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The in vitro effect of antimicrobial photodynamic therapy on *Candida* and *Staphylococcus* biofilms

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Background/aim: This study was designed to evaluate the effect of antimicrobial photodynamic treatment (APDT) in a biofilm model using combinations of various dyes (rose bengal, riboflavin, and methylene blue) as photosensitizers and light sources (LED and UVA) against staphylococcal and candidal biofilms.

Materials and methods: Sterile microtiter plates were used for the development and quantification of the biofilms. APDT was carried out using combinations of the light sources and dyes. The percentage of the growth inhibition was then calculated using a spectrophotometer. The broth media in the wells were aspirated, wells were stained with crystal violet, and optical density values were measured spectrophotometrically. SEM analysis of the impact of APDT on bacterial and fungal biofilms was also performed.

Results: The experiments showed that the most efficacious combination was red LED + methylene blue against both staphylococcal and candidal biofilms. A marked inhibition (45.4%) was detected on both *C. albicans* and *C. parapsilosis* biofilms. Red LED + methylene blue was also effective on *S. aureus* and *S. epidermidis* biofilms. SEM images suggested that the number of adherent cells and biofilm mass were markedly reduced after APDT treatment.

Conclusion: Although the results of this study indicated the in vitro efficacy of APDT, it might also be a promising technique for the control of biofilm growth within intravenous catheters.

Key words: Antimicrobial photodynamic therapy, biofilm, *Candida*, *Staphylococcus*

1. Introduction

Antimicrobial photodynamic treatment (APDT) has emerged in recent years as an adjunctive to the conventional antimicrobial therapeutic modality for the treatment of different types of bacterial and fungal infections. Photodynamic antimicrobial therapy represents an alternative antibacterial, antifungal, and antiviral treatment against drug-resistant organisms (1). It is a novel approach based on the interaction of a nontoxic photosensitizer and a harmless low-energy light source. The combination of these two factors in the presence of oxygen results in the creation of reactive oxygen species and triggers a cascade of biological events that leads to apoptosis and the death of microorganisms. This antimicrobial approach may help to destroy the microbial populations in biofilms. The biofilm matrix surrounding microbial cells makes them tolerant to harsh conditions and resistant to antimicrobial treatments. Hence, it is critically important to design or screen antibiofilm practices that can effectively minimize and eradicate biofilm-related catheter infections (2).

Staphylococcus spp., *Enterococcus* spp., gram-negative bacilli, and *Candida* spp. are the most commonly encountered microorganisms in catheter-related bloodstream infections (CRBSIs). Poor clinical response to antimicrobial therapy is related to the biofilm production among these microorganisms. Clinical practice guidelines recommend the removal of the catheter or antimicrobial lock therapy in certain conditions (3). APDT is a promising salvage therapy for CRBSIs because it can help to prevent biofilm formation and rapidly reduce the bacterial and candidal load from the biofilms developed on the surface of the intravenous catheters (1,3–5).

In this study, the in vitro effectiveness of photodynamic therapy in the reduction of biofilms grown in microplate wells and on glass slide surfaces was evaluated. A biofilm model was constructed to assess the effect of

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photodynamic therapy using rose bengal (RB), riboflavin (RBF), and methylene blue (MB) as the photosensitizer agents combined with UVA and LED light sources against staphylococcal and candidal biofilms.

2. Materials and methods

2.1. Bacterial and fungal strains

Staphylococcus aureus ATCC 35556, *Staphylococcus epidermidis* ATCC 35984, *Candida albicans* ATCC 90028, and *Candida parapsilosis* ATCC 96142 were used in this study as reference strains for biofilm production. Fungal strains were cultivated on Sabouraud dextrose agar plates and bacterial strains were grown on Mueller Hinton agar plates for 24 h at 35 °C under aerobic conditions. Overnight cultures of each strain diluted in sterile saline were used to prepare the test suspensions. The turbidity of each suspension was adjusted to the equivalent of 3×10^6 CFU/mL for *Candida* spp. and 3×10^8 CFU/mL for *Staphylococcus* spp. using the McFarland turbidity standards (2,4,5).

