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Antimicrobial Photodynamic Therapy on Drug-resistant *Pseudomonas aeruginosa*-induced Infection. An *In Vivo* Study†

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ABSTRACT

***Pseudomonas aeruginosa* is considered one of the most important pathogens that represent life-threatening risk in nosocomial environments, mainly in patients with severe burns. Antimicrobial photodynamic therapy (aPDT) has been effective to kill bacteria. The purpose of this study was to develop a burn wound and bloodstream infection model and verify aPDT effects on it. *In vitro*, we tested two wavelengths (blue and red LEDs) on a clinical isolate of *P. aeruginosa* strain with resistance to multiple antibiotics using HB:La^{+3} as photosensitizer. Verapamil® associated to aPDT was also studied. *In vivo*, *P. aeruginosa*-infected burned mice were submitted to aPDT. Bacterial counting was performed on local infection and bloodstream. Survival time of animals was also monitored. In this study, aPDT was effective to reduce *P. aeruginosa in vitro*. In addition, Verapamil® assay showed that HB:La^{+3} is not recognized by ATP-binding cassete (ABC) efflux pump mechanism. In the *in vivo* study, aPDT was able to reduce bacterial load in burn wounds, delay bacteremia and keep the bacterial levels in blood 2–3 logs lower compared with an untreated group. Mice survival was increased on 24 h. Thus, this result suggests that aPDT may also be a novel prophylactic treatment in the care of burned patients.**

INTRODUCTION

Infections caused by opportunistic pathogens are the main cause of morbidity and mortality in immunocompromised patients (1). Patients with severe burns present an immunosuppression condition and consequently higher susceptibility to infections due to the destruction of the cutaneous barrier that acts as protection against external agents and owing to the fact that coagulated and denatured proteins on the wound site provide an optimal environment to microbial growth (2,3). Accidents by burns deserve special attention as they are one of the most common forms of trauma. Septicemia caused by burns continues to be a great concern causing over 50% of burn-related deaths in hospital environments (4).

A burn wound infection can evolve easily to high levels of bacteremia and consequently sepsis, leading these patients to death (5). Bloodstream infections are a major cause of morbidity and mortality in thermally injured patients. Studies show that microorganisms isolated from burned patients in specialized centers present an increasing resistance to antibiotics, therefore this type of infection becomes very dangerous and difficult to treat. In this setting, new alternatives of treatment are required.

Antimicrobial photodynamic therapy (aPDT) involves the killing of organisms by light in the presence of a nontoxic photoactivable dye or photosensitizer (PS). Excitation of the PS by absorption of light of appropriate wavelength converts the PS to its photoactive triplet state, which in the presence of oxygen will then generate reactive oxygen species, such as singlet oxygen and superoxide, resulting in cell death (6). Antimicrobial PDT has been suggested as an alternative approach for treating local infections as it has been shown that a wide range of microorganisms, including resistant bacteria, viruses and yeasts, can be killed by aPDT (7,8).

Pseudomonas aeruginosa is a common nosocomial pathogen that causes infections with a high mortality rate mainly due to the high intrinsic resistance of microorganism to many antimicrobials and the development of increased multidrug resistance in healthcare facilities (9). Antimicrobial PDT is a new approach for the treatment of drug-resistant bacterial infections and the eradication *in vitro* and *in vivo* of *P. aeruginosa* using lethal photosensitization has been reported in the literature (10–14). Indeed, Hamblin *et al.* developed a mouse model to test the efficacy of aPDT against infected wounds induced by bioluminescent *P. aeruginosa* (12); however, so far, the effect of aPDT on MDR *P. aeruginosa*-infected burns *in vivo* has not been widely investigated.

