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Antimicrobial photodynamic inactivation of *Staphylococcus aureus* biofilms in bone specimens using methylene blue, toluidine blue ortho and malachite green: An in vitro study

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ABSTRACT

Objective: To evaluate the *in vitro* effectiveness of APDI with a 660 nm laser combined with methylene blue (MB), toluidine blue ortho (TBO) and malachite green (MG) dyes to inactivate *Staphylococcus aureus* (ATCC 25923) biofilms in compact and cancellous bone specimens.
Methods: Eighty specimens of compact and 80 of cancellous bone were contaminated with a standard suspension of the microorganism and incubated for 14 days at 37 °C to form biofilms. After this period, the specimens were divided into groups ($n = 10$) according to established treatment: PS-L− (control – no treatment); PSmb + L−, PStbo + L−, PSmg + L− (only MB, TBO or MG for 5 min in the dark); PS-L+ (only laser irradiation for 180 s); and APDImb, APDItbo and APDImg (APDI with MB, TBO or MG for 180 s). The findings were statistically analyzed by ANOVA at 5% significance levels.
Results: All experimental treatments showed significant reduction of log CFU/mL *S. aureus* biofilms when compared with the control group for compact and cancellous bones specimens; the APDI group's treatment was more effective. The APDI carried out for the compact specimens showed better results when compared with cancellous specimens at all times of application. For the group of compact bone, APDImg showed greater reductions in CFU/mL (4.46 log 10). In the group of cancellous bone, the greatest reductions were found in the APDImb group (3.06 log 10).
Conclusion: APDI with methylene blue, toluidine blue ortho and malachite green dyes and a 660 nm laser proved to be effective in the inactivation of *S. aureus* biofilms formed in compact and cancellous bone.

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1. Introduction

Bacterial infection of the maxillofacial area, denominated osteomyelitis, is a highly debilitating condition that results in significant morbidity and health care costs, and it is notoriously difficult to treat.[1,2] It is caused by intraosseous bacterial spread. Although most cases are secondary to odontogenic infections (teeth extraction, pulp necrosis or periodontal infection),[3] hematogenous osteomyelitis may also occur. The typical pathogenic organisms are the *Staphylococcus* species (*Staphylococcus aureus* in 50% of cases),[1,2] the *Peptostreptococcus* species and *Pseudomonas aeruginosa*, among others. Less common causes, including fungal infections, mycobacterial infections, syphilis, and actinomycosis, must also be considered.[4,5] The essentials of the treatment of osteomyelitis involve early diagnosis, drainage of pus, bacteriologic culture and antibiotic sensitivity testing, appropriate antibiotic therapy, supportive therapy, surgical debridement and reconstruction if necessary.[1,2]

Another relevant factor in bone infections is microorganism's development of resistance to antimicrobial agents, which in this case arises from prolonged antibiotic therapy and the abandonment of treatment owing to the reduction in the symptoms by the chronification of the process.[6–8] The emergence of antibiotic-resistant *S. aureus* and the presence of a complex network of bacterial biofilm hinder the efficacy of conventional systemic antibiotics.[9] Biofilms formed by *S. aureus* are communities embedded in a matrix of extracellular polymers that offers advantages for the microorganism, which is mainly composed of intercellular adhesion polysaccharides, which reduce the penetration of antimicrobial agents and increase the protection against the host immune system.[10–12] In addition, biofilm has the ability to produce extracellular enzymes, which have shown very aggressive behavior.[13] In fact, bacterial resistance has become a severe global health problem, and the general concern about the future of antibacterial chemotherapy has prompted a search for novel antimicrobial agents with new mechanisms of action.[14]

An alternative therapy that has been indicated for the treatment of infections is antimicrobial photodynamic inactivation (APDI), which combines a nontoxic photosensitizer (PS) with harmless visible light at the correct wavelength to excite the PS to its reactive triplet state, which will then generate reactive oxygen species, such as singlet oxygen and superoxide, that are toxic to cells.[22] In recent years, APDI has been proposed as an alternative treatment for localized bacterial infections in response to the problem of antibiotic resistance.[15,16] APDI provides significant advantages over existing antimicrobial therapies. It appears to be equally effective at destroying multi-drug-resistant microbes and native strains, it acts remarkably more quickly against microorganisms than do antimicrobials, and, furthermore, there is no reported evidence for APDI-resistant mechanisms.[17]

The aim of this study was to evaluate the *in vitro* effectiveness of APDI with a 660 nm laser combined with methylene blue (MB), toluidine blue ortho (TBO) and malachite green (MG) dyes to inactivate *S. aureus* (ATCC 25923) biofilms in compact and cancellous bone specimens.

