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RESEARCH

Evaluation of antimicrobial photodynamic therapy with erythrosine and blue light emitting diode for inactivation of *Aggregatibacter actinomycetemcomitans*

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Abstract

This study aims to analyze the effect of antimicrobial photodynamic therapy on *Aggregatibacter actinomycetemcomitans* using erythrosine as a photosensitizer and a blue light emitting-diode as a light source. Inoculum samples of *A. actinomycetemcomitans* with PBS were used in each of the groups, being the control group (C); light group (L) corresponding to light emitting-diode irradiation for 300 s; photosensitizing group (0) without irradiation; and the aPDT groups with different irradiation times (aPDT20) with 20s of irradiation; (aPDT40) with 40s of irradiation; (aPDT60) with 60s of irradiation; (aPDT180) with 180s; and (aPDT300) with 300s. Samples were used to determine colony forming units (CFU). Aliquots of 10 µL were plated through six serial dilutions on brain-heart infusion agar in Petri dishes. The plates were incubated at 37 °C for a period of up to 24–48 h under microaerophilic conditions to evaluate the total bacteria recovered. After this period, CFUs were counted, and the data was subjected to one-way analysis of variance. When aPDT was performed for 180 and 300 s, the mean log10 (CFU/ml) was equal to 0. In the aPDT60 group, a significant yet incomplete microbial reduction was observed. SEM images confirmed that membrane integrity was maintained, indicating that aPDT induced cellular alterations without causing membrane disruption. Antimicrobial photodynamic therapy employing erythrosine as a photosensitizer and blue light emitting-diode light-curing unit for composite resin polymerization used in dental practices demonstrated significant antimicrobial efficacy against *A. actinomycetemcomitans*, a principal pathogen in periodontitis, under the evaluated experimental conditions.

Keywords Erythrosine · Photobiomodulation · *Aggregatibacter actinomycetemcomitans* · Microbiological techniques · Photodynamic antimicrobial chemotherapy (PACT) · Scanning electron microscope

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Introduction

Mild periodontitis affects an estimated 45–50% of the adult population and over 60% of individuals aged 65 years or older. Periodontitis is a chronic inflammatory disease characterized by the progressive destruction of the supporting structures of the teeth, driven by an altered host immune-inflammatory response to bacterial pathogens within the dental biofilm [1]. The gold standard treatment for periodontitis involves mechanical scaling and root planning. However, in severe infection or advanced disease, adjunctive systemic antibiotic therapy may be indicated to improve therapeutic outcomes and control the microbial load [2].

Aggregatibacter actinomycetemcomitans is the key pathogen in aggressive periodontitis, which is characterized by the rapid destruction of tooth-supporting tissues, leading to tooth loss. The use of antibiotics as an adjunct to mechanical treatment of periodontitis can lead to the development of bacterial antimicrobial resistance [3]. Antimicrobial photodynamic therapy (aPDT) has become an effective, low-cost alternative for eliminating microorganisms responsible for periodontal disease [4]. In aPDT, the photosensitizer (PS), a non-toxic dye, is first introduced to the target site to ensure direct interaction with the microbial focus. Following a predetermined incubation period to allow binding and distribution within the area, the application of a specific wavelength of light activates the photosensitizer, triggering the photochemical reaction that generates reactive oxygen species (ROS), thereby inducing microbial inactivation [5, 6].

ROS can be formed either through electron transfer (type I reaction) or through energy transfer (type II reaction). When produced in sufficient amounts, can cause microbial cell death. For periodontitis treatment, the use of aPDT facilitates access to complex dental anatomy, such as bifurcation regions, and can be employed selectively for microorganisms without toxicity to host tissues. Furthermore, it results in immediate microbial death and is unlikely to lead to microbial resistance [7–9].