Within this context, this study was divided into two phases: initially, we performed an *in vitro* assay and tested the viability of the use of aPDT in a clinical isolate of *P. aeruginosa* strain using hypocrellin B: lanthanum (HB:La^{+3}), which has shown to be an successful photosensitizer (15). After that, we developed a burn wound infection model in mice and investigated whether aPDT could reduce *P. aeruginosa* in the

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infected site. Finally, we examined if aPDT applied in a single or double session could prevent bloodstream infection and increase survival in these animals.

MATERIALS AND METHODS

Microbial strains. Pseudomonas aeruginosa strain used in this study was isolated from a patient with septicemia following procedures defined by CLSI (Clinical Laboratory Standards Institute). Antimicrobial susceptibility test showed resistance to 12 types of antimicrobials, including reference drugs as cefotaxime, cefepime, meropenem and ciprofloxacin (Table 1). Cells were cultured in tryptic soy broth (TSB) at 37°C, and used for experiments in mid log growth phase to an optical density at λ = 600 nm of 0.6–0.8, which corresponds to 10^8 colony forming unit (CFU mL^{-1}; 16). This bacterial suspension was centrifuged, washed with phosphate buffered saline (PBS), and then resuspended in PBS at a density of 3×10^8 CFU mL^{-1}.

Photosensitizer and light source. Hypocrellin B with lanthanide ions (HB:La^{+3}) was synthesized and characterized at Applied Biomedical Optics Laboratory (UNIFESP/SP, Brazil; Fig. 1). Stock solutions of HB:La^{+3} at 1 mM were dissolved in PBS to a final concentration of 10 μM. Light-emitting diode (LED; Eccofibras/São Carlos, Brazil) emitting at λ = 460 ± 20 nm and a LED (MMOptics/ São Carlos, Brazil) emitting at λ = 645 ± 10 nm, both with 225 mW, (I = 200 mW cm^{-2}) were used.

Photodynamic inactivation in vitro. HB:La^{+3} (10 μM) was used with a blue and red LED in separated groups. Preirradiation time was 30 s and illuminations were carried out in 96-well plate for 1 min (12 J cm^{-2}), 2 min (24 J cm^{-2}), 4 min (48 J cm^{-2}), 6 min (72 J cm^{-2}) and 8 min (96 J cm^{-2}). The experiments were performed in triplicate.

HB:La^{+3} associated to efflux pump inhibitor on P. aeruginosa. *Pseudomonas aeruginosa* cells were treated with verapamil (Vp), a known calcium channel blocker and pump inhibitor (17), (Sigma–Aldrich, MO) to verify if HB:La^{+3} could be a substrate of efflux pumps. Final concentration of 10 μg L^{-1} Vp was added to bacterial culture, which was incubated for 30 min at 37°C. The cells were harvested and washed in deionized water. Thereafter, they were suspended in 10 μM of HB:La^{+3} for 5 min and illuminated with red LED at fluences of 24, 48, 72 and 96 J cm^{-2}. The experiments were performed in triplicate.

We used methylene blue (MB-150 μM) as a control since it has been recognized as a substrate for efflux pumps (18). Preirradiation time was 5 min and a diode laser at λ = 660 nm and intensity of 133 mW cm^{-2} was used to irradiate the cells. The cells were pretreated with Vp as described earlier.

Mouse model of full-thickness thermal burns. Adult female BALB/c mice (age 3–4 months; body mass of about 25 g) were used in the study. All the animals were housed one per cage, maintained on a 12 h light and 12 h dark cycle, and had access to food and water *ad libitum*. The procedures were approved by Institutional Ethic Committee on Research Animal Care. The mice were anesthetized by intraperitoneal injection of ketamine (90 mg kg^{-1}) and xylasine (10 mg kg^{-1}) cocktail and received buthorfanol tartrate (2 mg kg^{-1}) subcutaneously for pain relief. The dorsal surface was shaved and burns were created by applying a preheated (95°C) steel device against dorsal surface of the mice for 10 s (nonlethal, full thickness and third degree burns). The device area was 6–6.5 cm^2, corresponding to a burn of 14.5–15.7% of the total body surface area according to Meeh's formula (19).

