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ORIGINAL ARTICLE

In vitro effectiveness of antimicrobial photodynamic therapy (APDT) using a 660 nm laser and malachite green dye in *Staphylococcus aureus* biofilms arranged on compact and cancellous bone specimens

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Abstract The aim of this study was to evaluate the in vitro effectiveness of antimicrobial photodynamic therapy (APDT) using a 660 nm visible laser combined with malachite green (MG) dye in the inactivation of *Staphylococcus aureus* (ATCC 25923) biofilms formed within compact and cancellous bone specimens. Specimens of 80 compact bones and 80 cancellous bones were contaminated with a standard suspension of *S. aureus* and incubated for 14 days at 37 °C to allow for the formation of biofilms. The specimens were divided into the following groups (n=10) according to the treatment conditions: PS−L−(control — no treatment), PS+L−(only MG for 5 min), PS−L+90 (only laser irradiation for 90 s), PS−L+180 (only laser irradiation for 180 s), PS−L+300 (only laser irradiation for 300 s), APDT90 (APDT for 90 s), APDT180 (APDT for 180 s), and APDT300 (APDT for 300 s). The findings were statistically analyzed using an ANOVA 5 %. All of the experimental groups were significantly different from the control group for both the compact and cancellous bone specimens. The compact bone specimens that received APDT treatment (for either 90, 180, or 300 s) showed reductions in the log10 CFU/ml of *S. aureus* by a magnitude of 4 log10. Cancellous bone specimens treated with 300 s of APDT showed the highest efficacy, and these specimens had a reduction in *S. aureus* CFU/ml by a factor of 3 log10. APDT treatment using these proposed parameters in combination with MG was effective at inactivating *S. aureus* biofilms in compact and cancellous bone specimens.

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Introduction

Staphylococcus aureus is the most important representative of the staphylococcal group of bacteria and causes clinically relevant infections in immunocompetent patients. It lives commensally on human skin, nares, and mucosal surfaces and is an opportunistic pathogen capable of infecting, invading, persisting, and replicating in many human tissues [1]. Several virulence factors, including coagulase, catalase, clumping factor A, and cytolytic toxins, are responsible for the pathogenicity of *S. aureus* [2]. The colonization rate of *S. aureus* in the normal population is between 10 and 40 %; approximately 10 % of the population is permanently colonized. Higher carrier rates have been described in hospitalized patients, particularly, in elderly individuals who are at risk for nosocomial infections due to immunosenescence, poor nutrition, multiple comorbidities, or impaired wound healing [3]. The ability of staphylococci to survive under different environmental conditions allows the bacteria to proliferate and colonize the tissues, leading to serious complications including sepsis, amputation, and death [4].

Microorganisms found in nature thrive by adhering to both living and inanimate surfaces through the formation of biofilms. Biofilms have been shown to be involved in approximately 80 % of all infections. The dense and protected environment of the film houses bacteria that have significantly different properties from free-floating bacteria of the same species and biofilms have been shown to be resistant (by 1,000-fold) to detergents, antiseptics, and antibiotics [5].

Biofilms formed by *S. aureus* are communities embedded in a matrix of extracellular polymers, mainly composed of intercellular adhesion polysaccharides, that offers advantages to the microorganism. The matrix reduces the penetration of antimicrobial agents and offers enhanced protection against the host immune system [6, 7]. Additionally, the biofilm has the ability to produce extracellular enzymes, which increase the aggressive behavior of the bacterium [8].

The emergence of antibiotic-resistant *S. aureus* and the presence of a complex network of bacterial biofilm have limited the effectiveness of conventional systemic antibiotics [9]. Indeed, bacterial resistance has become a severe global health problem, and the general concern regarding the future of antibacterial chemotherapy has prompted the search for novel antimicrobial agents with new mechanisms of action [4].

An alternative therapy that has been indicated for the treatment of infections is termed antimicrobial photodynamic therapy (APDT). APDT combines the use of a nontoxic photosensitizer (PS) of harmless light at the correct wavelength to excite the PS to its reactive triplet state, which then generates reactive oxygen species, such as singlet oxygen and superoxide; these reactive oxygen species are toxic to cells [10]. In recent years, APDT has been proposed as an alternative treatment for localized bacterial infections in response to the problem of antibiotic resistance [11]. APDT provides significant advantages over existing antimicrobial therapies. It appears equally effective at killing multi-drug resistant microbes and bacterial native strains. Furthermore, APDT acts much more rapidly against microorganisms than antimicrobials, and no evidence of APDT resistance mechanisms has been reported to date [12].

