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Bactericidal effect of malachite green and red laser on *Actinobacillus actinomycetemcomitans*

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Abstract

The aim of this study was to investigate the ability of malachite green (MG) combined with a low-power red laser to kill *Actinobacillus actinomycetemcomitans* and to investigate MG photodegradation after photodynamic therapy (PDT) by optical absorption spectroscopy. The etiology of periodontal disease is that microorganisms form a bacterial biofilm on the surface of the teeth. It is an infectious disease and *A. actinomycetemcomitans* is considered an important agent in biofilm ecology. Instead of using antibiotics, PDT is an alternative approach to eradicate bacteria. Cultures of *A. actinomycetemcomitans* were exposed to a 30 mW diode red laser, in the presence or absence of MG. A group of cultures was treated in dark conditions in the presence of MG (0.01% w/v) for 5 min. In the presence of MG, two exposure times for laser irradiation were used: $t = 3$ min (energy dose = 5.4 J/cm^2), and $t = 5$ min (energy dose = 9 J/cm^2). The samples were diluted and bacterial colonies were counted and converted into colony forming units. Absorption spectra of the bacterial suspensions, MG, MG-stained bacterial suspensions, and photosensitized bacterial suspensions were obtained. *A. actinomycetemcomitans* can be photoinactivated by a red laser in the presence of MG. Significant differences were observed between the two energy doses used ($p < 0.05$). Red laser alone and MG alone were not able to kill bacteria. Optical absorption showed that MG is photobleached after irradiation. These results indicate that *A. actinomycetemcomitans* can be photosensitized by red laser combined with MG and that the dye is photodegraded following irradiation.

Keywords: Photodynamic therapy; Diode laser; Malachite green; Oral bacteria; Periodontitis; Photosensitizers

1. Introduction

Actinobacillus actinomycetemcomitans is a Gram-negative, non-spore forming, non-motile, facultative anaerobic coccobacillus. There is strong evidence implicating *A. actinomycetemcomitans* as the causative agent of localized aggressive periodontitis, a disease characterized by rapid destruction of tooth-supporting tissues. This organism possesses a large number of virulence factors with a wide range of activities which enable it to colonize the oral cavity, invade periodontal tissues, evade host defenses, initiate connective tissue destruction and interfere with tissue repair [1].

Periodontitis is an inflammation of the gingiva and the adjacent attachment apparatus and is characterized by loss of connective tissue attachment and alveolar bone. The primary etiology is bacterial plaque, which can initiate destruction of the gingival tissues and periodontal

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attachment apparatus [2,3]. It may be subclassified based upon etiology, clinical presentation, or associated complicating factors [4]. The microbial etiology of periodontitis has been extensively studied and it is not associated with a single microorganism, but is a consortium of bacteria participating in the initiation and progression of periodontitis. For periodontopathic bacteria to cause periodontal diseases, it is essential that they are able to colonize subgingival pockets and produce virulence factors that directly damage host tissue [5,6]. Studies have demonstrated an association between periodontal disease severity and risk of coronary heart disease and stroke. Periodontal disease, once established, provides a biological burden of endotoxin (lipopolysaccharide) and inflammatory cytokines (especially IL-1 beta, PGE(2), and TNF-alpha), which serve to initiate and exacerbate atherogenesis and thromboembolic events [7]. Moreover, this oral inflammatory disease is associated with loss of metabolic control in diabetic patients [8]. Sometimes, the use of systemic antibiotics as an adjunct in the treatment of periodontal disease has been necessary. However, overuse of antibiotics has been a major culprit in the production of drug-resistant organisms [9–12].

Bacteria may also be killed by visible light in the presence of a sensitizing dye. Many dyes have inherent antibacterial effects, however, only during irradiation is the photodynamic bactericidal effect elicited. Local infections such as those that occur within the oral cavity may be potential targets for antibacterial photodynamic therapy. The supra- and subgingival biofilms on tooth surfaces should be easily accessible for flushing with the dye and for activating them with light. Thus, periodontal diseases are promising applications [13]. Cultures of *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *A. actinomycetemcomitans* were treated with a range of photosensitizers and then exposed to light. Toluidine Blue O (25 μg/mL) and Methylene Blue (25 μg/mL) were effective lethal photosensitizers of all three target organisms, enabling substantial light dose-related reductions in viable counts [14]. This technique may be useful as a means of eliminating periodontopathogenic bacteria from diseased sites [13,15–23].