Establishment of infection. The infecting bacterium inoculum containing 3×10^9 CFU mL^{-1} was prepared from stock cultures. Samples were brought to room temperature, cultured in tryptic soy agar (TSA) and growth at 37°C overnight. Infection was induced immediately after burn creation by a subcutaneously inoculation of 100 μL of *P. aeruginosa* in sterile PBS (19).

HB:La^{+3} lethality test. To verify if HB:La^{+3} could be lethal to animals in tested conditions, 20 mice were submitted to burning and HB:La^{+3} (100 μL to 10 μM) was inoculated under the burn and illuminated directly with blue LED and red LED, both with 24 J cm^{-2}. All the animals were observed for 7 days.

Antimicrobial PDT in burn wounds and bacterial counting in local tissue. HB:La^{+3} (100 μL) was added 30 min after infection. After 5 min to allow HB:La^{+3} to bind and penetrate into bacteria, the wounds were illuminated separately with blue LED or red LED (24 J cm^{-2}).

Mice were killed by cervical dislocation immediately after treatment to measure the quantity of bacteria in local burn tissues and verify local proliferation. Burn wound tissues were cut and homogenized in 1 mL of PBS. Number of *P. aeruginosa* in 1 g of tissue (wet weight) was determined by serial dilution plate count in triplicate on TSA (triptic soy agar). Tissues surrounding the entire burn area were excised using a sterile surgical scissor. The depth of the skin biopsy extended all the way to the panniculus carnosus muscle on the back so all epidermal and dermal components were removed. Immediately following excision, the tissues were weighted and grinded with 1 mL of sterile PBS, the aliquots were serially diluted in PBS to give dilutions of 10^{-1} to 10^{-5} times the original concentrations and streaked horizontally on TSA plates as described by Jett *et al*. (20). Plates were incubated at 37°C overnight.

Antimicrobial PDT in burn wounds and bacterial load in bloodstream. Antimicrobial PDT was performed as described elsehwere in a single (30 min after infection) and double session (15 and 30 min after infection) to verify if aPDT in burn wounds can avoid or delay bacteremia. In all experiments, the light source was placed vertically in contact with the animal skin, which was protected with sterile plastic

Table 1. Antimicrobials tested on clinical isolate of *Pseudomonas aeruginosa*.

Antimicrobial	S	R	I
Aztreonam			X
Cefepime		X	
Cefotaxime		X	
Ceftriaxone		X	
Ceftazidime		X	
Ciprofloxacin		X	
Chloramphenicol		X	
Gentamicin		X	
Imipenem		X	
Meropenem		X	
Piperacillin/tazobactam		X	
Polymyxin B	X		
Sulfamethoxazole/trimethoprim		X	
Ticarcillin/clavulanic acid		X	

S, susceptible; R, resistent; I, intermediate.

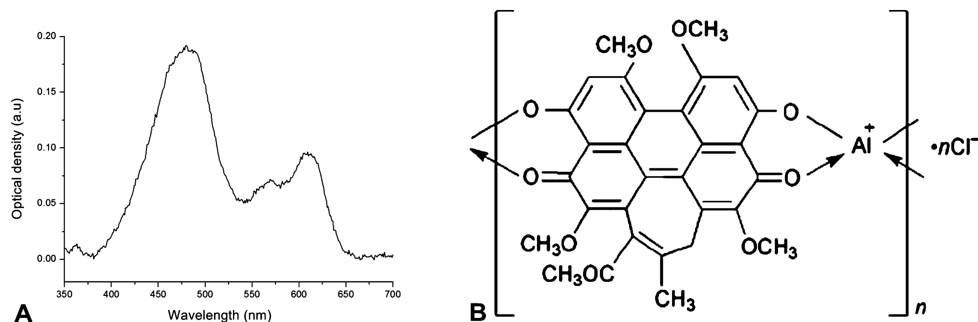

Figure 1. (A) Absorbance spectrum of hypocrellin B: lanthanum (HB:La^{+3}); (B) Molecular structure of HB:La^{+3}.

