 0.25% sodium dodecyl sulfate (SDS) in phosphate-buffered saline (PBS) or in PBS alone. APDT was carried out with 100 µM MB under laser radiation (PhotolaseIII, DMC, Brazil) at ʎ = 660 nm and parameters as following (P = 100 mW; I = 250 mW/cm^2, and doses of 15, 45 and 75 J/cm^2).

Results: Following APDT, PBS groups of *A. actinomycetemcomitans* presented 4 Logs of microbial death after 5 min irradiation. However, there was no bacterial reduction in SDS groups. On the other hand, *P. gingivalis* was sensitive to APDT in the presence of 0.25% SDS with 2 logs reduction from dark toxicity.

Conclusion: The presence of 0.25% SDS can lead to different responses depending on the different microbial species.

Keywords: Periodontopathogenic bacteria, Surfactant vehicle, Photodynamic antimicrobial chemotherapy (PACT), Periodontitis, Photosensitizer.

1. Introduction

Antimicrobial photodynamic therapy (APDT), also known as photodynamic antimicrobial chemotherapy (PACT), was used in periodontal treatment based on scaling and root planing, as an adjunct form to decrease the bacterial load in the periodontal pocket and avoid using systemic antimicrobials (1–3). APDT for periodontitis consists of the topical administration of a photosensitizer (PS) that is distributed within the periodontal pockets with accumulation on microbial biofilms. Activation of the PS *in situ* by light exposure using a laser or LED with the appropriate dosimetric parameters, leads to electronic excitation and either subsequent electron or energy transfer to oxygen in the immediate environment and the production of reactive oxygen species (ROS) (4). Such species, typically singlet oxygen, are highly damaging to simple microbial cells, including bacteria (2,5,6). The PS most used in dentistry is methylene blue (MB), which is a planar heterocyclic dye derived from phenothiazine (formed by two benzene rings connected by a nitrogen atom and a sulfur atom). Due to its planar nature, MB can aggregate depending on the medium in which it is found and its concentration. The aggregation state determines the types of photochemical reactions that occur, and these affect the efficiency of treatment with APDT. The presence of dimers or monomers is noticeable through the visible absorption spectrum, with the dimer showing absorption at 590 nm and the monomer at 665 nm (3,7). The dimer / monomer ratio is commonly used to characterize the degree of aggregation of methylene blue in a given medium (8–11).The application of MB in a solution of 0.25% w/v sodium dodecyl sulfate (SDS, surfactant vehicle) in phosphate-buffered saline (PBS), is intended to increase killing of the Gram-negative bacteria *A. actinomycetemcomitans* and *P. gingivalis* when compared

to methylene blue in phosphate-buffered saline (PBS). SDS acts as surfactant agent and has been safely used in oral formulations such as mouthwashes and toothpastes (12). SDS has a bacterial membrane-dissolving effect and is also able to decrease the molecular aggregation of the photosensitizer, increasing the amount of monomers and decreasing the amount of dimers (9,13). Previous studies showed that SDS with APDT on periodontal bacteria produced a significant bacterial reduction. However, more studies are necessary to elucidate the effect of the SDS component on bacterial inactivation (14). This study aims to compare the action of antimicrobial photodynamic chemotherapy using the photosensitizer in SDS and PBS, quantifying the microbial death of *A. actinomycetemcomitans* and *P. gingivalis*.

2. Materials and Methods

Experimental procedures were performed with *A. actinomycetemcomitans* ATCC 29523 and *P. gingivalis* ATCC 33577 cultured under anaerobiosis in brain heart infusion (BHI) broth medium and incubated at 37 ° C for a period of 48 h.

Photosensitizer - Methylene blue (Sigma-Aldrich, St Louis, USA) was used as photosensitizer. The powder was diluted in distilled water to a concentration of 10 mM, the solution was filtered through a sterile 0.22 µm membrane (Milipore, SP, Brazil) and stored at 5 ^{o}C protected from light. For the experiment, the mother solution was diluted 1/100 with distilled water to generate final concentration of 100 µM MB. In a plastic tube (Eppendorf), 1 mL PBS and 20 µL methylene blue at 100 µM were vortexed to homogenize the contents, thus creating a master solution. In two other plastic tubes, 500 µL of the main solution and 500 µL of

PBS were deposited and in the other 500 µL of the main solution and 500 µL of 0.5% SDS. The two plastic tubes were vortexed, thus forming two inocula containing the photosensitizer used during the experiment.

Optical UV-Visible absorption spectrum (200 - 800 nm) was obtained using a spectrophotometer (Shimadzu, Kyoto, Japan) to certify monomer and dimer ratio of 100 µM MB in PBS or 0.25% SDS with a 2 mm optical path quartz cuvette.

Light source - A diode laser (Photon Lase III, DMC, São Carlos, Brazil) was used for irradiation with the following parameters on table 1.

