Photosensitization of *Aggregatibacter actinomycetemcomitans* with methylene blue: A microbiological and spectroscopic study

Aécio M. Yamada Júnior [a]; Renato A. Prates[a]; Silvana Cai[b]; Martha S. Ribeiro[a,*]
[a]Center for Lasers and Applications, Instituto de Pesquisas Energéticas e Nucleares, IPEN/CNEN-SP, 05508-000, São Paulo, Brazil
[b]Microbiology Department, Instituto de Ciências Biomédicas, Universidade de São Paulo, ICB/USP, 05508-900, São Paulo, Brazil

ABSTRACT

The aim of this study was to determinate the efficiency of methylene blue (MB) to kill cultures of *Aggregatibacter actinomycetemcomitans* under red light and to investigate MB photobleaching by optical absorption spectroscopy. Bacteria were diluted in aqueous solution, putted in glass tubes and distributed in 5 groups: (L-MB-) control group; (L+MB-) laser alone by 5min; (L-MB+) MB alone through 5min; (3L+MB+) MB+laser 3min; (5L+MB+) MB+laser 5min. Laser parameters were P=30mW, λ=660nm, E=9J in 5min and E=5.4J in 3min. The samples were diluted and bacterial colonies were counted and converted into colony forming units (CFU). Absorption spectra of the MB-stained bacterial suspension and photosensitized bacterial suspension were obtained. Groups L-MB-, L+MB-, and L-MB+ did not show a decrease in CFU/mL. L+MB+ groups showed a significant decrease in CFU/mL but no statistically significant differences were observed between 3min and 5min. Spectroscopy showed that MB is photodegraded after irradiation and that dimer species are more notably consumed than monomeric species. These results suggest that MB is a suitable photosensitizer to reduce *A. actinomycetemcomitans*, and that 3min of irradiation are enough to produce a significant effect. Due to the spectral changes observed on MB solution after irradiation a type I mechanism may be involved.

Keywords: bacterial photoinactivation, low-power laser light, methylene blue photobleaching, photodynamic therapy, red light

INTRODUCTION

Aggregatibacter actinomycetemcomitans is one of the most important bacteria in the aggressive periodontal disease etiology. Frequently, *A. actinomycetemcomitans* has been found in more than 85% of patients with aggressive periodontitis and approximately 31% of patients with chronic periodontitis[1]. Studies have demonstrated an association between periodontal disease severity and risk of coronary heart disease and stroke[2,3].

Periodontal disease, once established, provides a biological burden of endotoxin and inflammatory cytokines, which serve to initiate and exacerbate atherogenesis and thromboembolic events[2]. The goals of periodontal therapy have consequently been directed towards eliminating *A. actinomycetemcomitans* from the oral cavity[4] . The use of systemic antibiotics as an adjunctive in the treatment of periodontal disease has been necessary. However, over-use of antibiotics has been a major culprit in the production of drug-resistant organisms. Therefore, the application of an alternative method to eradicate bacteria from periodontal pockets is highly desirable.

Photodynamic therapy (PDT), which involves the use of low power lasers with appropriate wavelength to kill cells or microorganisms previously treated with a photosensitizer drug, is an athermal alternative approach. The excited photosensitizer reacts with the substrate, mostly oxygen or water, to produce highly reactive oxygen species, as free radicals and/or singlet oxygen. These compounds cause injury and death of microorganisms. The selective action of PDT is one of the most important characteristics of this therapy, which is deadly for microorganisms prior than normal cells[5,6,7].

[*] marthasr@usp.br; phone: 55-11-31339197; fax: 55-11-31339374; www.ipen.br

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Culture of *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *A. actinomycetemcomitans* were treated with a range of photosensitizers and then exposed to light. Toluidine blue and methylene blue were effective lethal photosensitizers of all three target organisms, enabling substantial light dose-related reductions in viable counts[8]. Therefore, this technique may be useful as a means of eliminating periodontopathogenic bacteria from diseased sites.

Methylene Blue (MB) shows strong absorption at the red end of the visible spectrum and presents an easy transit through the cellular membrane in Gram-positive as well as Gram-negative bacterial species[9]. Furthermore, it is suggested that photobleaching processes of MB proceeds with different mechanisms[10].

The purpose of this study was to determinate the efficiency of MB to kill cultures of *Aggregatibacter actinomycetemcomitans* under red light and to investigate MB photobleaching by optical absorption spectroscopy.