film. To measure the quantity of bacteria in bloodstream, the mice were bled by the retro-orbital plexus 7, 10, 15, 18 and 22 h postinfection in all groups. Numbers of CFU mL^{-1} in blood were determined by serial dilution plate count in triplicate on TSA. A 200 μL of blood were collected and placed into 1.8 mL of TSB with sodium sulfonate. After serial dilution from 10^{-1} to 10^{-4} times the original concentration, 10 μL aliquots of each dilution were streaked onto an agar plate in triplicate and incubated to 37°C for 12 h to allow colony growth.

Mouse survival follow-up and statistical analysis. The survival of the animals of the previous experiments was monitored after aPDT. A log-rank test verified the significance of difference. For the other experiments, bacterial colonies were counted and converted into CFU for statistical analysis. Values are given as means, and error bars are standard deviations. Statistical comparison between means was carried out using one-way analysis of variance (ANOVA). Mean comparisons were performed with the Tukey's test. Significance was established when $P < 0.05$.

RESULTS

Susceptibility of *P. aeruginosa* to aPDT

Results for lethal photosensitization of a clinically isolated *P. aerugionosa* using $HB:La^{+3}$ are shown in Fig. 2. After 2 min, corresponding to 24 J cm^{-2} LED fluence, *ca* 5 logs of killing were achieved. This statistically significant decrease in bacterial load ($P < 0.05$) persisted until 8 min of irradiation (96 J cm^{-2}). No statistically significant differences were observed on decline of bacterial load between blue and red LED. Control group (no irradiation and photosensitizer), LEDs groups (only blue or red irradiation by 8 min) and $HB:La^{+3}$ group (only 10 μM photosensitizer for 8 min) did not show any bactericidal effect.

Efflux pump inhibitor on *P. aeruginosa*

In Fig. 3, we show the effects of aPDT on bacterial reduction using $HB:La^{+3}$ and MB associated to Vp. After 24 J cm^{-2} light dose had been delivered (2 min), there was a statistically significant reduction of about 5 logs for $HB:La^{+3}$ group, as well as $HB:La^{+3}$ combined to Vp ($P < 0.05$). This reduction was kept until 96 J cm^{-2} (8 min) for both groups. No statistically significant differences were observed between $HB:La^{+3}$ group and $HB:La^{+3}$ associated to Vp. On the other hand, using MB and Vp, we observe a statistically significant increase in bacterial killing of about 1 log compared with MB alone ($P < 0.05$).

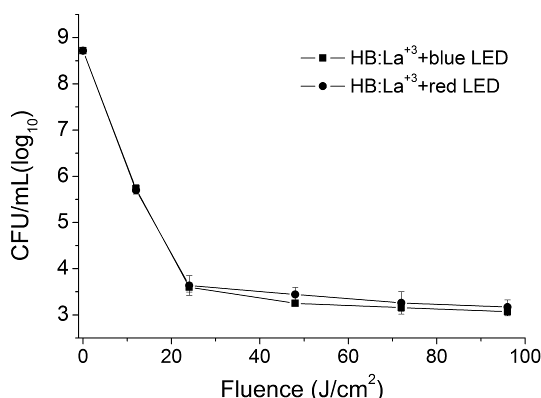

Figure 2. Effect *in vitro* of $HB:La^{+3}$ on the reduction of *P. aeruginosa* exposed to blue and red LED depending on the exposure time (LED intensity: 200 mW cm^{-2}). Bars represent SD.

Mouse model of third degree thermal burns and establishment of infection

In this *in vivo* study, we established the infection after full thickness, third degree burns confirmation by histology. We verified that mice submitted only to burns or bacterial inoculation survived the whole experimental period, whereas mice submitted to burns and contamination died within 18 h. We then tested $HB:La^{+3}$ lethality following aPDT and observed that burned not infected mice that received $HB:La^{+3}$ alone or associated to blue and red LED survived during the 7 day observation period.

