



PHOTOSENSITIZATION OF IN VITRO BIOFILMS FORMED ON DENTURE BASE RESIN

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Statement of problem. Proper sterilization or disinfection of removable prostheses and surgical guides has been problematic in dental practice because of the absence of simple and low-cost techniques that do not cause damage to acrylic resins.

Purpose. The purpose of this study was to study the effect of photodynamic therapy against *Streptococcus mutans*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans* biofilms formed on acrylic resin specimens.

Material and methods. The specimens were sterilized in ethylene oxide gas and submitted to in vitro biofilm growth. The photodynamic therapy consisted of the application of 0.05% methylene blue (P+) conjugated to irradiation with a light-emitting-diode of 630 nm and 150 mW (L+). The specimens were randomly divided into groups (n=5): negative control (P-L-); stained and irradiated at 10 J/cm² (P+L+ 10); stained and irradiated at 30 J/cm² (P+L+ 30); stained and not irradiated (P+L-); not stained and irradiated at 10 J/cm² (P-L+ 10); not stained and irradiated at 30 J/cm² (P-L+ 30); and gold standard (GS), sterilized. Afterward, the specimens were submitted to contact with culture medium agar for 10 minutes in petri plates, which were incubated for 48 hours at 37°C. The number of colony-forming units was obtained, and the data were expressed according to scores (1=0; 2=1-10; 3=11-100; 4=101-1000) and analyzed by the Friedman and Dunn tests ($\alpha=.05$).

Results. *Streptococcus mutans* was sensitized by (P+L-); *P aeruginosa* and *C albicans* were also sensitized by the dye but showed a slight microbial reduction with (P+L+ 30), as did *S aureus* ($P>.05$); *E coli* presented an initial score of 3 and achieved a bacterial reduction to score 2 with (P+L+ 30) ($P=.039$).

Conclusions. Photodynamic therapy was effective in reducing *E coli* counts on biofilms formed on acrylic resin specimens. The inhibition of microorganism growth tended to be directly proportional to the amount of energy provided by the light-emitting diode. (J Prosthet Dent 2014;112:632-637)

CLINICAL IMPLICATIONS

Photodynamic therapy promises to be an effective method for sterilizing acrylic resin. Future developments should allow clinicians to use this technique to sterilize removable prostheses and surgical guides quickly, with no damage to the acrylic resin.

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Photodynamic therapy (PDT), also called photodynamic inactivation or antimicrobial photodynamic chemotherapy, is a therapeutic modality that has shown promise in the inactivation of pathogenic microorganisms.¹⁻⁵ Reports date back over 100 years on the photodynamic effects of chemical compounds against microorganisms,⁶ which were reduced by the popularization of sulfonamides and penicillin. This technique is also used in oncology to effectively treat cancerous lesions through the induction of irreversible damage to the neoplastic tissue cells.⁷⁻¹⁰ Given the difficulties posed by the emergence of antimicrobial resistance, researchers have been seeking alternative solutions for the treatment of oral infections. Some studies have demonstrated the potential of PDT in the inactivation of microorganisms, including some types of viruses, bacteria, and fungi.^{5,11,12}

PDT is the association of a photosensitizing agent to a light source in order to cause microbial cell necrosis and death. The action takes place when the photosensitizing dye absorbs photons and passes electrons to an excited state. In the presence of a substrate such as oxygen, the photosensitizer transfers energy to the substrate in order to return to its natural state. Short-lived highly reactive molecules such as singlet oxygen are formed, which can cause serious damage to cellular components of microorganisms.⁶ Methylene blue in different concentrations and conjugated to a red light source such as a laser or light-emitting diode (LED) has shown antimicrobial efficacy.¹³⁻¹⁶