Malachite green (MG) shows strong absorption at the red end of the visible spectrum [24] and presents an easy transit through the cellular membrane in Gram-positive as well as Gram-negative bacterial species. This cationic dye, which belongs to the triarylmethane family (that also includes Crystal Violet and Victoria Blue), could be used as a potential photosensitizer since it promotes dissipation of the cell membrane potential [25]. Periodontists use 0.1% w/v MG to stain and visualize dental plaque [26]. However, despite its use in clinical practice, to the best of our knowledge there are no reports of the use of MG as a photosensitizer in oral applications. The aim of this study was to investigate the ability of MG associated with a low-power red laser to kill *A. actinomycetemcomitans* as well as to investigate MG photodegradation after PDT by optical absorption spectroscopy.

2. Materials and methods

A 30 mW GaAlAs diode laser (Unit Kondortech—São Carlos, Brazil) with wavelength of 660 nm was used in this study. Bacterial suspensions were irradiated from the bottom of the glass tube. The laser beam passed under all suspensions.

A. actinomycetemcomitans strains were subcultured in Tryptic Soy Agar (TSA) containing 6 g/L yeast extract (Fig. 1) and were 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 (1.5×10^8 CFU/mL), 900 μL aliquots were taken and distributed into five glass tubes. The control group (L−PS−) was untreated by either a laser or a photosensitizer; in the laser group (L+PS−), the bacterial suspension was irradiated for 5 min with an energy dose of 9 J/cm^2 in the absence of the photosensitizer; in the MG group (L−PS+), malachite green was added to the suspension to a final concentration of 0.01% w/v for 5 min in dark conditions; in the photodynamic therapy groups, 0.01% w/v MG was added to the bacterial suspension for 5 min (pre-irradiation time) and subsequently treated with laser for 3 min (L+3PS+), and 5 min (L+5PS+) with energy doses of 5.4 and 9 J/cm^2, respectively.

One-hundred microliters of PBS (pH 7.2) was added to groups L−PS− and L+PS− and the same volume of 0.1% MG was added to groups L−PS+, L+3PS+ and L+5PS+, resulting in a final concentration of 0.01% w/v. The estimated cell concentration decreased to 1.35×10^8 CFU/mL in all groups.

After irradiation procedures, each group was serially diluted and bacterial suspensions were spread over the surfaces of Tryptic Soy Agar (TSA) Petri dishes in triplicate. The groups were incubated for a further 48 h at 37°C in a cabinet containing 5–10% CO_2 atmosphere. The 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 of the processes that occur during photoinactivation of *A. actinomycetemcomitans* combined with MG, and changes in MG solution before and after the irradiation, UV–vis optical absorption spectrometry was performed using a CARY OLIS-17 Inc. (On-Line Instrument Systems, Inc., Bogart, GA, USA) spectrophotometer. Room temperature was maintained at 25 °C and 0.01% w/v MG diluted in PBS, pH 7.2, was put into a quartz cell with a 1 mm light path. A fractionated irradiation was performed for 3 min and spectra were obtained after 30, 60, 90, 120, 150 and 180 s. Furthermore, the UV–vis optical absorption of the bacterial suspensions in PBS, pH 7.2, and 0.01% w/v MG was performed before and following laser irradiation. The spectra were analyzed