Antimicrobial PDT in burns and bacterial load *in situ*

No bacterial decrease was observed in burned mice only irradiated or inoculated with $HB:La^{+3}$ as well as in burned mice without treatment (control group). However, aPDT using $HB:La^{+3}$ combined to blue or red LEDs showed about 2 logs of bacterial reduction (Fig. 4). In fact, the initial mean value of

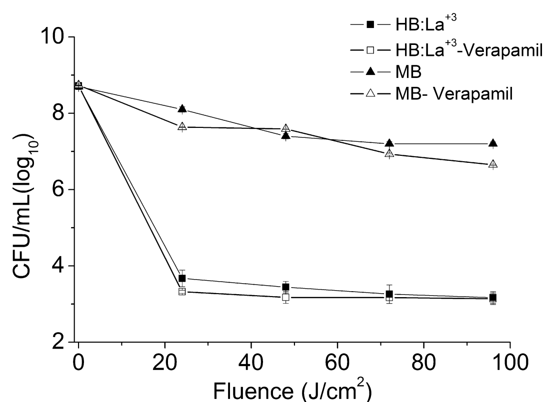

Figure 3. Effect *in vitro* of $HB:La^{+3}$ and MB associated to verapamil on the reduction of *P. aeruginosa* exposed to red LED (LED intensity: 200 mW cm^{-2}) and red laser (laser intensity 133 mW cm^{-2}), respectively, depending on the exposure time. Bars represent SD.

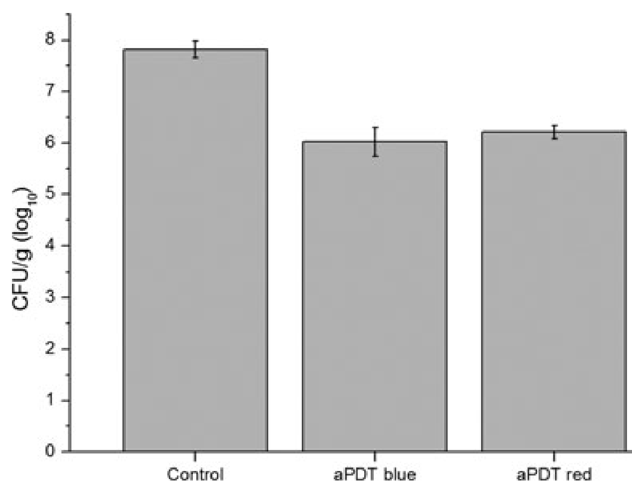

Figure 4. Comparison of viability of bacterial cells recovered *in situ* from untreated infected burns with those treated by blue or red aPDT. Each data point is a mean ± SD of five animals.

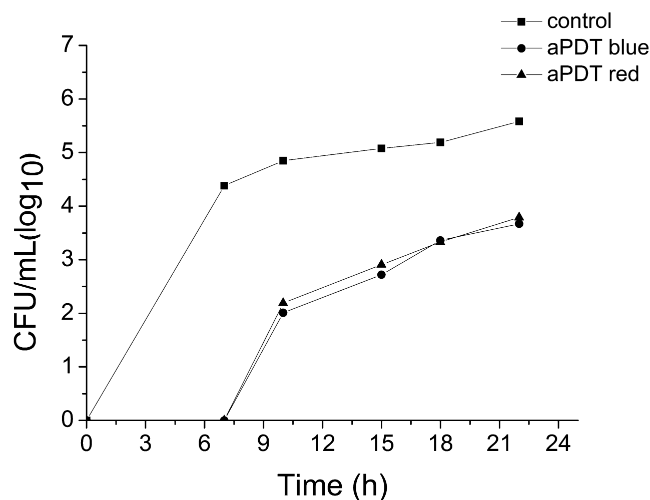

Figure 5. Comparison of viability of bacterial cells recovered from blood of untreated mice with those treated by blue or red aPDT at one session. Each data point is a mean ± SD of five animals.

infectious load collected from five animals was 7.8 logs. Immediately after aPDT, the mean infectious burden was significantly reduced to 6.0 and 6.2 logs for blue and red LED, respectively. Despite statistically significant differences between control and treated groups ($P < 0.05$), no significant differences were detected between blue and red LEDs. These animals were submitted only to one session of aPDT.