Because of the mechanism of PDT, it is unlikely that an organism develops resistance to this type of therapy.^{6,17} Other advantages may also be observed with the use of photodynamic inactivation, including the selectivity of the photosensitizer, focalization of light on the region of interest only, possibility of repeating the therapy without cumulative toxic effects, noninvasiveness, and low risk.³ PDT has been used effectively in dentistry in the areas of cariology,

periodontology, endodontics, and oral pathology.^{16,18-26}

In prosthodontic treatment, a reduction in the number of microorganisms would improve the control of cross-infection between the dental office and dental laboratory. Strict asepsis is needed for immediate dentures and the surgical guides used in implant dentistry. Removable dental prostheses and surgical guides are fabricated from acrylic resin, which can be sterilized by ethylene oxide gas, gamma rays, immersion in a 2% glutaraldehyde solution for 10 hours, or microwave irradiation. Ethylene oxide gas and gamma rays are used in hospitals and have a high cost. The main disadvantage of 2% glutaraldehyde is the long immersion time required. Although promising, microwave irradiation has not been shown to provide 100% microbial elimination and may adversely affect the dimensional stability and hardness of the resin.²⁷⁻²⁹ The use of different concentrations of sodium hypochlorite and 70% ethanol have been reported for disinfection of acrylic resins.³⁰⁻³² The immersion of acrylic resin in sodium hypochlorite for 10 minutes increased the surface roughness and decreased flexural strength, favoring the adhesion of microorganisms on the denture surface. Immersion in 70% ethanol for 15 minutes caused qualitative changes in the acrylic resin surface, as observed by scanning electron microscope.³³

The search for a simple alternative method of disinfection or sterilization of acrylic resin devices is of interest to clinical dentistry because of problems of cross-infection between the laboratory and dental office and the use of nonsterile immediate dentures and surgical guides.³¹ In addition, a wide range of potentially pathogenic microorganisms has been found in previously worn dentures.³⁴ PDT has proved to be an efficient, low-cost option to solve these problems.^{5,6,24} This in vitro study aimed to evaluate the antimicrobial efficacy of PDT against 5 different groups of microorganisms (*Streptococcus mutans*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida*

albicans) inoculated onto acrylic resin specimens to simulate the contaminated prosthetic parts of acrylic resin devices used in prosthodontics and implantology. The null hypothesis was that methylene blue at 0.05% would not be effective as a photosensitizing agent associated with a LED of red wavelength (630 nm) at 10 and 30 J/cm².

MATERIAL AND METHODS

A power analysis was performed with software (BioEstat 5.3; Mamirauá Institute) to determine sample size. Data were obtained from a pilot study, including the difference between the means of the P-L- and P+L+ groups (=1.2), error standard deviation (=0.12), and number of treatments (=7) ($\alpha=0.05$). This test stated that 4 specimens per group were sufficient for a 90% probability of the statistical analysis to reject the null hypothesis (H_0) when the H_0 was actually false (power of the test=.90). Therefore, 5 specimens per group ($n=5$) were used.

A representative heat-polymerized acrylic resin (Lucitone 550; Dentsply Ind Com Ltd) which is used for the fabrication of complete dentures, partial removable dentures, and surgical guides for implants was selected for this study. Acrylic resin formers (15 mm×15 mm×4 mm), were embedded in metal flasks (Jon #6; Jon) with Type III dental stone (Herodent; Vigodent S/A Ind Com).

After the formers had been removed, the acrylic resin was mixed (21 g powder, 10 mL liquid), placed in the mold in the plastic phase, and polymerized according to the manufacturer's instructions (immersion in water at 73°C for 90 minutes and at 100°C for 30 minutes). After bench cooling and deflasking, the test specimens were immersed in distilled water at 50°C for 24 hours to eliminate residual monomer. The surfaces of each specimen were finished with 150-, 220-, 400-, 600-, 1200-, and 2000-grit abrasive paper (Norton Indústria Brasileira) and polished with wet rag wheels (Invicta), felt cones, and canvas polishing disks with pumice slurry followed

by calcium carbonate. The polished specimens were randomly distributed by drawing lots into groups ($n=5$), sealed in surgical-grade paper envelopes (Veda Max; Zermatt Ind Com Ltda), and sterilized with ethylene oxide gas (White Martins) in sterilizer equipment (UN.SUPR; Sercon).

Methylene blue (Vetec Fine Chemicals Ltd) at a concentration of 0.05% was selected as the photosensitizer. The light source was a red LED of 630 nm and output power of 150 mW (MMOptics Ltda), with variable energy intensities of 10 J/cm^2 and 30 J/cm^2 . The blue photosensitizer was coupled with the red LED because of the compatibility of the wavelength of this light source with the absorption band of the dye.