The aim of this study was to evaluate the in vitro effectiveness of APDT using a 660 nm visible red laser combined with malachite green dye for the inactivation of *S. aureus* (ATCC 25923) biofilms formed in compact and cancellous bone specimens.

Materials and methods

Bone specimens

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

This study was approved by the Animal Research Ethics Committee (UNESP Dental School, São José dos Campos-SP, Brazil) under protocol number 05/2008-PA/CEP. The study strictly followed all of the ethical principles set forth by the Declaration of Helsinki (2000).

Microorganism

A reference strain [American Type Culture Collection (ATCC)] of *S. aureus* (ATCC 25923) was used to generate single-species biofilms in bone tissues for the evaluation of the proposed treatments. A microbial suspension containing 10× 6 cells/ml was prepared by seeding a 24-h culture onto Mannitol agar (Difco, Detroit, MI, USA) using a spectrophotometry.

In vitro formation of single-species biofilms

Compact and cancellous bones were used as substrates for growing in vitro *S. aureus* single-species biofilms. To perform the experiment, the specimens were distributed according to type (80 compact and 80 cancellous specimens) into 160 24-well flat-bottom microtiter plates. The plates were organized according to the experimental groups (eight plates with ten compact bone specimens and eight plates with ten cancellous bone specimens). In each plate, ten wells were filled with 2 ml of sterile tryptic soy broth (TSB, Difco) and inoculated with 0.1 ml of the *S. aureus* standard suspension. The 24-well flat-bottom microtiter plates were incubated for 14 days at 37 °C. The humidity and nutrition conditions were evaluated daily.

Photosensitizer and laser source

Malachite green (MG; Sigma-Aldrich Corp., St. Louis, MO, USA) was used as a photosensitizer. A stock solution was prepared by dissolving the powder in sterile distilled water at a concentration of 0.1 mg/ml, followed by filtration through a 0.22 μm membrane filter (Millipore, São Paulo, Brazil). After filtration, the dye solution was stored in the dark.

An indium–gallium–aluminum–phosphide (InGaAlP) laser (Photon lase III, DMC Equipments, São Carlos, Brazil) with a wavelength of 660 nm was used as the light source for all of the experiments. The output power was 40 mW with an energy dose of 3.6, 7.2, and 12 J and a fluence of 128, 257, and 428.5 J/cm^2. The biofilms were illuminated by exposing the entire area of the bone specimen (0.1 cm^2) for 90, 180 or 300 s at a distance of 2 cm from the output of the beam to the specimen; this resulted in 400 mW/cm^2 of irradiance.

Experimental conditions

Each bone type was subdivided into eight groups containing ten specimens each. These groups were determined based on the type of treatment: PS−L−(control — no treatment), PS+L−(only MG for 5 min in the dark), PS−L+90 (only laser irradiation for 90 s), PS−L+180 (only laser irradiation for

180 s), PS−L+300 (only laser irradiation for 300 s), APDT90 (APDT for 90 s), APDT180 (APDT for 180 s), and APDT300 (APDT for 300 s).

After treatments, each specimen was transferred to a sterile 2.5 ml polypropylene microtube containing 1 ml of sterile saline and was shaken in a shaker apparatus for one minute. This allowed for the formation of initial suspensions, and 1:10, 1:100, and 1:1000 dilutions were made. The initial suspension and dilutions were plated in duplicate on Petri dishes containing tryptic soy agar (TSA, Difco) culture medium. The plates were incubated at 37 °C for 24 h. After the incubation period, those plates containing from 30 to 300 colonies were used to calculate the colony forming units per milliliter (CFU/ml) using a colonies counter device (Spencer, Santo André, São Paulo, Brazil).