Antimicrobial PDT in burns and bacterial load in bloodstream

Figure 5 demonstrates the number of viable cells of *P. aeruginosa* in bloodstream after aPDT on infected burns. As can be observed, 7 h after bacterial inoculation, the control group presented an average of about 6 logs of bacterial load in the bloodstream, while aPDT blue and aPDT red groups did not present any bacterial growth at this time. Antimicrobial PDT blue and aPDT red groups showed bacteremia only 10 h after bacterial inoculation, and the bacteria levels were about 2 logs lower compared with control group at this time. A 15 h after bacterial inoculation, control group presented 7.1 logs of bacterial burden, whereas aPDT blue and aPDT red groups maintained about 4.8 logs. After 18 h, although bacterial levels in aPDT groups increased to 5.5 logs, they were kept 2 logs lower than the control group. This behavior was observed up to 22 h after the bacterial inoculation. There was not any statistically significant difference when control, HB:La^{+3} alone and LED alone groups were compared among them as well as between aPDT blue and aPDT red groups. Nonetheless, significant differences were detected when aPDT groups were compared with control, HB:La^{+3} and LEDs groups ($P < 0.05$).

We then investigated if the observation above described one session of aPDT delayed bacteremia in mice and retained *ca* 2 logs bacterial load level lower than control group, could be more effective when two sessions of aPDT were applied. Disappointingly, two sessions of aPDT were not successful to avoid bacteremia and reverse bacterial load in bloodstream (Fig. 6). However, bacterial recovering following two sessions of aPDT was significantly lower than control group (about 3 logs, $P < 0.05$; compare Figs. 5 and 6).

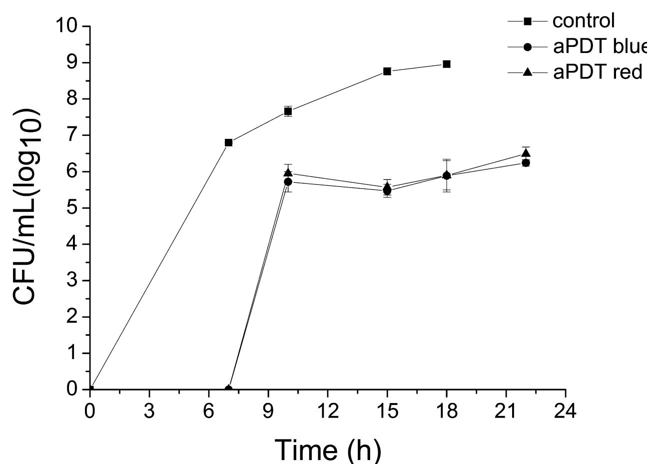

Figure 6. Comparison of viability of bacterial cells recovered from blood of untreated mice with those treated by blue or red aPDT at two sessions. Each data point is a mean ± SD of five animals.

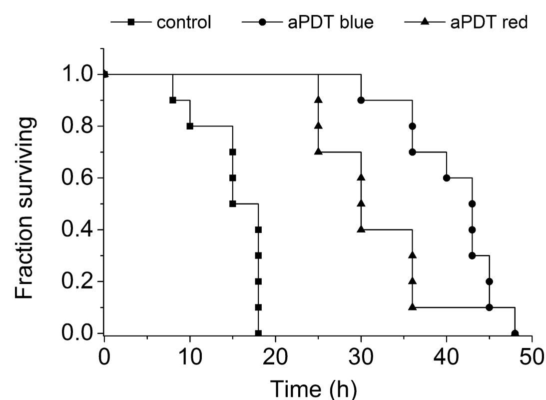

Figure 7. Survival plot for blue and red aPDT after one and two treatments, and for untreated mice.