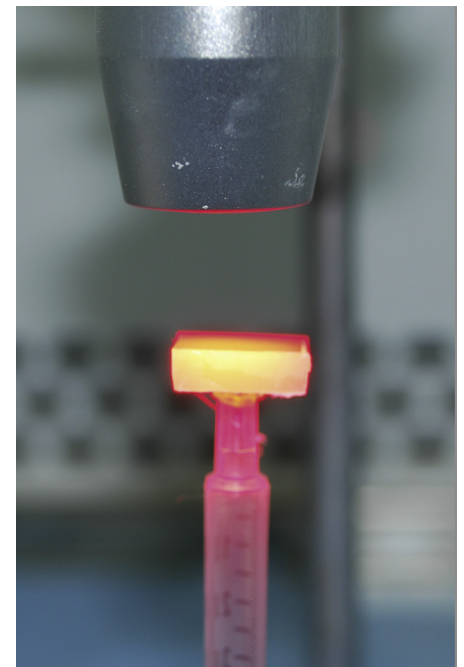
The microorganisms tested were *Streptococcus mutans*, provenance American Type Culture Collection (ATCC) 25175, and *Staphylococcus aureus* ATCC 25923, both with the morphotintorial characteristics of gram-positive cocci; *Escherichia coli* ATCC 11205 and *Pseudomonas aeruginosa* ATCC 10145, both with the morphotintorial characteristics of gram-negative cocci; and *Candida albicans* ATCC 001, yeast. These microorganisms underwent processes of activation, resuspension, and readout in a spectrophotometer (Ultrospec 1100 pro; Amersham Biosciences) in order to obtain a culture medium broth (brain-heart infusion broth; Acumedia) with a microbial inoculum of 1% at 0.5 McFarland scale, corresponding to 10^6 colony-forming units (CFU)/mL.^{5,11,12,35} The absorbances used were 0.08, at a wavelength of 590 nm for *Streptococcus mutans*, between 0.08 and 0.10 at a wavelength of 652 nm for *Staphylococcus aureus*, 1.43 at a wavelength of 600 nm for *Escherichia coli*, 0.64 at a wavelength of 600 nm for *Pseudomonas aeruginosa*, and 0.28 at a wavelength of 530 nm for *Candida albicans*. Specimen contamination consisted of their immersion in 15 mL of contaminated culture medium broth in individual falcon-type tubes. After this, the tubes containing the immersed test specimens were incubated at 37°C for 48 hours.

For each microorganism, the specimens were distributed into the following groups: P-L-, which were inoculated but not stained and not irradiated; P+L-, which were inoculated and stained but not irradiated; P-L+ 10, which were inoculated, not stained, but irradiated by the light source at 10 J/cm^2 ; P-L+ 30, which were inoculated, not stained, but irradiated by the light source at 30 J/cm^2 ; P+L+ 10, which were inoculated, stained, and irradiated at 10 J/cm^2 ; P+L+ 30, which were inoculated, stained, and irradiated at 30 J/cm^2 ; and GS (gold standard), which were sterilized in ethylene oxide gas. The P-L- group was the negative control to test the effectiveness of the contamination of the specimens, whereas the GS was the positive control and verified the effectiveness of previous sterilization with ethylene oxide gas. Each contaminated specimen was stained on all its surfaces with the 0.05% methylene blue. They were then irradiated with the red LED for the time necessary to achieve the levels of energy 10 J/cm^2 and 30 J/cm^2 , according to the experimental design (Fig. 1).

After the treatments, each specimen was placed in contact with the culture medium agar (brain-heart infusion broth and Sabouraud dextrose; Acumedia) in separate petri plates ($20 \times 100 \text{ mm}$). After 10 minutes, the specimens were discarded, and the plates were incubated at 37°C for 48 hours. Five petri plates per group were used. After the incubation period, the CFU were counted and the data were expressed according to the following scores: 1, corresponding to 0 CFU; 2, corresponding to 1 to 10 CFU; 3, corresponding to 11 to 100 CFU; and 4, corresponding to 101 to 1000 CFU. The scores for each group were statistically analyzed by the Friedman and Dunn multiple comparison tests ($\alpha=.05$).