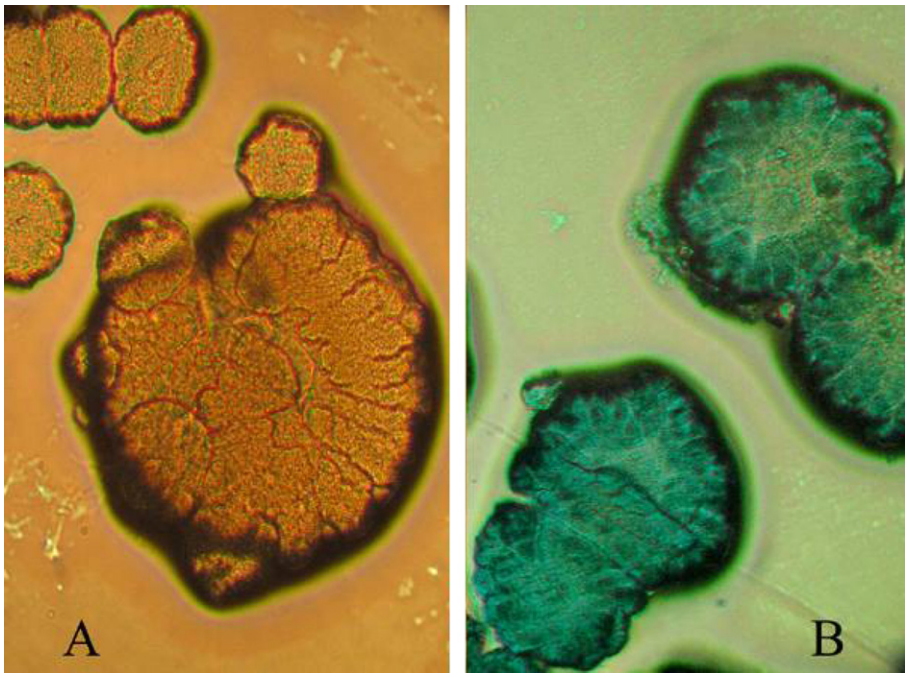

Fig. 1. (A) *A. actinomycetemcomitans* colonies in TSA medium after incubated in microaerofilic conditions at 37 °C for 48 h; and (B) bacterial colony stained by malachite green. Original magnification 100×.

with Olis-17 (On-Line Instrument Systems, Inc.) and Origin Lab 7.0 software (OriginLab Corporation, Northampton, MA, USA).

3. Results

Fig. 2 demonstrates that *A. actinomycetemcomitans* was eradicated to some extent by red laser light in the presence of MG. However, the degree of photoinactivation was dependent upon the energy dose. Neither the MG 0.01% w/v in the absence of light nor the laser light (energy dose = 9 J/cm^2) in the absence of MG had a statistically significant effect on the viable count of the bacterial suspension. On average, 1.35×10^8 cells/mL were detected in the groups L−PS−, L−PS+, L+PS−. As can be seen from Fig. 2, substantial kills of cells were obtained on irradiation with laser light in the presence of MG. The kills amounted to 97.2% of the original suspensions in group L+3PS+ and to 99.9% in group L+5PS+. Upon irradiation with red light, the CFU count was reduced by between $2\log_{10}$ (5.4 J/cm^2) and $3\log_{10}$ (9 J/cm^2). Statistically significant differences were observed between L+3PS+ and L+5PS+ ($p < 0.05$).

The UV–vis spectroscopic analysis showed a strong absorption band between 550 and 680 nm, as well as at 350–470 and 260–340 nm. The exposure to laser light appears to reduce the absorption peaks caused by the cationic dye (Fig. 3). A decrease in MG absorption as a function of irradiation time can be observed in the 618 nm peak in Fig. 4. Furthermore, UV absorption spectra of the irradiated isolated dye revealed a new band at 225–290 nm (Fig. 3).

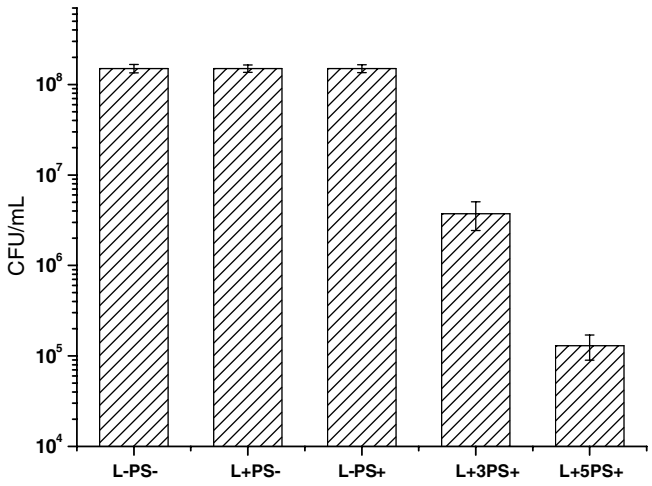

Fig. 2. Figure represents *A. actinomycetemcomitans* organisms viable number. (L−PS−) represents the control group that was untreated by either a laser or a photosensitizer; in the laser group (L+PS−), the bacterial suspension was irradiated for 5 min in the absence of the photosensitizer; in the MG group (L−PS+), malachite green was added to the suspension to a final concentration of 0.01% w/v for 5 min in dark conditions; in the photodynamic therapy groups, 0.01% w/v MG was added to the bacterial suspension for 5 min and subsequently treated with laser for 3 min (L+3PS+), and 5 min (L+5PS+). Bars represent standard errors of the means.