Survival analysis

We show in Fig. 7 fraction surviving curves for untreated and aPDT-treated mice. It can be observed that all animals of the control group died within 18 h of bacterial inoculation. In contrast, aPDT groups survived until 48 h following infection. An interesting remark is that aPDT-blue treated mice survived longer than aPDT-red treated mice. In fact, only 10% of the animals from aPDT red group survived within 36 h after infection, but in aPDT blue group 70% of the animals survived within this same period. However, despite significant differences between control and aPDT groups ($P < 0.05$), no statistically significant differences were observed between aPDT blue and aPDT red groups ($P > 0.05$).

DISCUSSION

In this study, we used a local and bloodstream murine model of a clinical isolate of *P. aeruginosa* to induce infection in burned mice. Our results indicate that the model was suitable for studying aPDT and showed success in delaying bacteremia and increasing survival in mice.

In previous pilot studies, we investigated *in vitro* and *in vivo* effects of methylene blue (MB) and toluidine blue (TBO), respectively (data not shown) and both photosensitizers showed a limiting bacterial killing activity. We then investigated the effects of HB:La^{+3} on *P. aeruginosa* susceptibility *in vitro*. Hypocrellins have been shown to act as photosensitizer agents because they exhibit rapid preparation, easy purification, low aggregation tendency, high quantum yield for singlet oxygen generation and fast clearance *in vivo* (21). In addition, HB:La^{+3} enhances singlet oxygen quantum yield, it does not present photobleaching and it was able to reduce 100% *Candida albicans* following 30 s of exposure to red or blue LED (22).

Our data show that 10 μM of HB:La^{+3} combined to blue or red LED achieved about 5 logs of killing after 2 min of irradiation without complete eradication, different than Toffoli *et al.* (22). It is well known that Gram-negative bacteria are more resistant to aPDT. In particular, *P. aeruginosa* is one of the Gram-negative bacteria more difficult to kill by aPDT (23,24). In fact, a few reports show a high index of *P. aeruginosa* killing using phenothiazines *in vitro* (13,14,23), and in those cases, higher photosensitizer concentrations (13,23) and longer exposure times (about 30 min; 13,14) are necessary. *P. aeruginosa* used in our study was recovered and isolated from a hemodynamic catheter of a sepsis patient, and it was resistant to 12 groups of antibiotics. This wild strain also produces more mucus compared to ATCC 27853 strain (data not shown). This characteristic can prevent HB:La^{+3} diffusion through the cell and can protect *P. aeruginosa* against aPDT (25). In addition, literature supports that efflux mechanisms are the major components of microbial resistance and inhibitors of efflux pumps could enhance bactericidal effect by aPDT (18,26).

The resistance-nodulation-cell division (RND) pump family plays a key role in resistance of *P. aeruginosa*. However, ABC pumps can also be presented in this Gram-negative bacterium in a small number (27). For this reason, we decided to verify this pump activity using methylene blue, a substrate for ABC pumps and verapamil (28). As expected, a small but statistically significant increase in bacterial killing was observed (about 1 log). We evaluated whether HB:La^{+3} could also be a substrate for ABC pumps. Contrary to MB, our results clearly demonstrated that Vp associated to HB:La^{+3} did not enhance *P. aeruginosa* killing *in vitro*. This finding suggests that HB:La^{+3} is not recognized by ABC efflux pumps.