2.2. Biofilm formation

Biofilm formation was evaluated and quantified using a 96-well polystyrene microtiter plate assay plus scanning electron microscopy (SEM). Biofilms were grown in the wells of sterile microplates and on the surface of 1 cm × 1 cm glass slides. Flat-bottom microplates (Corning) were inoculated with aliquots of 20 µL of bacteria and yeast cell suspensions and 180 µL of Mueller Hinton broth (MHB). The microplates were incubated at 37 °C for 72 h in an orbital shaker at 150 rpm to ensure biofilm formation. In addition, aliquots of 1 mL of bacterial and fungal suspensions were inoculated into 6-well culture plates (Thermo Fisher Scientific) containing 1 mL of MHB for SEM analysis. Glass slides were then placed into each of these wells. After incubation for 72 h, these glass slides were used for SEM analysis (2,4,5).

2.3. Light sources and photosensitizers

The light sources used in the treatment of biofilms were one UVA lamp, with a wavelength of 370 nm, and two LED lamps, one emitting light in the red spectrum at 660 nm and the other in the green spectrum at 518 nm. Photosensitizers, obtained from Sigma-Aldrich (St. Louis, MO, USA), were watery solutions of RBF (1 g/1 L, 0.1%, in distilled water), used in conjunction with UVA; MB (25 µg/mL), used in conjunction with red LED; and RB (1 g/ 1 L, 0.1%, in distilled water), used in conjunction with blue LED (2,6).

2.4. Photodynamic treatment of biofilms and antibiofilm assay

After 72 h of incubation with resulting biofilm formation, 10 µL of RBF (0.1%), 10 µL of MB (25 µg/mL), and 10 µL of RB (0.1%) suspensions were added to the wells of the microplates. Biofilm control plates were left without an antimicrobial drug for the formation of biofilms. In order to prevent microbial growth and biofilm formation in negative controls, amphotericin B was added to the wells of the microplates containing *Candida* spp. and teicoplanin was added to the wells of the microplates containing *Staphylococcus* spp. Microplates were then exposed to the corresponding light sources for 5 min. Intensity of the light spot was measured with a power meter (PM200, Thorlabs GmbH, Dachau, Germany). The applied PDT was 0.77 mW and energy fluency was set to 233 mJ/cm^2 when irradiated for 300 s (mW (J/s) × s = mJ).

Biofilm formation on the surface of microplates and the antibiofilm effect of APDT were evaluated using a quantitative crystal violet assay as described previously (7). Briefly, the culture broth media in the wells of the microplates was aspirated gently, and wells were washed twice with distilled water. After the media and microbial cells were discarded, the remaining biofilm was stained with crystal violet for 1 min. Biofilm formation was then quantified by measuring the optical density values in each well using a spectrometer at 630 nm. The biofilm inhibition index was calculated for each well using the following formula: Biofilm inhibition index: (untreated control biofilm OD – treated biofilm OD) / untreated control biofilm OD × 100. The effect of APDT on the treated biofilms were determined in comparison with the untreated biofilms.

2.5. Photodynamic inactivation of planktonic cells

To observe the impact of PDT on biofilm formation by bacteria and fungi, survival of the microbial cells after the treatment was also analyzed in addition to the quantification of biofilm by crystal violet assay. The viability of bacteria and fungi was analyzed by counting the numbers of CFU/mL after plating on appropriate culture media. Briefly, 10^8 CFU/mL suspensions of bacteria (*S. aureus* and *S. epidermidis*) and 10^6 CFU/mL suspensions of fungi (*C. albicans* and *C. parapsilosis*) in phosphate-buffered saline were incubated with each of the photoactivators in the dark for 15 min and were then exposed to different light sources. After 300 s of treatment, aliquots of 10 µL of cell suspensions were inoculated onto SDA and MHA plates and incubated at 37 °C for 24 h. Following 24 h of growth, colonies were counted, expressed in CFU/mL, and the effects of APDT on the count reduction (log CFU/mL) of bacterial and fungal cells were determined (2).