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Table 1 - APDT irradiation parameters used in the experiments

Parameters	
Wavelength (nm)	660.52 ± 0.71
Mode	Continuous
Output power	100 mW
Polarization	Random
Irradiated area	0.4 cm^2
Irradiance at the well	250 mW/cm^2
Irradiation time	60, 180, 300 s
Energy	6, 18, and 30 J
Radiant Exposure	15, 45, and 75 J/cm^2

Preparation of the suspension

The periodontopathogenic bacteria *Aggregatibacter actinomycetemcomitans* ATCC 29523 and *Porphyromonas gingivalis* ATCC 33577 were grown in BHI broth in a 15 mL plastic tube under anaerobiosis at a temperature regulated at 37°C for a period of 48 hours. After preparing the inoculum, it was placed in a centrifuge for 5 min to remove the supernatant liquid and then supplemented with

8 mL of PBS. It was vortexed to homogenize the content. To reduce the concentration of bacteria, a 150 µL aliquot of this inoculum was poured into another 15 mL plastic tube containing 8 mL of PBS, thus making the standard suspension.

In vitro antimicrobial photodynamic chemotherapy

The inoculum of *A. actinomycetemcomitans* (ATCC 29523) and *P. gingivalis* (ATCC 33577) were used in two experimental groups, the PBS group and the SDS group. Within these groups were 6 subgroups, the control group (no light or PS) was composed of bacteria in PBS suspension or bacteria in SDS suspension; laser group (laser irradiation for 5 min), photosensitizer group (PS) without irradiation, and APDT group with PS and irradiation times of 1, 3 and 5 min (29). In the PBS group, the photosensitizer methylene blue at a concentration of 100 µM in aqueous solution was used and in the SDS group, methylene blue at a concentration of 100 µM in 0.25% sodium dodecyl sulfate was used. In a 96-well plate, the wells corresponding to each experimental group were marked, where each well contained 180 µL of PBS to subsequently perform a serial dilution (10^{-1} to 10^{-5} times the original concentration). Another 96-well plate was used exclusively for the irradiation of the subgroups and the homogenization of 100 µL of the bacterial inoculum with 100 µL PBS or SDS. Each well was treated according to group and 20 µL sample was removed to perform 10-fold serial dilution. 10-microliter aliquots were then seeded on blood agar plates. The plates were incubated anaerobically in a bacteriological oven with the temperature regulated at 37°C for a period of 48 h to allow colony forming unit (CFU) growth to be counted for analysis. This experiment was performed separately 3 times.

Statistical analysis

The Kolmogorov-Smirnov test was used to analyze the data distribution and it was followed by two-way ANOVA test. Tukey test was used for comparison of means and groups were considered statistically different when $p<0.05$.

3. Results

After making the 100 µM methylene blue solution in PBS or 0.25% SDS, it was found that MB + SDS changed the color when compared to the inoculum with MB with PBS, presenting a lighter blue color. To assess this phenomenon, optical absorption spectrophotometry was used (figure 1). This demonstrates that the MB with SDS sample showed a lower dimerization process (peak 660 nm of the blue line) and a greater formation of monomer compared to the MB. With this we can verify that the photosensitizer MB with SDS showed greater absorption at 660 nm, when compared with MB + PBS (fig. 1).

<Enter figure 1>

After counting and analyzing the colony forming units in triplicate with the inoculum of *A. actinomycetemcomitans* ATCC 29523, the two types of treatment (APDT in aqueous solution and APDT in SDS) were compared and it was observed that APDT using methylene blue in aqueous solution produced a much higher bacterial kill when compared with APDT in 0.25% SDS. It was observed that the only groups that showed a statistical difference were the laser groups (with a $p = 6.7x10^{-6}$), the APDT group 3 min (with a $p = 3.68x10^{-8}$) and the APDT group 5 min (showing a $p = 1.28x10^{-8}$). The rest had p-values > 0.05. The control

group had a p of 0.94, the FS group (represented as 0 in the graph below) had a p of 0.12, the APDT1 group had a p of 0.38 (fig. 2A).

For *P. gingivalis* ATCC 33577, it was observed that APDT in both aqueous solution and 0.25% SDS showed significant bacterial death. However, in SDS all bacterial colonies were eliminated within 1 minute of irradiation, whereas in PBS this elimination only occurred after 5 min. The Control, laser and 0 groups showed a microbial reduction of approximately 3 logs difference between MB with SDS and MB. The result shows an aspect of toxicity of MB with SDS in our experimental conditions for *P. gingivalis*. Figure 2B, shows the microbial reduction as a function of the irradiation time for both media (fig. 2B).