The next set of experiments aimed to develop an animal model of infection to investigate the effects of aPDT using HB:La^{+3} combined to blue or red LEDs on *P. aeruginosa*-infected burns. It is worth mentioning that, in a pilot study, we inoculated a clinical isolate of *P. aeruginosa* onto the burns, following methodology reported by Huang *et al.* (13). However, in this case, it was not possible to establish septicemia. Hence, we decided to inject bacterial cells subcutaneously (19). Once the mouse model of infection was established, we tested HB:La^{+3} lethality *in vivo*. Our findings did not show any lethal effect on mice by the use of HB:La^{+3} since only infected mice died within 18 h.

We also observed that mice aPDT-treated showed a statistically significantly lower bacterial burden on burn wounds and in bloodstream. *In situ*, bacterial burden was reduced by 2 logs following aPDT, using blue or red LED. Consequently, aPDT delayed bacteremia and kept bacterial load in the bloodstream lower than untreated group until the end of the experiment. Double session of aPDT diminished even more bacteria in the bloodstream, but survival of aPDT-treated mice in single or double session was similar.

In common with our results, mice decease was not avoided when excisional wounds infected with *P. aeruginosa* were treated with BF6-mediated aPDT (29). On the other hand, in a less-aggressive *P. aeruginosa* wound infection, 90% of mice survived following aPDT treatment using a poly-L-lysine-c_{e6} conjugate (12). In another reported study, similar bacterial reduction *in situ* (more than 2 logs) was observed when burn wounds infected with *P. aeruginosa* were treated with MB-PDT (13).

Pseudomonas aeruginosa is a highly virulent and invasive bacterium that rapidly reaches the bloodstream causing sepsis, but previous studies have demonstrated that aPDT can inactivate *P. aeruginosa* virulence factors (10,30,31). We suggest that inactivation of virulence factors responsible for bacterial invasion and tissue damage is contributing to a better response of mice face to infection and, for this reason, it was possible to delay bacteremia, maintaining lower bacterial levels in bloodstream and increasing mice survival. Nonetheless, despite bacterial load in bloodstream has been diminished even more (about 1 log) with two sessions of aPDT on burn wound, the interval for the second treatment was not enough to prevent recurrence and reduce bacterial load in the blood to a level acceptable to avoid decease.

Regarding the light source, blue and red LEDs showed the same behavior in bacterial viable cells recovered from mice blood. Interestingly, despite no statistically significant differences between aPDT red and aPDT blue groups ($P = 0.0502$), our results showed that aPDT-blue treated mice started to die 5 h later than aPDT-red treated mice. Furthermore, survival in aPDT red group was 60% less than in aPDT blue group 36 h after infection. Although we do not have data to support it, this increase in the survival of aPDT-blue treated mice may result from two effects: bacterial death or host defense. As absorption of HB:La^{+3} is more intense in blue region (see Fig. 1), a plausible hypothesis for our results is that more PS molecules are activated following blue LED irradiation with consequent sublethal damage to bacteria. Indeed, it has been reported that the oxidative damage promoted by sublethal photodynamic inactivation inhibits virulence determinants and reduces *in vivo* pathogenicity of *Candida albicans* (32). Further studies are necessary to confirm our proposition.

From the data presented in this study, it can be concluded that killing *in vitro* of *P. aeruginosa* using HB:La^{+3} associated to blue LED or red LED was similar, and ABC efflux pump inhibitor did not increase killing when associated to HB:La^{+3}. The burn model used in this study induced a third degree burn and it was possible to develop local and disseminated infection by subcutaneous inoculation of a clinical isolate of *P. aeruginosa* with resistance to multiple antibiotics. HB:La^{+3} under the parameters tested was not noxious to mice.

CONCLUSION

Put together, these findings suggest that aPDT could be an alternative approach for the treatment of *P. aeruginosa*-infected burns, as it was able to reduce *P. aeruginosa in situ*,

delay bacteremia, keep bacterial levels in bloodstream lower compared with untreated group and double the mice life expectancy. A prophylactic treatment that would delay bacteremia in such way that medical intervention was more efficient is a novel interesting approach for aPDT.

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