2.6. Scanning electron microscopy

SEM analysis was used in order to visualize the effect of APDT on grown bacterial and fungal biofilms. Briefly, biofilms were grown on 1 cm × 1 cm sterile glass slides as described above in 6-well culture plates. After the photodynamic treatment, slides were removed, initially fixed using 2.5% glutaraldehyde + 2% formaldehyde in

phosphate buffer for 2 h at 4 °C, and were then rinsed in distilled water. A series of ethanol washes (20%, 40%, 60%, 80%, and 100%) for 30 s was used for dehydration of the glass slides. Glass slides were then dried in air for 24 h and examined by SEM (8).

3. Results

3.1. Estimation of antibiofilm treatment

Biofilm formation and antibiofilm activity were assessed using crystal violet staining assay. In comparison with the biofilm controls, it was observed that red LED + MB combination reduced biofilm formation by all of the *Candida* and *Staphylococcus* strains, as shown in Table 1. In contrast, RBF + UV treatment showed only minimal effect on the test strains, whereas the effect of green LED + RB was found to be variable among strains.

Biofilm inhibition index values were calculated for each well. Table 2 depicts the biofilm inhibition indexes. As shown in Table 2, the group in which red LED + MB was used showed a higher biofilm inhibition index in all of the microorganisms. The red LED + MB combination reduced biofilm formation by 45.4% both in *C. albicans* and *C. parapsilosis.* The groups to which RBF + UV was applied showed the lowest biofilm inhibition when compared with other groups. RBF + UV showed no effect on biofilm formation in *C. parapsilosis* and only minimally reduced the formation of biofilm in *S. aureus* and *S. epidermidis*, and by 24.5% in *C. albicans*. On the other hand, the biofilm inhibition effect of the green LED + RB combination was found to be variable among strains. An inhibitory effect of green LED + RB was observed for *S. aureus* and *C. albicans*, while no effect was found in other groups. Of the light sources and photosensitizer combinations, the red LED + MB combination showed the most promising effect on all microorganisms.

In the negative control groups in which amphotericin B or teicoplanin was used, unexpected results were observed, showing biofilm formation to some extent (Table 1). This unexpected result demonstrated that although amphotericin B and teicoplanin were used in order to observe the inhibitory effect of these drugs on biofilm formation, biofilms still developed to some extent in both the *Candida* and *Staphylococcus* strains, indicating that they could not completely prevent biofilm growth.

3.2. Planktonic cell assay results

The antimicrobial activity of APDT on planktonic cells was evaluated by counting colony numbers and estimating log reductions. As shown in Figure 1, red LED + MB and RBF + UV combinations markedly reduced the number of surviving cells in both *Candida* and *Staphylococcus* strains.

Table 1. Mean optical density values obtained by crystal violet assay after APDT.

	Mean OD values of biofilm formed wells			
	C. albicans	*C. parapsilosis*	*S. aureus*	*S. epidermidis*
Biofilm controls	0.110	0.110	0.160	0.100
Negative controls	0.140	0.120	0.100	0.080
Red LED + MB	0.060	0.060	0.040	0.040
Green LED + RB	0.085	0.158	0.238	0.037
RBF + UV	0.083	0.110	0.147	0.091

Light-emitting diode (LED), methylene blue (MB), rose bengal (RB),riboflavin (RBF).

Table 2. Biofilm inhibition index values after APDT estimated for *Candida* and *Staphylococcus* strains.

	Biofilm inhibition index			
	C. albicans	*C. parapsilosis*	*S. aureus*	*S. epidermidis*
Negative controls	–27.2	–9	37.5	27.2
Red LED + MB	45.4	45.4	75	60
Green LED + RB	22.7	–43.6	–48.7	63
RBF + UV	24.5	0	8.1	9

Light-emitting diode (LED), methylene blue (MB), rose bengal (RB),riboflavin (RBF).