2. Materials and methods

2.1. Bone specimens

Eighty specimens of compact bone and 80 specimens of cancellous bone were obtained from the diaphysis and epiphysis regions, respectively, from bovine tibiae with the use of carborundum discs (Carborundum Abrasives SA, Recife, Pernambuco, Brazil), resulting in specimens with dimensions of 5 mm × 2 mm × 2 mm. The specimens were rinsed in saline (0.85% NaCl) and sterilized at 121 °C for 15 min.

2.2. Microorganism

A reference strain [American Type Culture Collection (ATCC)] of *S. aureus* (ATCC 25923) was used to form single-species biofilm in the bone tissues to evaluate the effectiveness of the proposed treatments. A microbial suspension containing 10^6 cells/mL was prepared using a 24-h culture seeded onto Mannitol agar (Difco, Detroit, MI, USA) using spectrophotometry.

2.3. In vitro formation of single-species biofilms

Compact and cancellous bones were used as substrates to form *in vitro S. aureus* single-species biofilms. To perform the experiment, the specimens were distributed by type (80 compact and 80 cancellous). We prepared 160 24-well flat-bottom microtiter plates corresponding to the experimental groups (8 plates with 10 specimens each of compact bone and 8 plates with 10 specimens each of cancellous bone). In each plate, ten wells were filled with 2 mL of sterile TSB (Tryptic Soy Broth, Difco, Detroit, MI, USA) and inoculated with 0.1 mL of *S. aureus* standard suspension. The 24-well flat-bottom microtiter plates were incubated for fourteen days at 37 °C. The humidity and nutrition conditions were evaluated daily.

2.4. Photosensitizer and laser source

MB, TBO and MG (Sigma–Aldrich Corp., St. Louis, MO, USA) were used as photosensitizers. A stock solution was prepared by dissolving the powder in sterile distilled water to obtain the concentration of 0.1 mg/mL and filtering it through a 0.22-μm membrane filter (Millipore, São Paulo, Brazil). After filtration, the dyes solutions were stored in the dark.

The light source used was an indium–gallium–aluminium–phosphide (InGaAlP) laser (Photon lase III, DMC Equipments, São Carlos, Brazil) with a wavelength of 660 nm. The output power was 40 mW, the energy dose was 7.2 J and the fluence of 257 J/cm^2 measured in the output of the light beam from the device tip. The biofilms illuminated the entire area of the bone specimen (0.1 cm^2) for a period of 180 s at a distance of 2 cm from the output of the beam to the specimen, resulting in 400 mW/cm^2 of irradiance.

2.5. Experimental conditions

For each bone type, subdivisions were performed in 8 groups with 10 specimens, each according to the type of treatment

to be administered: PS-L− (control, no treatment); PSmb + L−, PStbo + L−, PSmg + L− (only MB, TBO or MG for 5 min in the dark); PS-L+ (only laser irradiation for 180 s) and APDTmb, APDTtbo and APDTmg (APDT with MB, TBO or MG for 180 s).

After the treatments, each specimen was transferred to a sterile 2.5 mL polypropylene microtube containing 1 mL of sterile saline and shaken in a shaker apparatus for 1 min to obtain an initial suspension. From the initial suspension, decimal dilutions of 10^{-1}, 10^{-2} and 10^{-3} were made. The initial suspension and the other dilutions were plated in duplicate on Petri dishes containing a TSA (Tryptic Soy Agar, Difco, Detroit, MI, USA) culture medium. The plates were incubated at 37 °C for 24 h. Then, the incubation was performed, counting the colonies that formed units per millilitre (CFU/mL).

2.6. Statistical analysis

The logarithm of CFU/mL (CFU/mL log 10) was calculated. Adherence to the assumptions of normality and homoscedasticity were verified using the Kolmogorov–Smirnov normality test, and a normal distribution among the sample could be observed. Through ANOVA 5% comparisons were made between experimental treatments within the same group and between the CFU/mL means of the experimental treatments with the APDI groups.