Statistical analysis

The logarithm of CFU/ml (log10 CFU/ml) was calculated for each specimen. Adherence to the assumptions of normality and homoscedasticity was verified using the Kolmogorov–Smirnov normality test, and the data exhibited a normal distribution. Using an ANOVA with an alpha value of 5 %, comparisons were made between experimental treatments within the same bone specimen group, and comparisons of the mean CFU/ml of the APDT experimental treatments were also evaluated between the bone specimen groups.

Results

For the compact bone treatment groups (Fig. 1), all of the treatment groups were significantly different from the control group ($p<0.05$), and the various treatments all led to a reduction in the *S. aureus* log10 CFU/ml. The PS group (PS+L−) had a significant reduction in *S. aureus* log10 CFU/ml compared with groups in which the laser was applied alone for 90, 180, or 300 s (PS−L+90, PS−L+180, or PS−L+300, respectively). For laser groups application for 90, 180, or 300 s (PS−L+90, PS−L+180, or PS−L+300, respectively), the results showed smaller reductions in *S. aureus* log10 CFU/ml (0.95 to 1.34 log10 magnitude decrease) compared to the other experimental groups. Among these groups, the PS−L+90 and PS−L+300 showed more significant reductions in *S. aureus* log10 CFU/ml compared with PS−L+180 group ($p=0.002$ and $p=0.000$, respectively). Groups receiving APDT for 90, 180, or 300 s showed the greatest reductions in *S. aureus* log10 CFU/ml (a 4 log10 magnitude decrease), and these groups were significantly different compared to the other experimental groups. APDT180 and APDT300 showed no significant difference between them ($p=0.344$), but showed greater reductions in *S. aureus* log10 CFU/ml compared to APDT90 group.

The same results were observed for the cancellous bone treatment groups (Fig. 2), and all of the experimental groups differed from the control group ($p<0.05$). The PS group (PS+L−) had a significant reduction in *S. aureus* log10 CFU/ml compared with groups in which the laser was applied alone for 90, 180, or 300 s (PS−L+90, PS−L+180, or PS−L+300, respectively) and the APDT groups for 90 or 180 s. In laser groups application for 90, 180, or 300 s (PS−L+90, PS−L+180, or PS−L+300, respectively), the results showed smaller reductions in *S. aureus* log10 CFU/ml (0.41 to 1.02 log10 magnitude decrease) compared to the other experimental groups. Among these groups, the PS−L+90 group showed more significant reductions in *S. aureus* log10 CFU/ml compared with PS−L+180 ($p=0.000$) and PS−L+300 ($p=0.020$) groups. The group that showed the greatest reduction in *S. aureus* log10 CFU/ml was the APDT300 treatment group (reduction of 3 log10), and this difference was statistically significant compared to the other treatment groups.

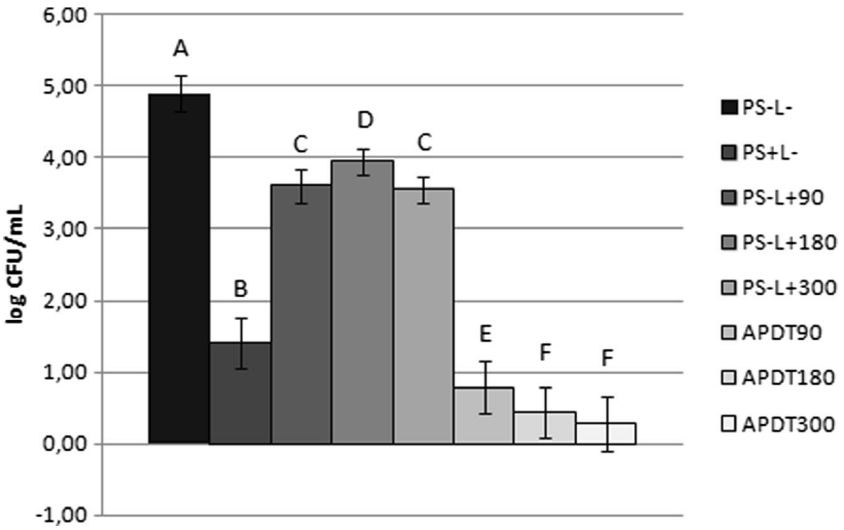

Fig. 1 The means, expressed in units of *Staphylococcus aureus* log10 CFU/ml, and standard deviations (*error bars*) were obtained using an ANOVA with an alpha value of 5 % for the experimental groups of the compact bone samples. *PS−L−* control — no treatment, *PS+L−* only MG for 5 min in the dark, *PS−L+90* only laser irradiation for 90 s, *PS−L+180* only laser irradiation for 180 s, *PS−L+300* only laser irradiation for 300 s, *APDT90* APDT for 90 s, *APDT180* APDT for 180 s, *APDT300* APDT for 300 s. Values followed by different letters differ statistically