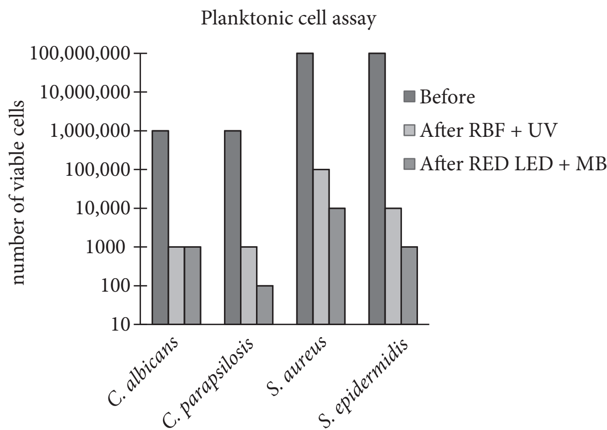

Figure 1. Colony numbers of *Candida* and *Staphylococcus* strains after APDT. Riboflavin (RBF), light-emitting diode (LED), methylene blue (MB).

In all planktonic treatment groups with red LED + MB and UV + RBF, there were more than 3 log10 reductions in the number of bacteria and fungi. Treatment with red LED + MB was the most prominent, resulting in a 4 log10 or greater kill in *Staphylococcus* strains.

3.3. Analysis of biofilm structure under scanning electron microscope

In the second part of the experiment, SEM analysis was used to observe the impact of APDT on 72-h bacterial and fungal biofilms. After biofilms were grown on glass slides in 6-well culture plates as described above, the APDT procedure was carried out. After treatment, slides were removed and evaluated by SEM. The biofilms treated with or without APDT are shown in Figure 2. SEM images revealed a change in biofilm formation after treatment with APDT. As shown in Figure 2, it was clearly observed that the number of adherent cells on the surface of glass slides was markedly reduced after treatment.

4. Discussion

This study focused on the investigation of the efficacy of APDT on biofilms formed by *Staphylococcus* and *Candida* species. Three different photosensitizers and two different light sources with various combinations between them were evaluated. It was found that the most efficacious combination was red LED + MB against both the *Staphylococcus* and the *Candida* strains used in the experiments. A marked inhibition (45.4%) was detected for both *C. albicans* and *C. parapsilosis* biofilms. Red LED + MB treatment was also very effective on *S. aureus* and *S. epidermidis* biofilms (75% and 60%, respectively). The efficacy of green LED + RB was found to be variable. It revealed 22.7% inhibition on *C. albicans* biofilm, but had no effect on *C. parapsilosis* biofilm. Green LED + RB did not show any inhibitory effect on *S. aureus* biofilm; however, it inhibited the biofilm formed by *S. epidermidis* (63%). RBF + UV treatment resulted in 24.5% inhibition of *C. albicans* biofilm, while no effect was detected for *C. parapsilosis* and bacterial biofilms.

The increase in the survival and resistance of microorganisms organized in biofilms demonstrates that alternative, safer, and effective antimicrobial strategies are urgently needed (9). Bacterial and fungal biofilm-related infections are difficult to eradicate since the effective penetration of antimicrobials through the biofilm is limited. APDT provides an opportunity to act locally, causing selective damage to target cells (2). Studies on the APDT approach to combat microbial biofilms have proven that it can be effective against biofilm-producing bacteria and fungi (especially *Staphylococcus* spp. and *Candida* spp.) (10). It was reported that APDT mediated by MB promoted an average reduction of 2.81 log10 CFU in *S. mutans* biofilms, as well as an average reduction of 3.29 log10 CFU in *S. aureus* biofilms (11).

Several studies evaluated the antibacterial effect of APDT; however, the number of studies about the antibiofilm efficacy of APDT is comparatively low. Published studies were mainly in the dentistry area, addressing the effect of APDT on oral cavity bacteria. Only three photoantimicrobial agents (MB, toluidine blue O, and indocyanine green) have so far received clinical approval in dentistry as an adjuvant approach (9).