RESULTS

The negative control of the *S mutans* group of specimens (P-L-) was scored between 3 and 4; when treated only



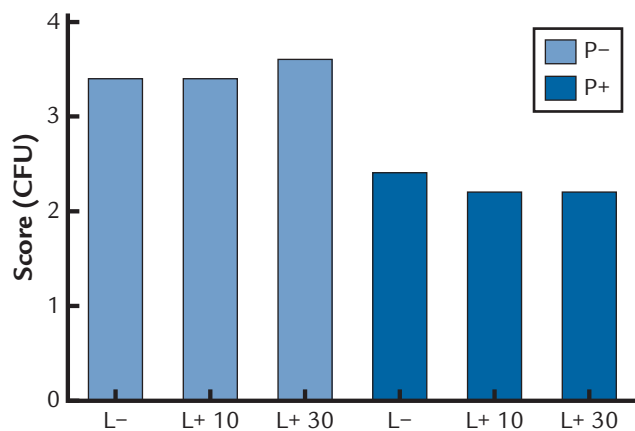
1 Application of PDT on acrylic resin specimen.

with the light (P-L+), no effect was observed. The use of methylene blue alone (P+L-) decreased the scores to a range of 3 to 2, and adding irradiation (P+L+) did not result in an additional effect ($P=.37$) (Fig. 2).

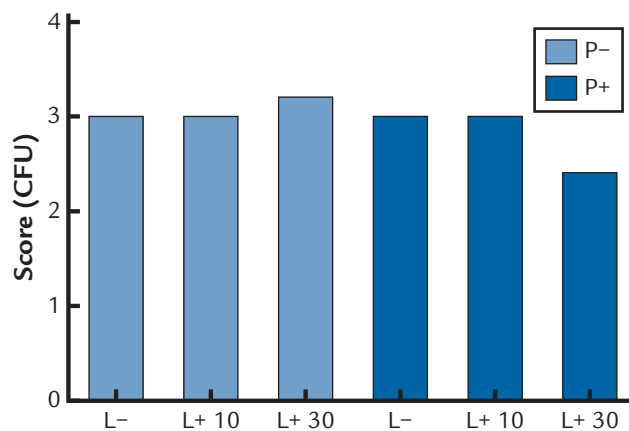
The negative control of the *S aureus* group of specimens (P-L-) was scored 3; when treated only with light (P-L+) or photosensitizer (P+L-), no effect was observed; the PDT with irradiation at 10 J/cm^2 (P+L+ 10) had no effect either, whereas the PDT with irradiation at 30 J/cm^2 (P+L+ 30) slightly decreased the CFU count to a score range of 3 to 2, which was not statistically significant ($P=.12$) (Fig. 3).

The negative control of the *E coli* group of specimens was scored 3; when treated only with light (P-L+) or photosensitizer (P+L-), no effect was observed; the PDT with irradiation at 10 J/cm^2 (P+L+ 10) had no effect either, whereas the PDT with irradiation at 30 J/cm^2 (P+L+ 30) significantly decreased the CFU count to score 2 ($P=.04$) (Fig. 4).

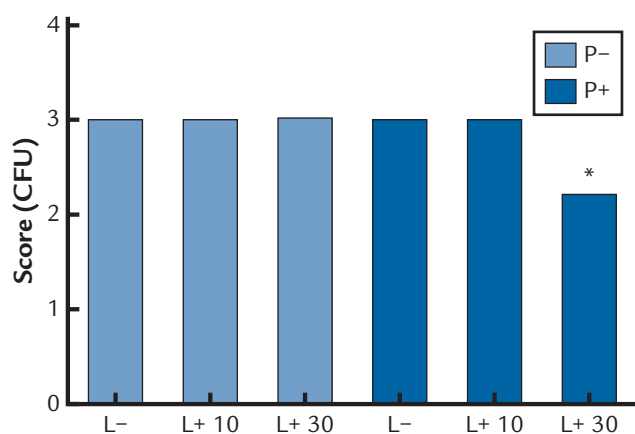
The negative control of the *P aeruginosa* group of specimens was scored 3; when treated only with light (P-L+), a slight decrease of CFU count was