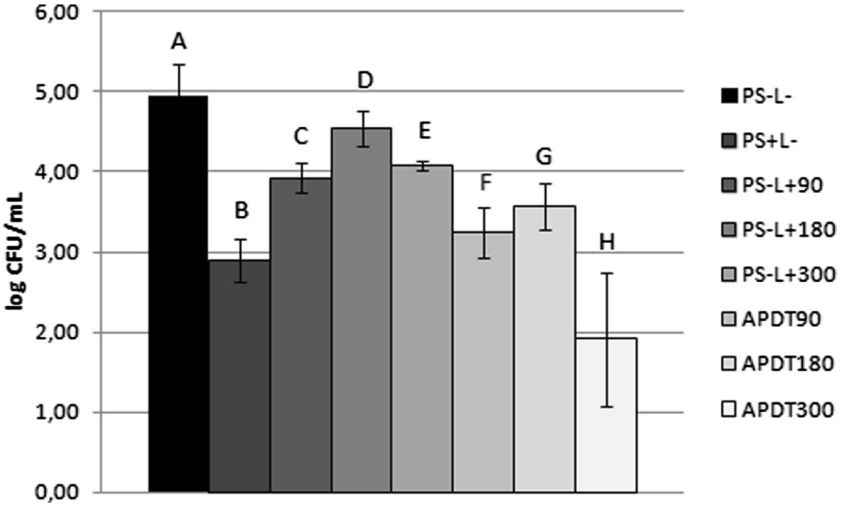

Fig. 2 The means, expressed in units of *Staphylococcus aureus* log10 CFU/ml, and standard deviations (*error bars*) were obtained using an ANOVA with an alpha value of 5 % for the experimental groups of the cancellous bone samples. *PS−L−*control — no treatment, *PS+L−* only MG for 5 min in the dark, *PS−L+90* only laser irradiation for 90 s, *PS−L+180* only laser irradiation for 180 s, *PS−L+300* only laser irradiation for 300 s, *APDT90* APDT for 90 s, *APDT180* APDT for 180 s, *APDT300* APDT for 300 s. Values followed by different letters differ statistically

The APDT treatment groups (90, 180, and 300 s) conducted in compact and cancellous bone (Fig. 3) were compared, and the compact bone APDT treatments were more effective than the cancellous bone APDT treatments ($p<0.05$).

Discussion

Given the once perceived miraculous nature of penicillin, it is not surprising that resistant strains of bacteria were observed within a year of its introduction into the clinical setting [13]. Indeed, the clamor of the public for antibiotics that could treat every conceivable disease also partially explains the incorrect and/or over-prescription of penicillin by harassed general practitioners, with occurred over the past half century. It is now appreciated that antibiotics must be used more responsibly if mankind is to retain some chemotherapeutic advantage over its microbial colonists. To maximize this advantage, however, other approaches to microbial disinfection must be adopted alongside to more traditional methods [14]. APDT using low-intensity lasers has been evaluated as an alternative method for antimicrobial treatment because, unlike antibiotics and antimicrobials, the development of resistance appears to be unlikely, as singlet oxygen and free radicals in microbial cells interact with multiple cell structures and affect different metabolic pathways [15, 16].

The organization of microbial cells within the different layers of the biofilm may provide some protection to the microorganisms within the inner layers, contributing to the bacterial resistance to conventional drugs [17]. In vitro susceptibility tests in model biofilms have revealed significant microorganism survival after treatment with antibiotics [18].