3. Results

For the compact bone groups (Fig. 1), all experimental treatments significantly reduced the CFU/mL log 10 of *S. aureus* when compared with the control group, in which no treatment was given ($p < 0.05$). The groups in which MB, TBO and MG were used for 5 min (PSmb + L−, PStbo + L− and PSmg + L−) showed significant differences compared with the group to which only laser was applied (PS-L+), showing higher rates of reduction in the CFU/mL log 10 of *S. aureus*. For the groups to which APDI was applied with different dyes, all differed statistically ($p < 0.05$) when compared with the PS-L+ group, but in comparison with groups in which dyes were used alone (PSmb + L−, PStbo + L− and PSmg + L−), it was observed that APDImb differed statistically from the group PSmb + L− ($p = 0.00$) but not from the PStbo + L− and PSmg + L− groups ($p = 0.082$ and $p = 0.749$, respectively). The APDItbo group presented greater reduction in the CFU/mL log 10 of *S. aureus* compared with PSmb + L− ($p = 0.004$), but when it was compared with PStbo + L−+ and PSmg + L−, only the isolated application of these dyes showed more greatly reduced log 10s of *S. aureus* ($p = 0.025$ and $p = 0.004$, respectively). In groups in which the APDI was used, APDImg showed the best results in reducing the CFU/mL log 10 (4.46 log 10) of *S. aureus*.

For the cancellous bone groups (Fig. 2), similar results were observed, *i.e.*, all experimental groups differed from the control group ($p < 0.05$) except for the PSmb + L− group ($p = 0.234$). Comparing the group in which the laser was applied alone (PS-L+) with groups of dyes (PSmb + L−, PStbo + L− and PSmg + L−) revealed that there was no difference with the group PSmb + L− group ($p = 0.922$) but there was a significant difference with the PStbo + L− and PSmg + L− groups ($p = 0.00$), which showed greater reductions in the CFU/mL log 10 of *S. aureus* than PS-L+. For the APDI groups, it was observed that APDImb showed the greatest reduction in the CFU/mL log 10 (3.06 log 10) compared with APDItbo and APDImg.

Fig. 3 shows the comparison between the groups of APDI in compact and cancellous bone with the same dyes. It was observed that there was a statistically significant difference ($p = 0.00$) between the groups of compact and cancellous bone,

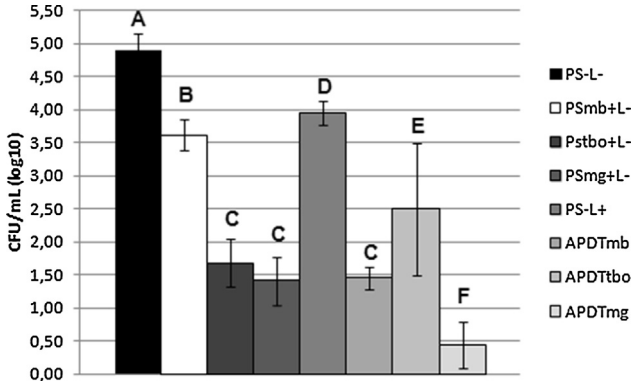

Fig. 1 – The means, expressed in units of *S. aureus* log 10 CFU/mL, and standard deviations (error bars) were obtained using an ANOVA with an alpha value of 5% for the experimental groups of compact bone samples. PS-L− = control, no treatment; PSmb + L− = only MB for 5 min in the dark; PStbo + L− = only TBO for 5 min in the dark; PSmg + L− = only MG for 5 min in the dark; PS-L+ = only laser irradiation for 180 s; APDTmb = APDT associated with MB; APDTtbo = APDT associated with TBO; and APDTmg = APDT associated with MG. Values followed by different letters differ statistically.