2- MATERIAL AND METHODS

A 30mW GaAlAs diode laser with λ= 660nm was used in this study. Bacterial suspensions were irradiated from the bottom of the glass tube. The laser beam passed through all suspension. *A. actinomycetemcomitans* strains were subcultured in Tryptic Soy Agar (TSA) containing 6 g/L yeast extract and incubated under microaerophilic conditions, at 37°C for 48 h. Catalase and morphologic Gram-test were used to confirm species identification. The strains were diluted in an optical density No. 0.5 McFarland standard solution (1.5×10^8CFU/mL). Nine hundred-μL aliquots were taken and distributed into five glass tubes. The control group (L-MB-) was untreated by either a laser or a photosensitizer; in the laser group (L+MB-), the bacterial suspension was irradiated for 5min with an energy dose of 9J/cm^2 in the absence of the photosensitizer; in the MB group (L-MB+), methylene blue was added to the suspension to a final concentration of 0.01% w/V for 5min in dark conditions; in the PDT groups, 0.01% w/v MB was added to the bacterial suspension for 5 min (pre-irradiation time) and subsequently treated by laser for 3min (3L+MB+), and 5min (5L+MB+) with energy doses of 5.4J/cm^2 and 9J/cm^2, respectively.

One-hundred μL of PBS (pH 7.2) was added to groups L-MB- and L+MB- and the same volume of MB 0.1% was added to groups L-MB+, L3+MB+ and L5+MB+, resulting in a final concentration of 0.01%w/V. The estimated cell concentration decreased to 1.35×10^8CFU/mL in all groups. After irradiation procedures, each group was serially diluted and bacterial suspensions were spread over the surfaces of TSA Petri dishes in triplicate. The groups were incubated for a further 48h at 37°C in a cabinet containing 5–10% CO_2 atmosphere. Bacterial colonies were counted and converted into colony forming units (CFU) for analysis. Statistical analysis of the experimental data was performed using one-way analysis of variance (ANOVA) and the Student's t-test. Significance was accepted at $p<0.05$.

To gain an understanding about the processes that occur during photoinactivation of *A. actinomycetemcomitans* combined with MB, optical absorption spectrometry was performed using a CARY OLIS-17 Inc. (On-Line Instrument Systems, Inc., Bogart, GA, USA) spectrophotometer. Optical absorption spectra of the bacterial suspension in PBS, and 0.01% MB-stained bacterial suspension were taken before and following laser irradiation. Room temperature was maintained at 25°C and samples were put into a quartz cell with a 1mm of light path.

3- RESULTS

The effect of MB-mediated photosensitization on bacterial viability is illustrated in Figure 1. Neither the irradiation of laser alone (L+MB-) nor the incubation com MB alone (L-MB+) showed a significant bactericidal effect on the viability of *A. actinomycetemcomitans*. The treatment with MB in the absence of laser irradiation did not cause significant reduction in the viability of the tested bacterial culture. This finding suggests that there is no direct toxicity of MB 0.01% for 5min. By the other hand, PDT groups showed a significant decrease on the viability of *A. actinomycetemcomitans*. The percentage of bacterial reduction for PDT groups was 99.78% (L3+MB+) and 99.85% (L5+MB+). No statistically significant differences were observed between 3min and 5 min of irradiation.

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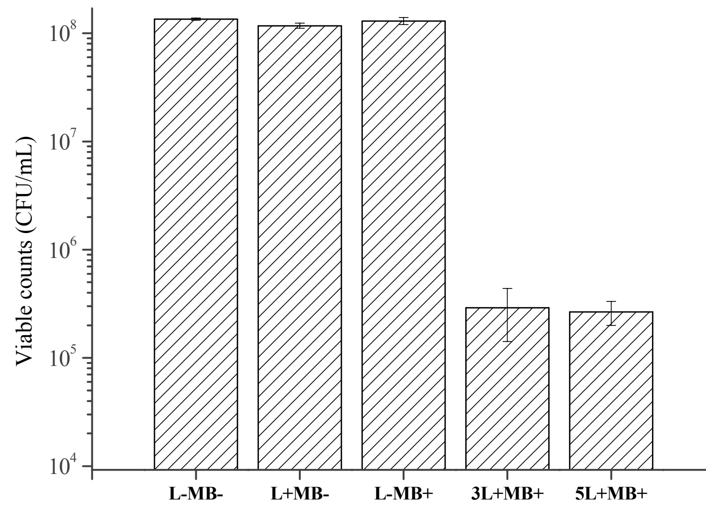

Fig. 1- Bactericidal effect of MB-mediated photosensitization on *A. actinomycetemcomitans*. Data represent mean values (n=3) and error bars represent standard deviation.