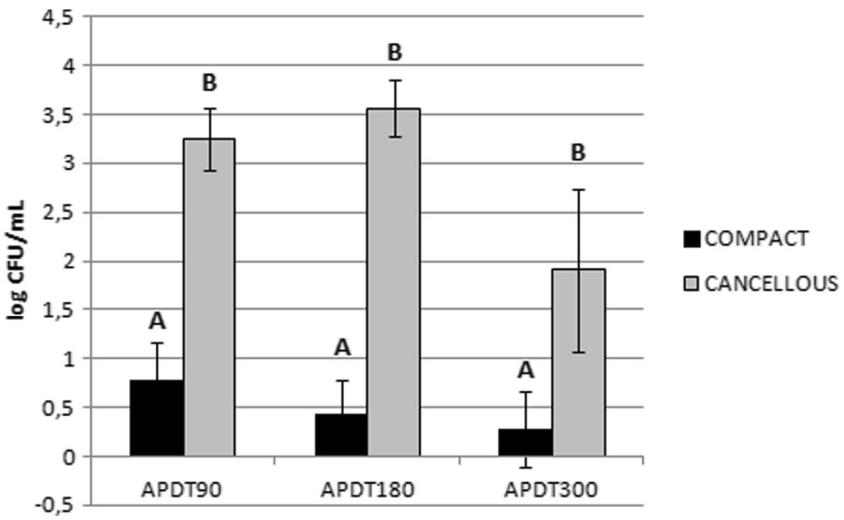

Fig. 3 The means, expressed in units of *Staphylococcus aureus* log10 CFU/ml, and standard deviations (*error bars*) were obtained using an ANOVA with an alpha value of 5 % for the APDT treatment groups for the compact and cancellous bone samples. *APDT90* APDT for 90 s, *APDT180* APDT for 180 s, *APDT300* APDT for 300 s. Values followed by different letters differ statistically

Drug resistance can include target alterations, overexpressed efflux capabilities, and drug inactivation. However, the fact remains that none of these drug resistance capabilities of resistant organisms include the singlet oxygen radical that is generated by APDT [19].

APDT has been extensively studied [1, 4–6, 8, 10–12, 20–22], and it has exhibited positive results in most studies. However, reduced susceptibility to APDT has been demonstrated when microorganisms are organized in biofilms [23]. In contrast to these findings, Hajin et al. [1], Simonetti et al. [4], Vilela et al. [6], Pereira et al. [8], and Miyabe et al. [23] have shown great APDT effectiveness against *S. aureus* biofilms compared to control groups. Similar results were observed in this study, in which APDT combined with MG was significantly different from all of the other treatment groups. These results may be explained by the high chemical reactivity of the photosensitizer which ensures a direct effect on other non-cellular biomolecules where these interactions occur. Thus, the polysaccharides involved in the extracellular polymeric substance (EPS) of the bacterial biofilm are equally susceptible to photo damage by APDT [24]. Several studies in the literature have evaluated the effects of antimicrobial agents on biofilms, and APDT has been included in these studies [6, 8, 9, 14]. In these previous experiments, the biofilms were formed on solid artifacts and, most commonly, acrylic discs. In our present work, we chose to use bone tissue specimens to promote the formation of biofilms on a biological tissue, simulating the challenges that could be encountered in vivo due to the anatomical and structural aspects of these tissues.

MG dye has been used to visualize bacterial biofilms on teeth, both as a support tool for counseling patients with periodontal diseases on oral hygiene and for professional quantification of the biofilm index. Additionally, MG is easily transported across the cell membrane in both Gram-positive and Gram-negative bacteria [25].

Vilela et al. [6] reported that APDT combined with MG reduced the *S. aureus* biofilm CFU/ml by a factor of 1.63 log10. In this study, reductions of *S. aureus* biofilm CFU/ml of approximately 4.62 log10-fold were observed for the compact bone groups, and reductions of approximately 3.04 log10-fold were observed for the cancellous bone groups. These differences may be due to the different laser parameters used in this study. There are few reports in the literature evaluating the efficacy of MG as a photosensitizer for APDT, and these limited studies were performed on planktonic cultures. Prates et al. [25] evaluated MG dye (300 mM) as an APDT photosensitizer against planktonic cultures of *Actinobacillus actinomycetemcomitans*; this study used an excitation wavelength of 660 nm and an energy dose of 9 J/cm^2. They observed microbial reductions ranging from 2 log10-fold to 3 log10-fold. Junqueira et al. [26] investigated the photodynamic antimicrobial effects of the MG photosensitizer on planktonic cultures of *Staphylococcus*, *Enterobacteriaceae*, and *Candida* using an in vitro approach. The *Staphylococcus*, *Enterobacteriaceae*, and *Candida* strains were sensitive to APDT combined with MG. Microbial reductions of approximately 7 log10-fold for *Staphylococcus*, 6 log10-fold for enterobacteria, and 0.5 log10-fold for the genus *Candida* were obtained.