Various dyes have been utilized in aPDT, including methylene blue (MB), toluidine blue O (TBO), indocyanine green, the natural photosensitizer curcumin, and erythrosine (ERY). Erythrosine is a red dye approved by the FDA for use in food products and dentistry. It has been used as a bacterial oral biofilm disclosing agent in both aqueous solutions and chewable tablets. Belonging to the xanthene class of cyclic compounds, erythrosine absorbs light from the visible spectrum and can initiate photochemical reactions. Its effectiveness against microorganisms outside the oral flora is well-established [10, 11]. Studies have demonstrated its effectiveness against microorganisms such as *Streptococcus mutans*, *Lactobacillus casei*, and *A. actinomycetemcomitans*, with dental curing lamps used as the light source, and further in vitro investigations have explored the combination of blue light-emitting diode (LED) and erythrosine in aPDT [10, 12–15].

Erythrosine exhibits a maximum light absorption within the wavelength range of 450–550 nm, with a peak at 532 nm [16]. This characteristic is integral to its role in photochemical reactions. Furthermore, erythrosine, at concentrations ranging from 9 to 25 mM, is utilized as a dental biofilm disclosing agent [11, 17, 18]. Significantly, erythrosine is regarded as a suitable photosensitizer for aPDT due to its regulatory approval for use in the oral cavity and its lack of direct toxicity to patients [11, 17]. Although the concentration of erythrosine as a disclosing agent is 250 times higher than its concentration as a photosensitizer used in this assay, further studies are needed to determine a safe concentration that does not cause tooth staining.

Erythrosine has been studied at concentrations ranging from 10 to 250 µM, utilizing illumination sources with varying emission bandwidths, including LEDs, tungsten lamps, and lasers. Several oral microorganisms, such as *Streptococcus mutans*, *Lactobacillus casei*, and *Candida albicans*, have been employed as microbial models in these evaluations [13]. Choi et al.. demonstrated in their study that erythrosine at concentrations between 20 and 40 µM is effective in inactivating *Streptococcus mutans* during aPDT [19]. In the study conducted by Goulart R et al.., the most effective parameters were identified as 1.0 mmol/L of ERY combined with an energy dose of 2 J/cm². This was achieved using a dental blue light-curing unit with halogen light emitting at an intensity of 350–500 mW/cm² and a wavelength range of 400–500 nm. These conditions resulted in a 77% reduction in microbial viability and produced the largest areas devoid of cellular aggregate formation [15]. A recent study evaluated the photodynamic effects of curcumin, nano-micelle curcumin, and erythrosine on *Lactobacillus casei* biofilms using a dental blue LED curing device. The findings indicated that aPDT with erythrosine at a concentration of 100 µmol/L or nano-micelle curcumin at 3 g/L effectively eradicated L. casei biofilms, highlighting their potential for clinical application [20, 21].

The use of a ERY combined with blue LED as light source represents a practical and cost-effective approach for managing periodontitis. Due to the widespread availability, compact size, and portability of blue LEDs commonly employed in dental clinics for resin photopolymerization, this method can be readily incorporated into routine clinical protocols, increasing its feasibility and potential for broader clinical application. Additionally, this approach may improve accessibility, providing a more affordable alternative to traditional periodontal therapies for a larger number of patients.

This study aims to evaluate the effect of antimicrobial photodynamic therapy on *A. actinomycetemcomitans* using erythrosine as a photosensitizer and a blue LED as the light source, addressing the scarcity of references to this procedure in the current literature.

Materials and methods

Inoculum preparation

A. actinomycetemcomitans (ATCC 29523) cells were subcultured in brain heart Infusion (BHI) broth under microaerophilic conditions (5–10% CO_2), at 37ºC for 48 h. Broth culture was centrifuged for 3 min at 876 g to remove the medium. Cells were suspended in PBS and concentration of bacterial suspension was adjusted to approximately 2×10^8 CFU/mL (0.5 McFarland).

Photosensitizer preparation

Erythrosine Dye (Sigma Aldrich Chemical Co., Milwaukee, WI, USA) was used as a photosensitizer at 100 µM, which was previously determined in a pilot study. The dye stock solution was prepared by dissolving erythrosine powder in deionized water, which was subsequently filtered through a 0.22 µm sterile membrane (Millipore, SP, Brazil) and stored in microcentrifuge plastic tubes protected from light. The stock solution was prepared at a concentration a hundredfold higher than that used for microbiological tests.