Using UV–vis absorption spectrophotometry (Fig. 5), isolated bacterial cells in PBS, pH 7.2, suspension were investigated and an absorption band less than 250 nm was observed. Optical spectroscopy also showed that the dye in PBS presents a peak at 260 nm as well as at 350 nm. The dye in a cell suspension retained absorption

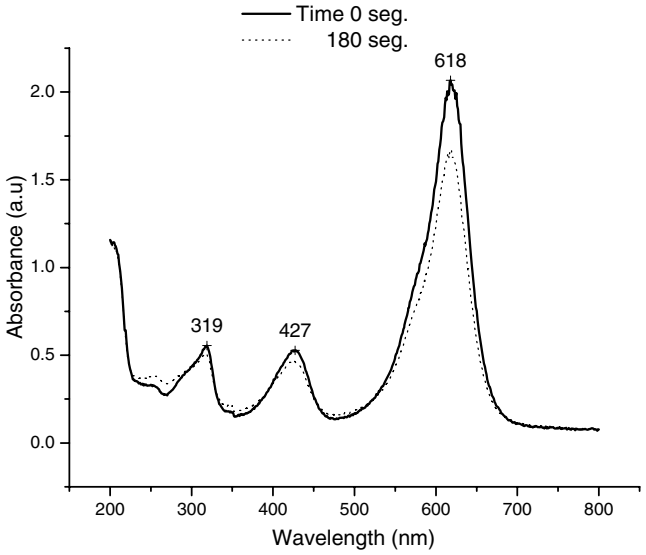

Fig. 3. Absorption spectra after irradiation with red laser ($\lambda = 660$ nm). Spectroscopic measurements were taken each 30 s of laser irradiation. Black line is the second 0 of irradiation and dot line represents 180 s of irradiation.

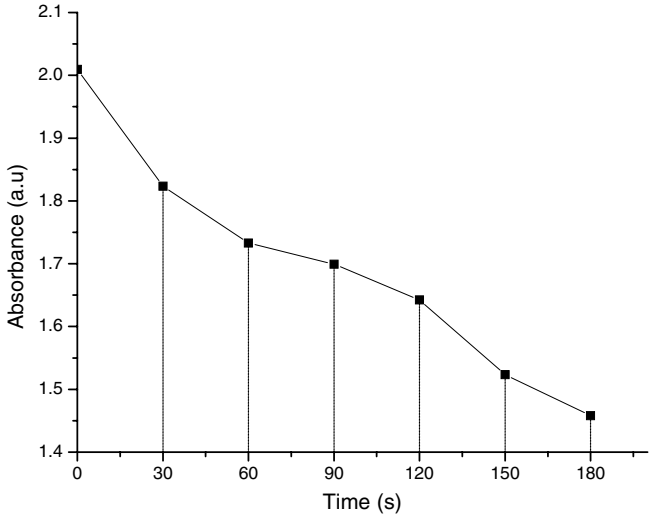

Fig. 4. Absorption in the 618 nm peak, during irradiation with red laser ($\lambda = 660$ nm). Spectroscopic measurements were taken each 30 s of laser irradiation.

peaks before irradiation. Exposure to light changed the suspension characteristics and the peaks observed before irradiation disappeared.

4. Discussion

The results of this study showed that 0.01% (w/v) malachite green combined with laser irradiation ($\lambda = 660$ nm) reduced *A. actinomycetemcomitans* by up to 99% of the colony forming units. This experiment demonstrated that this dye did not present any toxic effects against *A. actinomycetemcomitans* as MG 0.01% (w/v) added to the bacterial suspension without irradiation did not reduce the CFU number. In fact, MG can be used as a medium supplement to facilitate the CFU quantification of *A. actinomycetemcomitans* [27,28].