The UV-visible absorption spectrum of *A. actinomycetemcomitans* shows a strong absorption in the UV region, which is mainly contributed by DNA. MB, MB-stained bacterial suspension displays two bands in the UV region between 200 nm and 350 nm, and two bands in the red region, which are centered at λ= 607 nm (dimer) and λ= 663 nm (monomer) (Fig. 2). It is possible to observe that the microorganism retains the dye since absorbance decreases. A small bathochromic shift of the MB is also observable in the spectra. Photosensitized bacterial suspension following laser irradiation indicates that dimer species are more notably consumed than monomeric species.

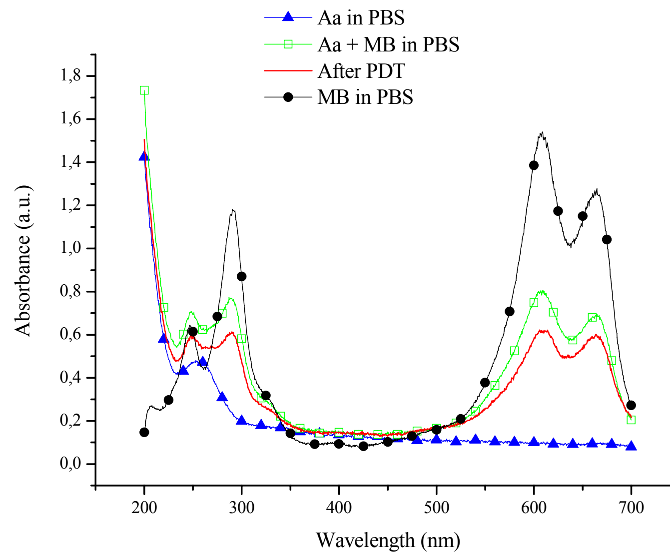

Fig. 2- Absorbance in the UV-Vis region of the MB solution with *A. actinomycetemcomitans*, before and after the photodynamic therapy for 5 min. Note that the dye in a cell suspension retained absorption peaks before irradiation and the exposure to light changed the suspension characteristics.

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4- DISCUSSION

During the last few years, there have been attempts to bring the concepts of PDT to the field of dentistry. Research groups have verified the lethal effect of laser radiation and photosensitizer on microorganisms associated with periodontitis and periimplantitis[11,12,13].

Successful PDT always involves the optimization of a large number of parameters. Obviously, selection of an effective photosensitizer is essential for the success of the technique. MB, which belongs to the phenothiazinium family, is a well-known photosensitizer. Some studies have clearly demonstrated the effect of MB against oral pathogenic bacteria[8,14].

The results of this study show that the exposure of *A. actinomycetemcomitans*, *in vitro*, to a red light in the presence of MB results in a decrease of its viability. Irradiation using a 660 nm–laser at 30mW for 3min or 5min with 0.01% MB was efficient to kill this Gram-negative bacterium. This finding agrees with Chan and Lai[14]. However, this result could not be true to use in vivo; Kömerik & Wilson have reported that the presence of organic materials (blood or nutrient broth), as well as saliva and serum, offer some protection to bacteria against lethal photosensitization[15].

The absorption spectra of MB+*A. actinomycetemcomitans* show a remarkable decrease of the MB peak intensity (hypochromic effect) in the presence of the bacterium and a slight bathochromic shift in the UV region. This phenomenon is suggested to be due to the strong interaction between the electronic states of the intercalated MB and DNA bases of the microorganism[10]. UV–Vis absorption spectroscopy also showed that after irradiation, the absorption bands of MB diminished (see fig. 2). This finding indicates that MB is photodegraded under red light. For MB, a decrease in the dimer/monomer ratio was observed suggesting that dimer species are more notably consumed than monomeric species. Due to the spectral changes observed on MB solution after irradiation, a type I mechanism to kill cells may be involved.

The results of this study suggest that *A. actinomycetemcomitans* can be killed by the appropriate laser-dye treatment combination; however, further *in vivo* evaluation is necessary. In oral cavity applications, for example, the complete isolation of the dye from the saliva and/or blood may not be possible.

5- CONCLUSION

These results suggest that MB is a suitable photosensitizer to reduce *A. actinomycetemcomitans*, and that 3min of irradiation are enough to produce a significant effect. Due to the spectral changes observed on MB solution after irradiation a type I mechanism could be implicated.

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