Previous studies have shown a reduction in the number of *S. aureus* cells by a magnitude of 0.97 log10, 0.97 log10, and 1.05 log10 following irradiation with a red LED (20 J/cm^2) or diode laser light (20 and 40 J/cm^2) in the absence of a photosensitizer, respectively [23, 27, 28]. In this study, groups receiving laser therapy at 660 nm for only 90, 180, or 300 s exhibited a reduction of *S. aureus* by approximately 0.95 log10 to 1.34 log10 for compact bone samples and a reduction of 0.41 log10 to 1.02 log10 for cancellous bone specimens; these results suggest that laser therapy has bactericidal action. Similar results were also observed by Hajin et al. [1]; this group observed a reduction in the number of CFU/ml of *S. aureus* by laser treatment alone. Conversely, Simonetti et al. [4], Vilela et al. [6], Pereira et al. [8] , Miyabe et al. [23], and Junqueira et al. [26], demonstrated divergent results; in these studies, laser application alone did not lead to a significant difference in microbial reduction compared to the control groups.

To evaluate the toxicity of MG in the dark, control assays in the absence of light exposure (PS+L−) were performed. At the concentration used in this study (0.1 mg/ml), MG was toxic to *S. aureus* biofilms, reducing *S. aureus* CFU/ml by a magnitude of 3.49 log10 for compact bone samples and 2.05 log10 for cancellous bone samples. Previous studies have shown that MG is not cytotoxic to planktonic cultures, bacterial biofilms [6, 8, 22], or yeasts cultures [8, 25]. However, Junqueira et al. [26] demonstrated that the addition of MG alone was cytotoxic to planktonic cultures of *Staphylococcus* and enterobacteria. In this work, the cancellous bone group that was treated with MG for 5 min showed greatest rates of reduction in *S. aureus* log10 CFU/ml compared with groups treated only with laser in the three application times (90, 180, and 300 s) and with respect to APDT for 90 or 180 s (APDT90 and APDT180). The possible explanation for the PS+L−group has been more effective than APDT90 and APDT180 groups would be the combination of two factors: the time of MG association with laser light must be greater than 90 and 180 s to reach higher levels of antimicrobial activity, and the anatomical structure of cancellous bone allows more adherence surface for the microorganism tested (*S. aureus*) difficulting the action of APDT in this tissue.

The cytotoxicity of phenothiazine photosensitizers on mammalian cells has also been widely studied [21, 29, 30]. These studies suggest that APDT in combination with methylene blue or toluidine blue is a safe antimicrobial treatment that spares normal tissues from the damaging effects of APDT treatment.

By examining the results of this study, it could be demonstrated that APDT was more effective in reducing the *S. aureus* log10 CFU/ml in compact bone samples than in cancellous bone samples. This observation can be explained by the capacity of light to reach all of the biofilm regions impregnated with the photosensitizing dye, thereby restricting the action of singlet oxygen within the entire biofilm. Additionally, the bonding surface of the microorganism varies between compact and cancellous bones due to the anatomical differences between the bone types.

Studies in the literature that use APDT to inhibit *S. aureus* planktonic cultures demonstrated the efficacy of this technique and suggested that APDT is a promising therapeutic option for the treatment of bacterial infections. However, there has been little research on APDT in the context of bactericidal biofilms attached to bone tissue in the literature, indicating that more studies on this topic should be conducted to establish protocols and parameters that can circumvent the known difficulties of this therapy. In this work, APDT in combination with MG and a 660 nm laser, with the proposed parameters, could effectively inactivate *S. aureus* biofilms arranged within bone specimens. Specifically, biofilms within compact bone that received APDT in combination with MG exhibited the greatest reduction in *S. aureus* log10 CFU/ml when compared to all of the treatment groups for both the compact or cancellous bone samples.

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