<Enter figure 2>

4. Discussion

Previous clinical studies using antimicrobial photodynamic therapy as an adjunct to periodontal treatment have reported divergent results in clinical situations, mainly because there is no standardization of irradiation parameters and because of the great diversity of clinical probe depths, most of the time involving deep pockets. In these cases, the light cannot reach deep enough into the periodontal pockets, preventing photodynamic action from occurring because of the requirement for the appropriate combination of light and photosensitizing agent (PS). Another important point is the low oxygen tension in these situations, which also limits APDT. This *in vitro* study was carried out in order to verify if the alteration of the formulation of the conventional photosensitizer would alter its photochemical characteristics to the point of having an impact APDT on its

efficiency, thus indicating the potential for clinically relevant benefits. In this study, the parameters used were selected from a bibliographic survey, the most cited in the literature being the data used and in pilot studies of our group (5,15–17). The causative etiological factor of periodontal disease is the presence of biofilm. This is more difficult to disrupt or destroy because it has a matrix of extracellular polysaccharides and has a complex structure with different bacterial colonies that interact with each other. Some clinical and *in vitro* studies have shown that antimicrobial photodynamic therapy is not effective in disorganizing the biofilm when compared to conventional treatment. Peron *et al.*, report that although the results suggest that APDT exhibits antimicrobial action in *P. gingivalis*, a non-uniformity in the protocol and the limited number of included studies lead to the conclusion that the bactericidal efficacy of APDT against periodontal pathogens remains uncertain. The results obtained in previous studies correspond to the results obtained here, because despite the presence of SDS potentiating the bactericidal effect on *P. gingivalis* during irradiation in the presence of methylene blue, it was toxic for the control, laser groups and PS (without irradiation) without presenting a plausible explanation for this event. Additionally, using APDT in aqueous solution there was a bactericidal effect, although only APDT with 5 min of irradiation showed 100% bacterial kill, while in SDS it occurred after 1 minute of irradiation. In *A. actinomycetemcomitans* it produced a bacterial kill of 4 logs in PBS solution while in SDS there was no significant decrease. It is possible that there was an interaction between the bacteria and the surfactant vehicle, changing its response to APDT. It may have happened due to the chosen concentration of SDS and by *A. actinomycetemcomitans* to present in its

cytoplasm an enzyme called catalase, which is capable of breaking up the molecules of hydrogen peroxide, transforming into H_2O + ½ O_2. These phenomena have no explanation, requiring further studies in the long term (5,17,18).

In this experiment, SDS produced a decrease in MB dimer formation, thus increasing the percentage of free monomer molecules, which reflects on the absorption at 660nm. In this situation, it is expected a better effect of APDT. Most clinical and *in vitro* studies that reported efficacy in APDT were in fungi, mainly in *C. albicans*, showing good results in the amount of fungal death. As in Da Collina *et al.*, who concluded that the use of 0.25% SDS was the only vehicle that improved the effectiveness of the methylene blue photosensitizer in APDT a planktonic culture of *C. albicans*. Few articles on periodontopathogenic microorganisms found that, within the parameters used, methylene blue-mediated APDT in a surfactant vehicle achieved significant levels of microbial reduction with 5 min of irradiation. Some authors suggest that *A. actinomycetemcomitans* can be inactivated by APDT mediated by methylene blue with differences depending on the time of exposure. There were no statistically significant differences between the time of 1 and 3 minutes of irradiation. On the other hand, 5 min of APDT showed 99.85% of bacterial reduction. Alvarenga *et al.*, also reported that APDT used as an adjuvant treatment showed effective short-term control of periodontitis infection. These results may have occurred due to the periodontal pockets having a clinical probing depth of 5 to 6 mm, being relatively shallow and thus presenting more oxygen in the subgingival portion (14,19–22)

APDT appears to be a promising option for reducing the amount of periodontopathogenic microorganisms in combination with conventional therapy. As it is controversial in the literature, further studies are needed to answer these questions regarding the response of *A. actinomycetemcomitans* and *P. gingivalis* and achieve a standardization of irradiation parameters.

5. Conclusions

A. actinomycetemcomitans showed bacterial reduction in APDT in aqueous solution, with a decrease of 4 logs during irradiation of 5 min. On the other hand, the APDT in 0.25% SDS did not present a significant reduction in the amount of bacterial death in the groups irradiated in the presence of MB. Under the parameters used in this experiment, the use of SDS did not improve the effect of APDT on *A. actinomycetemcomitans*.

For APDT in *P. gingivalis* with the use of methylene blue in aqueous solution, a bacterial reduction was observed from the moment that there was irradiation with the presence of the photosensitizer. APDT in 0.25% SDS showed toxicity in the control, laser and PS groups, and during irradiation with the use of photosensitizer, they presented 100% bacterial death in the first irradiation period. It is concluded that the presence of SDS can lead to different responses, depending on the bacterial species.

Acknowledgement

The authors thanks to UNINOVE to research support.

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Figure legends

Figure 1 Absorption spectra of photosensitizers MB and MB + SDS in PBS solvent.

Figure 2 A) Log_{10} mean and standard deviation (CFU/mL) of the *A. actinomycetemcomitans* groups in different media. (*) represents statistical difference between groups in PBS and SDS in PBS dilution. The intragroup statements are reported in the text. Stripe bars graphic represents the PBS group and black bars represents 0.25% SDS in PBS solvent groups. Fig 2 B) indicate *P. gingivalis* data. The patters and symbols are the same.