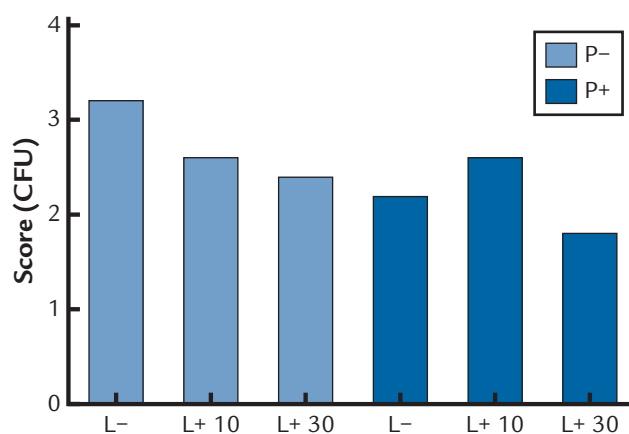
2 CFU count of *S. mutans* after treatments. P-, unstained groups; P+ stained groups; L-, unirradiated groups; L+, irradiated groups (J/cm^2).



3 CFU count of *S. aureus* after treatments. P-, unstained groups; P+ stained groups; L-, unirradiated groups; L+, irradiated groups (J/cm^2).



4 CFU count of *E. coli* after treatments. P-, unstained groups; P+ stained groups; L-, unirradiated groups; L+, irradiated groups (J/cm^2); * $P=0.0394$.



5 CFU count of *P. aeruginosa* after treatments. P-, unstained groups; P+ stained groups; L-, unirradiated groups; L+, irradiated groups (J/cm^2).

observed ($P=0.18$). The use of methylene blue alone (P+L-) decreased the scores to a range of 3 to 2, and adding irradiation (P+L+) did not result in an additional effect ($P=0.37$) (Fig. 5).

The negative control of the *C. albicans* group of specimens was scored 4; when treated only with light (P-L+), no effect was observed ($P=0.69$). The use of photosensitizer alone (P+L-) decreased the CFU count to score 3, and adding irradiation at $30 J/cm^2$ (P+L+ 30) resulted in a slight additional effect ($P=0.09$) (Fig. 6).

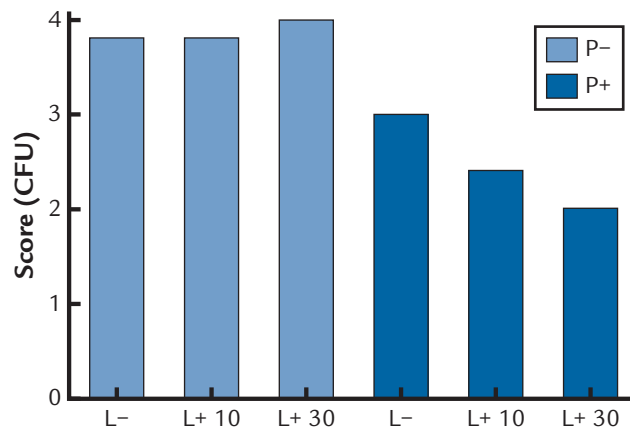
The GS groups, whose specimens were sterilized in ethylene oxide gas, showed no microbial growth. For this reason, this group was not used for statistical comparisons.

DISCUSSION

Data from this study led to partial rejection of the null hypothesis. Differences were observed among the microorganisms tested because the PDT presented different results for each microorganism.

Streptococcus mutans was sensitive to 0.05% methylene blue dye alone, showing a reduction in counts when only stained, but not associated with light. Rolim et al²⁶ reported that 327 μM methylene blue associated with a red LED at $24 J/cm^2$ was not effective against *S. mutans*, although Pereira et al⁵ had presented efficacy with 0.01 mg/mL methylene blue and InGaAlP red laser at $350 J/cm^2$ against this

microorganism. For specimens inoculated with *S. aureus*, group P+L+ 30 showed a reduction in rates of bacterial growth, but this was not statistically significant. These bacteria were not sensitive to methylene blue alone. The group P+L+ 30 for *E. coli* showed a significant reduction in the number of CFU. The specimens inoculated with *P. aeruginosa* also showed a sensitivity of this microorganism to the methylene blue dye, reducing the CFU count by a score of 1. In addition, they presented a slight sensitivity to the light alone. *C. albicans* did not present any sensitivity to the red LED but presented a sensitivity to methylene blue alone and conjugated with the red light at 10 and $30 J/cm^2$, showing a progressive but