Light source

Irradiation was carried out with an light emitted diode (LED) equipment (DMC, São Carlos, SP, Brazil) that emits a wavelength 470 nm ± 30 nm and a radiant power of 1000 mW. Samples were irradiated from the top of a 48-well microtiter plate with an irradiance of 500 mW/cm^2, irradiation times ranging from 20 to 300 s and respectively radiant exposure: 10, 20, 30, 90 and 150 J/cm^2 (Table 1).

Table 1 Dosimetry of the LED used in the experiments

Parametes	LED
Wavelength (nm)	470 ± 30
Operating mode	Continuos
Radiant power (mW)	1000
Beam area (cm^2)	2
Irradiance (mW/cm^2)	500
Exposure time (s)	20;40;60;180;300
Pre irradiation time (s)	60
Radiant exposure (J/cm^2)	10; 20; 30; 90; 150
Radiant energy (J)	10;20;30;90;150

Experimental groups

Inoculum samples of *A. actinomycetemcomitans* were used in each of the groups, repeated three times: control group (C); light group (L) corresponding to laser irradiation for 300 s; photosensitizing group (0) without irradiation; and the aPDT groups with different irradiation times: (PDT20) with 20 s; (PDT40) with 40 s and (PDT60) with 60 s; (PDT180) with 180 s; and (PDT300) with 300 s of irradiation.

Microbiological evaluation

Samples were used to determine colony forming units (CFU). After aPDT, cells suspension from each group was serially diluted from 10^{-1} to 10^{-6} times the original concentration. Aliquots of 10 µl from the six dilutions were plated on the surface of BHI agar petri dishes in triplicate. The plates were incubated at 37 °C for a period of up to 24–48 h under microaerophilic conditions. After this period, CFUs were counted, and the data were subjected to statistical analysis. These samples were analyzed by an independent evaluator who was blinded to the group assignments.

Evaluation through scanning electron microscope (SEM)

The aPDT60 group exhibited a significant but incomplete microbial reduction, providing an opportunity to observe progressive structural changes. Bacterial cells from these groups were fixed with 2.5% glutaraldehyde for 2 h. The cells were then washed in PBS and dehydrated in a graded ethanol series of 70%, 80%, 90%, and 100%, with each step lasting 10 min. The cells were then coated with a thin layer of gold and analyzed using SEM (FEI Quanta FEG 250, Hillsboro, Oregon, United States).

Statistical analysis

The study variable was the concentration of viable bacteria, and the results are presented as CFU/mL with the standard deviation of the means. Group comparisons were conducted using one-way analysis of variance (ANOVA), followed by Bonferroni post hoc testing. Statistical significance was considered at $p < 0.05$. All analyses were performed using IBM SPSS Statistics version 29.0.

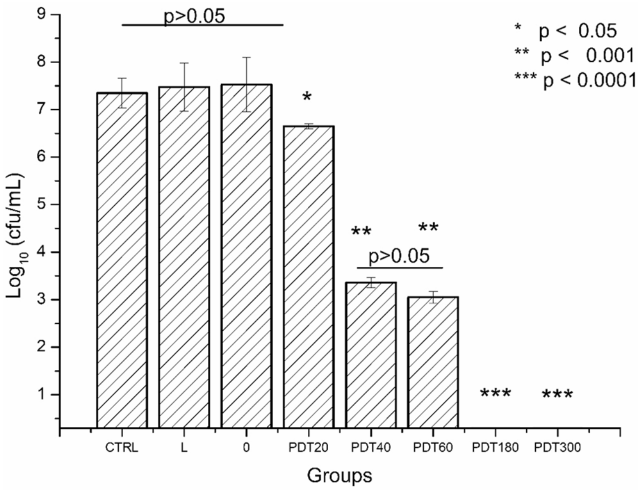

Fig. 1 Photodynamic inactivation of *A. actinomycetemcomitans*. Data represent mean values, with error bars indicating standard deviations. Asterisks indicate statistically significant differences compared to the control group ($p < 0.05$)