Catheter-related infections provide promising opportunities for the therapeutic approach of APDT since the association of biofilms and CRBSIs is clearly defined (12). The most common etiological agents of CRBSIs are *Staphylococcus* spp. and *Candida* spp. Current guidelines generally recommend the removal of infected catheters due to *S. aureus* and *Candida* species because of their higher mortality rates. On the other hand, it was reported that, in some cases, beside antibiotic lock therapy, APDT can provide a salvage for infected catheters (3).

Several studies demonstrated the effectiveness of APDT on planktonic cells. Perez-Laguna et al. (13) demonstrated that the photosensitizing agents RB and MB effectively inactivated *S. aureus* by a 6 log10 reduction in bacterial growth. The combination of RB and MB with the antibiotics mupirocin or linezolid showed a synergistic bactericidal effect on *S. aureus* in vitro. Vecchio et al. (14) showed the in vitro effect of MB-APDT against both *S. aureus* and MRSA using white light lamps and red LED lamps. In another study, De Oliveira et al. (15) evaluated the in vitro bactericidal effect of APDT on *S. aureus* (ATCC 25923) using different concentrations (100, 50, 25, 12.5, and 6.25 μg/mL) of the phenothiazine compound combined with LED light (λ 632 ± 2 nm) using varied energy densities (12, 9.6, 7.2, 4.8, and 2.4 J/cm^2). They