Previous studies on the effectiveness of PDT have focused on different microorganisms and photosensitizers. For example, a substantial bacterial reduction was observed on *F. nucleatum*, *P. gingivallis* and *A. actinomycetemcomitans* using Toluidine Blue O. Methylene Blue showed less efficiency on *A. actinomycetemcomitans*, which

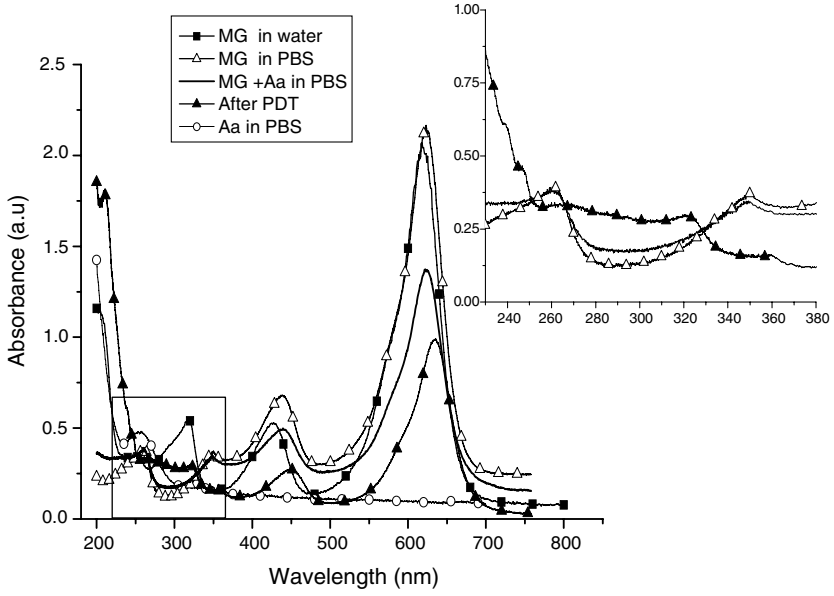

Fig. 5. The figure represents absorption spectra in the UV–vis region of the malachite green solution with or without *A. actinomycetemcomitans*, before and after the photodynamic therapy for 5 min. Note that the dye in a cell suspension retained absorption peaks before irradiation and the exposure to light changed the suspension characteristics. The peaks observed before irradiation disappear.

only presented a 64% bacterial reduction when compared to 92.6% and 99.9% for *P. gingivallis* and *F. nucleatum*, respectively [14]. In addition, *A. actinomycetemcomitans* and *F. nucleatum* were less sensitive to the therapy when compared with *P. gingivalis*, *P. intermedia* and *S. sanguis,* using three different wavelengths and Methylene Blue with the same parameters. Bacterial reductions differed from 78% to 95% depending on the energy dose used [20]. A more recent study using photosensitizers based on porphyrin skeleton and a red laser showed only a 62% reduction of *A. actinomycetemcomitans* [22]. On the other hand, our results showed a 99.9% reduction of *A. actinomycetemcomitans* with an energy dose of 9 J/cm^2 and 97.2% using 5.4 J/cm^2. It is important to note that the initial bacterial concentration in all of these studies was 10^8 CFU/mL. However, in this study about 1×10^6 bacterial cells still remain viable after treatment. This is most likely attributable to the light energy doses received by the bacterial suspensions in the glass tube. The diffusion of red light decreases in intensity logarithmically, so that some areas inside the tube would have received a lower light dose. In fact, this finding may not represent a subpopulation resistant to photolysis. When microorganisms were subjected to lethal photosensitization again, similar kills to those achieved with the original suspensions were found [29,30].

The photodynamic antibacterial action by triarylmethane dyes has already been investigated by other authors. Thus, Senda et al. [31] reported an inhibitory effect of bacterial biofilm formation in a human mouth using Crystal Violet combined with yellow light. In addition, *P. gingivalis* was also killed by the combination of Crystal Violet and yellow light in a rat model of subcutaneous abscess [32]. It is important to emphasize that the relationship between wavelength emitted by the light source and the absorption spectra of the photosensitizer is essential to develop photochemical and photophysical effects. A close relationship between absorption of the dye and wavelength must be observed in the use of photodynamic therapy. Thus, toxicity in the cell only occurs when the absorption band of the photosensitizer is resonant with the emitted radiation [20]. The light wavelength, photosensitizer absorbance, light energy, light intensity and exposure time may play a part in the results, since, to achieve the best results the photosensitizer has to be efficiently sensitized by the light source. In addition, there are other variables that can influence PDT applications in vivo, such as the presence of gingival cervicular fluid, blood and the structure of biofilm. Therefore, the parameters used in in vitro studies may not promote the same bacterial reduction in vivo [15,17,20,33,34]. The treatment effectiveness involves the optimization of a large number of parameters and the dynamics of the photoreactions must be more clearly understood [20].