6 CFU count of *C albicans* after treatments. P-, unstained groups; P+ stained groups; L-, unirradiated groups; L+, irradiated groups (J/cm^2).

statistically insignificant reduction in the CFU count. Pereira et al⁵ reported the efficacy of PDT against *S aureus* and *C albicans* biofilms by using higher concentration of dye and higher levels of light energy. This study sought to use a low dye concentration in order to avoid discoloration of the acrylic resin and a low-energy dose of LED to decrease the irradiation time required. However, these parameters were only significantly effective for *E coli* biofilm.

Among the controls for almost all microorganisms, groups P-L- and P-L+ showed a large number of CFU, whereas the GS did not show any microbial growth. This determined that there was a real single species biofilm formation in inoculated specimens, in accordance with other studies,^{5,12,35} and that the ethylene oxide gas sterilization was also effective. The isolated action of light source had almost no effect on the microorganisms, whereas the photosensitizer reduced the count of some microorganisms in the P+L- groups.

Wood et al¹⁷ reported that the action of erythrosine at a concentration of $22 \mu\text{M}$, without irradiation, had a negligible effect on *S mutans* compared to the control group, which was not stained and not irradiated. Mima et al²³ observed that Photogem $50 \text{ mg}/\text{L}$ and a blue LED, when not associated, also produced a negligible antimicrobial effect. Miyabe et al¹¹ reported that only laser irradiation or a 3 mM

concentration of methylene blue alone failed to reduce the count of *Staphylococcus* spp significantly. Costa et al¹² found the same results by using erythrosine 0.39 to $200 \mu\text{M}$ and laser 532 nm . The concentration of the photosensitizer, the light source, and the species of microorganisms may promote variation in results. A tendency was also observed toward a reduction in the microorganism count with an increase in the energy applied by the light source. This finding was in agreement with that of Queiroga et al,¹⁵ who reported a decrease in the *C albicans* count by raising the amount of energy provided by the red laser to $180 \text{ J}/\text{cm}^2$.

Some specimen groups presented intense microbial growth that resulted in colony aggregation, which made an exact count impossible. Therefore, because data do not allow a parametric description, an ordinal scale was used. This solution was taken, with some changes, from a study with a similar methodology performed by Paranhos et al.³⁵

The following limitations of the study are noted. Only the surface of the specimen that came into contact with the culture medium agar in the petri plates was light irradiated. The treatment of the whole specimen could have avoided possible contamination from other areas that had not been fully irradiated and may have provided more effective values of microbial death. Irradiation with the red LED of 150 mW ,

at energy intensity higher than $30 \text{ J}/\text{cm}^2$, would demand a long exposure time, which is not desirable. A more powerful machine in the same wavelength might have produced more effective results because energy is power multiplied by irradiation time. The inoculation in this study, although similar to other studies,^{5,11,12,35} was of great magnitude ($10^6 \text{ CFU}/\text{mL}$). A lower microbial load might have produced improved results.

Additional studies are necessary to establish a protocol for sterilizing acrylic resin specimens with PDT by evaluating higher amounts of energy in light sources of different wavelengths and lower concentrations of various photosensitizers. Clinical trials are also necessary. This study represents a starting point for a promising line of research.

CONCLUSIONS

On the basis of the results and within the limitations of this study, it can be concluded that PDT with 0.05% methylene blue as photosensitizer and a 630 nm LED significantly reduced the microbial count in the *E coli* biofilms formed on acrylic resin specimens. For *S mutans*, *S aureus*, *P aeruginosa*, and *C albicans* biofilms, an adjustment of protocol parameters is needed. Furthermore, the inhibition of microorganism growth tended to be directly proportional to the amount of energy provided by the light source: although $10 \text{ J}/\text{cm}^2$ was not effective, irradiation with $30 \text{ J}/\text{cm}^2$ showed more promising results.

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