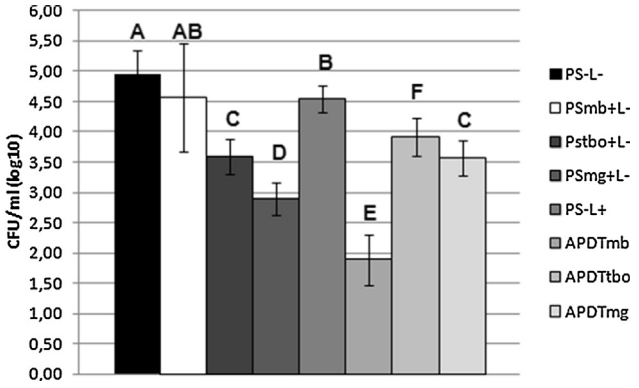

Fig. 2 – The means, expressed in units of *S. aureus* log 10 CFU/mL, and standard deviations (error bars) were obtained using an ANOVA with an alpha value of 5% for the experimental groups of cancellous bone samples. PS-L− = control, no treatment; PSmb + L− = only MB for 5 min in the dark; PStbo + L− = only TBO for 5 min in the dark; PSmg + L− = only MG for 5 min in the dark; PS-L+ = only laser irradiation for 180 s; APDTmb = APDT associated with MB; APDTtbo = APDT associated with TBO; and APDTmg = APDT associated with MG. Values followed by different letters differ statistically.

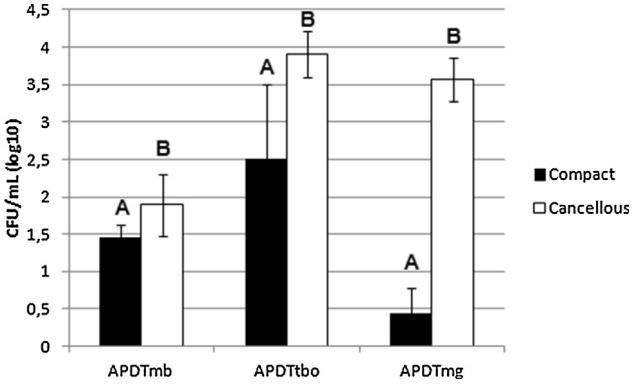

Fig. 3 – The means, expressed in units of *S. aureus* log 10 CFU/mL, and standard deviations (error bars) were obtained using an ANOVA with an alpha value of 5% for the APDT treatment groups for the compact and cancellous bone samples. APDTmb = APDT associated with MB; APDTtbo = APDT associated with TBO; and APDTmg = APDT associated with MG. Values followed by different letters differ statistically.

and the reductions in the CFU/mL log 10 were more significant in the groups of compact bone.

4. Discussion

Photodynamic therapy (PDT) was discovered over 100 years ago by Oskar Raab and Hermann Von Tappiener when they noticed that *Paramecium* spp. protozoas stained with acridine orange were destroyed upon exposure to bright light. Since then, PDT has primarily been developed as a treatment for cancer, ophthalmologic disorders and in dermatology. However, in recent years, interest in the antimicrobial effects of PDT has been revived, motivated by the rapidly increasing emergence of antibiotic resistance amongst pathogenic bacteria, and PDT has been proposed as a therapy for a large variety of localized infections been called antimicrobial photodynamic inactivation.[18–20] APDI provides significant advantages over existing antimicrobial therapies. It appears equally effective at destroying multi-drug-resistant microbes as it is with native strains, it acts remarkably more quickly against microorganisms than do antimicrobials, and, furthermore, there is no reported evidence for PDI-resistant mechanisms.[17]

S. aureus are known to possess a number of inherent traits that contribute to their high prevalence and virulence, yet perhaps their most effective barrier against host defences and antimicrobial agents is the propagation of biofilms. *S. aureus* produce a glycoprotein slime or glycocalyx biofilm that can adhere very effectively to host tissues and metallic implants, including pins and screws and prosthetic hips and joints, while at the same time providing protection to the embedded *S. aureus* cells.[6] Reduced susceptibility to APDI has been demonstrated when microorganisms are organized in biofilms.[21] In contrast to these findings, Vilella et al.,[10] Pereira et al.,[13] Simonetti et al.,[14] Hajin et al.[22] and Miyabe et al.[23] have demonstrated the significant effectiveness of this treatment against *S. aureus* biofilms when compared with control groups. Similar results were found in this study: the APDI with all three tested dyes yielded significantly different results from those for the other studied groups. A number of studies in the literature have investigated the action of antimicrobial agents in biofilms, including APDI.[9,10,13] In these studies, biofilms were formed on solid artefacts on most acrylic discs. In our work, we chose to use specimens of bone tissue in order to promote the formation of biofilms on biological tissue, simulating the challenges that could be encountered *in vivo* owing to the anatomical and structural aspects of these tissues.