Results

A killing curve was generated to determine the susceptibility of *A. actinomycetemcomitans* to ERY-aPDT, using five different exposure times (Fig. 1). The mean CFU/mL values of cells exposed only to light (L group: 7.5 ± 0.5 logs) showed no significant difference ($p > 0.05$) compared to cells not exposed to ERY or light (C group: 7.3 ± 0.3 logs). Similarly, no significant difference was observed between cells treated only with ERY (0 group: 7.5 ± 0.6 logs) and the C group. Light irradiation during 300 s and 100 µM of ERY did not affect bacteria viability. Irradiation in the presence of the photosensitizer for 20, 40, and 60 s resulted in a gradual reduction of viable cells, with values of 6.65 ± 0.1 logs, 3.36 ± 0.1 logs, and 3.04 ± 0.1 logs, respectively. ERY-aPDT with 180 s and 300 s of irradiation resulted in complete bacterial inactivation, showing a significant difference compared to the other groups ($p < 0.05$).

Scanning electron microscopy revealed the qualitative morphological characteristics of the tested microbial samples, which appeared as slightly elongated cocci with minimal extracellular material. The cells were adhered to each other, forming large clusters characteristic of the species, even in the absence of exopolysaccharides. After aPDT treatments, larger bacterial clusters were observed. These specimens displayed similar roughness on the outer cell wall, along with a higher accumulation of material between the cells. The bacterial cells did not exhibit cell wall rupture after aPDT treatment. They maintained their volume and integrity, indicating that cell morphology was not affected. Scanning electron microscopy analysis was conducted exclusively on the control group and the aPDT60 treatment group, as these exhibited the most significant results (Figs. 2 and 3).

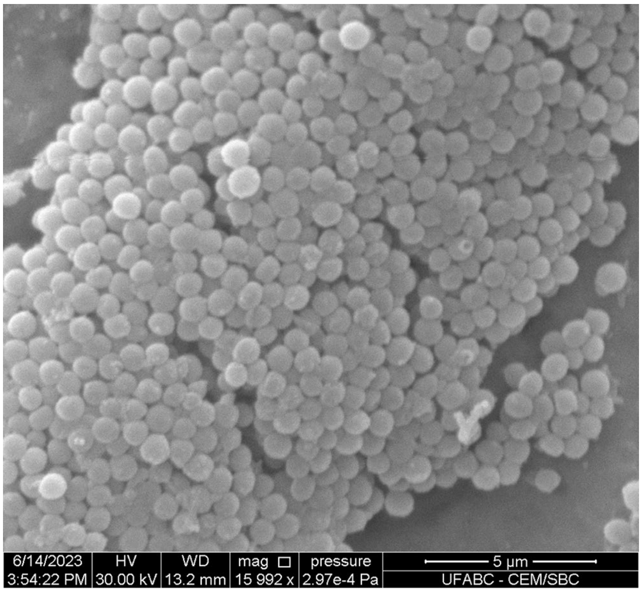

Fig. 2 *A. actinomycetemcomitans* was treated with aPDT using 100 µM ERY and 60 s blue light irradiation, cell morphology was not affected

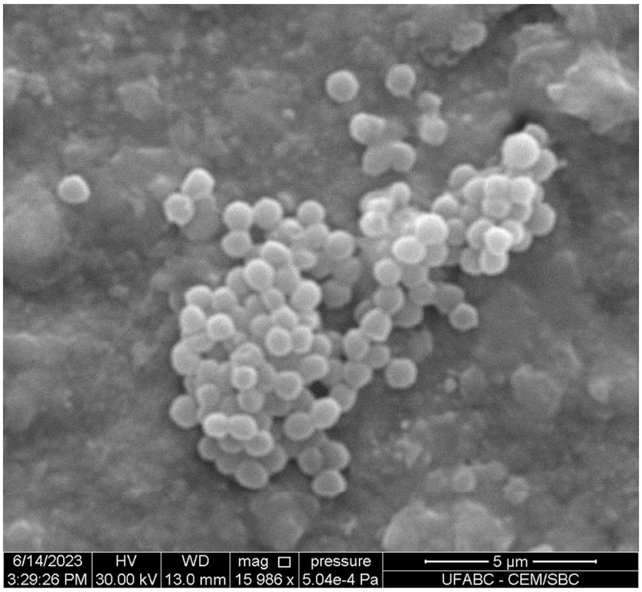

Fig. 3 The control group (0) showed intact membranes and clear adhesion proteins in *A. actinomycetemcomitans*, with no shape changes or deformations