UV–vis absorption spectroscopy showed that after irradiation, the absorption peaks of MG diminished (Fig. 3). This finding suggests that malachite green photodegrades with an increase in exposure time to the red light (Fig. 4). These absorption reductions were observed at 550–680 nm, as well as 350–470 and 260–340 nm wavelength bands. The exposure to light reduced the absorption peaks in these bands. Absorption bands were observed at 225–290 and 320–380 nm suggesting the formation of a new photoproduct (see insert in Fig. 5). This was previously investigated and identified as 4-dimethylaminobenzophenone [35].

The photodynamic activity of the photosensitizer is based on photooxidative reactions, which induce multiple consecutive biochemical and morphological reactions. When a photosensitizer molecule absorbs light of a resonant energy, it may undergo an electronic transition to the singlet excited state. Following the absorption of light, the photosensitizer, initially at the ground state, is activated to a short-lived excited state that may convert to a long-lived triplet state. This triplet state is the photoactive state, which may generate cytotoxic species like singlet oxygen. These reactive oxygen species are responsible for irreversible damage to cell membranes including protein modifications [36,37]. However, a very inefficient and almost undetectable intersystem crossing to the MG triplet state was observed in bovine serum albumin (BSA) bound to MG at 90 K [38]. Consequently, when bound to biopolymers under ordinary biological conditions, the electronic excitation of MG is not expected to produce substantial populations of triplets. A significant contribution of this excited state (^{3}MG*) to the overall photobleaching of this dye is not expected. This low triplet yield of MG implies that this dye is not expected to sensitize singlet oxygen to any significant extent in biological systems [39]. Therefore, MG can be expected to develop its photodamaging effects towards biopolymers primarily via the classical photosensitization mechanism type I (initiated by superoxide, hydroxyl anion and other oxygen radicals), with very little contribution from the type II mechanism (initiated by singlet oxygen) [35,40]. Interestingly, the mechanism of formation of the derivatives of MG makes this dye a potential photosensitizer agent for the treatment of hypoxic areas [39,41]. This fact could explain why this photosensitizer acts efficiently against *A. actinomycetemcomitans.* The type II mechanism involves the singlet oxygen that can be inactivated by bacterial virulence factors [42,43]. The type I mechanism, therefore, could be more effective on this microorganism. The free radical formed cannot be inactivated by bacterial virulence factors [44].

Bacterial cells were observed by spectroscopy and a UV absorption band less than 250 nm was shown (Fig. 5). This is compatible with bacterial DNA and RNA absorptions, as well as protein bands. The dye in a cell suspension retained the same UV absorption peak at 260 nm. Therefore, before irradiation, an interaction between the cells and the dye was assumed. Furthermore, following light exposure, changes in suspension characteristics showed a degradation of MG and photoproduct formation.

This study showed that an important periodontal bacterium can be photoinactivated by red light and malachite

green. This dye may be beneficial as a photosensitizer in periodontal treatment. However, clinical assays and double blind studies are still required to improve our understanding of lethal photosensitization as an adjunct to periodontal therapy.

In conclusion, malachite green, from triphenylmethane dyes, acted as a photosensitizer on *A. actinomycetemcomitans*. Successful bacterial inactivation following continuous laser radiation at $\lambda = 660$ nm was demonstrated at two energy doses (5.4 and 9 J/cm^2). Furthermore, the photosensitizer was degraded after the irradiation and new photoproducts were formed in suspension.

5. Abbreviations

CFU colony forming units
BSA bovine serum albumin
DNA deoxyribonucleic acid
GaAlAs gallium–aluminium–arsenide
Il-1 interleukin-1
L+3PS+ group photodynamic therapy for 3 min
L+5PS+ group photodynamic therapy for 5 min
L+PS− group laser alone
L−PS− control group
L−PS+ group photosensitizer alone
MG malachite green
PBS phosphate buffered saline
PDT photodynamic therapy
PGE2 prostaglandin E2
RNA ribonucleic acid
TNF tumor necrosis factor
TSA tryptic soy agar
UV ultraviolet
Vis visible

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