Pereira et al.[13] evaluated the effects of APDI using MB on the viability of single, dual and three-species biofilms formed by *S. aureus*, *Streptococcus mutans* and *Candida albicans* and verified that the reductions (log 10) for single-species biofilms were greater (2.32–3.29) than were those obtained for multiple-species biofilms (1.00–2.44). In our work, we found a 3.45 log 10 reduction in the CFU/mL of *S. aureus* biofilms for the compact bone and a 3.06 log 10 reduction for cancellous bone, showing clinical relevance and potential future use for this treatment.

At the present time, phenothiazinium salts such as TBO and MB are used clinically for antimicrobial treatments. The minimal toxicity of these dyes to human cells,[24] in addition to their ability to produce high quantum yields of singlet oxygen, has produced great interest in testing the potential of these dyes as photo-activated antimicrobial agents.[25] These compounds are cationic at physiological pH, which enables them to target the bacterial membranes of both Gram-positive and Gram-negative bacteria.[19] MB and TBO are effective PSs against a broad range of microorganisms, such as *Escherichia coli*, *Staphylococcus aureus*, *Streptococci*, *Listeria monocytogenes*, and *Vibrio vulnificus*.[26] MG is a member of the triarylmethane family, along with crystal violet, and it shows strong absorption of red light. MG has been used in dental practice to visualize dental biofilms and as a colorimetric test to evaluate dental erosions.[27,28] However, MG did not produce singlet oxygen, indicating that the antimicrobial activity of APDI may also be promoted by other reactive oxygen species.[29]

Many studies in the literature have evaluated the toxicity of photosensitizing dyes without combination with light, reporting an absence of cytotoxic effects against microorganisms.[10,13,16,23,30] In this study, the results showed that the isolated use of MB, TBO and MG at the proposed concentrations (0.1 mg/mL) reduced the CFU/mL log 10 of *S. aureus* when compared with the control group, showing a bactericidal effect. Similar effects were shown by Junqueira et al.[31] with MG and Peloi et al.[32] with MB.

In this study, we used a low-intensity laser at a wavelength of 660 nm because these light sources are the most used in dental clinics and the most available on the market. Red light sources (630–700 nm) have been used extensively in APDI because of their relatively long wavelengths, which can effectively penetrate biological tissues. The scientific literature reports that the interaction between these light sources and the photosensitizers that absorb at this wavelength, such as MB, TBO and MG, can result in significant microbial destruction.[32,33] In order to determine the bactericidal activity of the 660 nm laser application on the microorganism tested, groups in which isolated laser light were used in this study,

using the same parameters as those used in the APDI groups. It was observed that both the compact and the cancellous bone groups showed reductions in the CFU/mL log 10 of *S. aureus* compared with the control groups, showing a small bactericidal effect. This can result because some bacterial cells are known to synthesize high levels of endogenous porphyrins that act as endogenous photosensitizers. Similar results were also found by Hajin et al.,[22] who observed a reduction in the number of CFU/mL of *S. aureus* only with laser application. Already Pereira et al.,[13] Simonetti et al.[14] and Miyabe et al.[23] had shown divergent results in which a laser-only application did not result in any difference in microbial reduction compared with the control group.

With respect to the potential clinical use of APDI, Bisland et al.[6] reported that its versatility, with optimized drug-light regimens, state-of-the-art light sources and interstitial placement of optical fibres for light delivery, may allow for therapy superior to that proffered by antibiotics, one that is targeted specifically to the site of infection, thus minimizing the risk of collateral damage to 'friendly' host flora as can occur with systemic use of antibiotics, and one that is easily customized in real time to the specific stage and severity of the infection.

5. Conclusion

APDI using methylene blue, toluidine blue ortho and malachite green dyes and 660 nm laser light, used with the described protocols, proved to be effective in the inactivation of *S. aureus* biofilms formed in compact and cancellous bone.

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We have not received financial support.

Competing interests

The authors confirm that there are no known conflicts of interest for the manuscript.

Ethical approval

The study was approved by the Animal Research Ethics Committee (UNESP Dental School, São José dos Campos-SP) under protocol number 05/2008-PA/CEP.

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