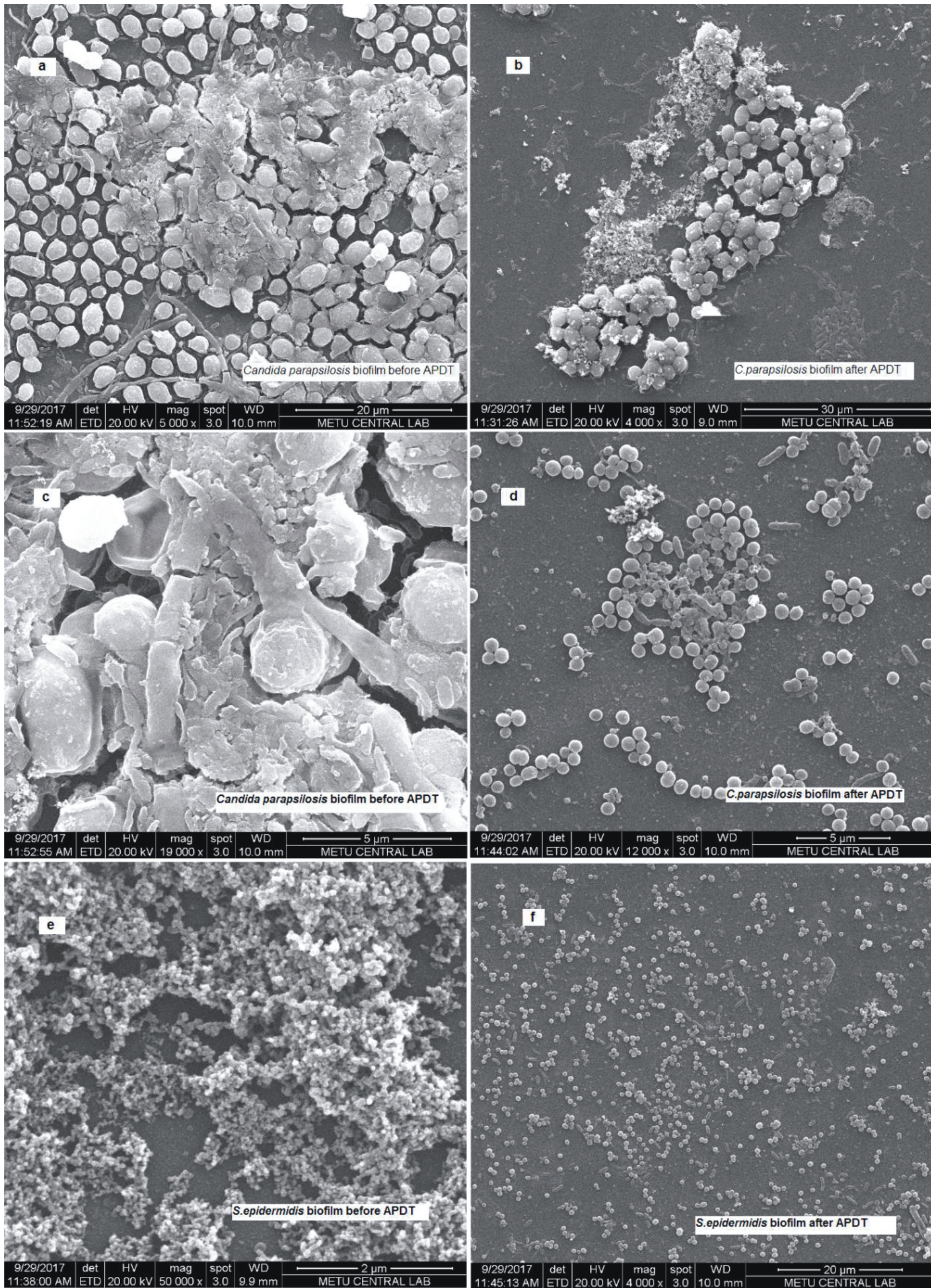

Figure 2. Scanning electron microscope images: a, c) *Candida parapsilosis* biofilm before APDT; b, d) *C. parapsilosis* biofilm after APDT; e) *S. epidermidis* biofilm before APDT; f) *S. epidermidis* biofilm after APDT.

showed that a single application of APDT, using energy density of 12 J/cm^2 associated either with 12.5 or 25 µg/mL of phenothiazine, resulted in higher in vitro inhibition of *S. aureus*.

The antimicrobial effect of a variety of photosensitizers on different pathogenic microorganisms including *Candida albicans* has been demonstrated by different authors. Some studies revealed high rates of reduction in the metabolic activity of biofilms formed from clinical isolates of *C. albicans*, *C. tropicalis*, and *C. glabrata* after exposure to APDT. Costa et al. (16) used RB and blue light LED for the photodynamic inactivation of planktonic cultures and biofilms of *C. albicans* and observed a reduction. They suggested that the combination of LED and RB exerted a photodynamic effect on *C. albicans* biofilm. Rossetti et al. (17) studied the effects of APDT using toluidine blue as a photosensitizing agent on the production of ROS, cell damage, and the ability of *C. albicans* to form biofilms.

APDT seems to be a promising alternative approach to conventional antibiotic therapy in response to the problem of antimicrobial resistance, especially in biofilm-based localized infections. The main advantages of APDT are the killing of microorganisms equally regardless of their intrinsic or acquired antibiotic resistance, no development of microbial resistance, and minimal damage to the host tissues (10). APDT has been widely used in various clinical conditions, such as cancers/tumors (skin, brain, lungs, pleura, gastrointestinal system, genitourinary system, head and neck), ophthalmic diseases, and cardiovascular diseases (18). APDT may help to prevent biofilm formation in intravenous catheters and may improve antibiofilm strategies. Certainly, the main goal is the minimization of catheter-related nosocomial infections.

Different LED light sources for APDT have been used in various studies. Even though there is no standardization of the wavelengths of LED light sources against various microorganisms, red LED with a wavelength of 620–660 nm yielded better results (6). In this study, three different LED light sources and photosensitizers have been chosen according to the previous studies and red LED (660 nm) and MB together were found to be more effective against both *S. aureus* (75%) and *Candida* spp. (45.4%).

In conclusion, the results of this study suggested that APDT had some ability to reduce the biofilm formation. APDT may have a role in the prevention or treatment of catheter-related bloodstream infections in the future. Further clinical studies are required to establish a safe and effective light dose for different body sites. The growing number of in vivo studies verifying future applications of various photosensitizers is encouraging and the key is to be brave enough to use them in clinical diseases.

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