Discussion

In the present study, the effect of antimicrobial photodynamic therapy on *A. actinomycetemcomitans* was evaluated, employing erythrosine, an oral biofilm disclosing agent, as the photosensitizer at 100 µ, and a blue LED, commonly used for photocuring composite resin, as the light source.

Erythrosine is already used in clinical practice, which facilitates the rapid availability of oral formulations containing this compound for clinical aPDT procedures. Thus, a specific erythrosine concentration was determined for testing under various irradiation parameters, with the goal of identifying the optimal dose for an effective clinical protocol. In the group treated with erythrosine, without light irradiation, no significant differences in CFU were observed. Consequently, it can be indirectly concluded that erythrosine did not exert a toxic effect on the *A. actinomycetemcomitans*, contrary to the findings of Merigo, et al., which showed an 82% inhibition compared to the negative control, and in line with others studies which did not demonstrate direct toxicity to host tissues [11, 22, 23].

However, when activated by blue light, erythrosine effectively undergoes photochemical reactions leading to cell death. Similarly, the study undertaken by Alsaif et al. employed a wavelength within the white light spectrum. Both studies utilized wavelengths that are not considered optimal for the photosensitizer, yet still yielded favorable outcomes [13].

Although blue LED technology is widely used in most dental offices today, a limitation of this device is the absence of a fiber optic system that allows its effective application in deeper periodontal pockets. However, it remains highly suitable for other clinical applications.

Several studies on aPDT have utilized LEDs and reported successful outcomes [24–26]. However, few have evaluated its effects on *A. actinomycetemcomitans*. This research assessed its efficacy against this bacterium and demonstrated bactericidal activity, with bacterial killing increasing proportionally to the duration of light exposure and complete bacterial eradication (10^8 CFU/mL) was achieved after 180 and 300 s of irradiation.

SEM images revealed no morphological differences between the cells in Group 0 and Group 60. This finding is supported by Sabino et al., who reported no signs of membrane rupture even after exposure to aPDT doses significantly higher than those required for complete microbial inactivation. This is likely due to protein damage being the primary mechanism of cell death, as it is clear that protein damage best correlates with cell killing [27].

It is well established that Gram-positive bacteria are more sensitive to singlet oxygen, whereas Gram-negative bacteria are more susceptible to hydroxyl radicals, corresponding to Type II and Type I reactive oxygen species (ROS) pathways in aPDT, respectively. However, this distinction was not considered in the present assays, which may represent a limitation of this study [28].

Although the results are promising, the required application time is excessively long from a clinical perspective. For effective clinical application, a shorter exposure time, such as 60–120 s, would be more feasible, particularly in scenarios requiring treatment of multiple sites.

Research has shown that blue light, within the wavelength range of 380–520 nm, is an effective option for aPDT [29, 30]. Likewise, the results of this study demonstrate that irradiation time is directly proportional to the reduction in CFU, leading to complete bacterial eradication. Based on the parameters used in this work, aPDT may be a promising adjunct to nonsurgical periodontal therapy for reducing periodontopathogenic microorganisms. However, given the controversies in the existing literature, further research is necessary to better understand the response of *A. actinomycetemcomitans* to this treatment.

Conclusion

Antimicrobial photodynamic therapy employing erythrosine at 100 µM as a photosensitizer and blue LED light demonstrated significant antimicrobial efficacy against *A. actinomycetemcomitans*, a principal pathogen in periodontitis, under the evaluated experimental conditions.

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Data availability The data are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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