Prevention of bloodstream infections by photodynamic inactivation of multi-resistant *Pseudomonas aeruginosa* in burn wounds

M. C. E. Hashimoto[*a], R. A. Prates[a], D.J. Toffoli[a], L. C. Courrol[a,b], M. S. Ribeiro[a]
[a]Center for Lasers and Applications, Instituto de Pesquisas Energéticas e Nucleares, IPEN–CNEN, Avenida Prof. Lineu Prestes, 2242 – Cidade Universitária, 05508-000 Sao Paulo/SP, Brazil.
[b]Departamento de Ciências Exatas e da Terra, Unifesp, Campus Diadema, Diadema, SP, Brazil.

ABSTRACT

Bloodstream infections are potentially life-threatening diseases. They can cause serious secondary infections, and may result in endocarditis, severe sepsis or toxic-shock syndrome. *Pseudomonas aeruginosa* is an opportunistic pathogen and one of the most important etiological factors responsible for nosocomial infections, mainly in immuno-compromised hosts, characteristic of patients with severe burns. Its multiresistance to antibiotics produces many therapeutic problems, and for this reason, the development of an alternative method to antibiotic therapy is needed. Photodynamic inactivation (PDI) may be an effective and alternative therapeutic option to prevent bloodstream infections in patients with severe burns. In this study we report the use of PDI to prevent bloodstream infections in mice with third-degree burns. Burns were produced on the back of the animals and they were infected with 10^9 cfu/mL of multi-resistant (MR) *P. aeruginosa*. Fifteen animals were divided into 3 groups: control, PDT blue and PDT red. PDT was performed thirty minutes after bacterial inoculation using 10μM HB:La^{+3} and a light-emitting diode (LED) emitting at λ=460nm±20nm and a LED emitting at λ=645 nm±10nm for 120s. Blood of mice were colected at 7h, 10h, 15h, 18h and 22h pos-infection (p.i.) for bacterial counting. Control group presented $1x10^4$ cfu/mL in bloodstream at 7h p.i. increasing to $1x10^6$ at 22h, while mice PDT-treated did not present any bacteria at 7h; only at 22h p.i. they presented $1x10^4$cfu/mL. These results suggest that HB:La^{+3} associated to blue LED or red LED is effective to delay and diminish MR *P.aeruginosa* bloodstream invasion in third-degree-burned mice.

Keywords: *Pseudomonas aeruginosa,* hypocrellin B, photodynamic therapy, LED, infected burn wound, bacteremia, septicemia.

1. INTRODUCTION

Septicemia caused by burns continues to be a great concern causing over 50% of burn deaths in hospital environments. Bacterial infection of traumatic and burn wounds is a chalenge for clinicians due to optimal environment for bacterial growth in burn wounds that contributes to development of infection. This localized proliferation may lead to systemic sepsis, which is often associated with a high degree of morbidity and mortality[1]. The extent of the burn injury itself and to secondary immunosuppression resulting from the thermal injury contributes for occurence of high levels of bacteremia that can evolves quickly to sepsis, leading these patients to death[2].

The treatment of severe burns is a long processs, and burn centers use a lot of wide-spectrum antibiotics, wich results in the development of antibiotic-resistant strains. Most studies show that the microorganisms isolated in the burns departament present an increasing resistance to antibiotics[3].

P. aeruginosa is a ubiquitous environmental bacteria tha can be found in a wide variety of natural habitats. Moreover, this bacterium is capable of infecting plants, animals and insects as the result of its extremely broad host range and the use of arsenal of virulence factors to cause serious infections. In the last decades *P. aeruginosa* has emerged as a major human opportunistic pathogen and a significant source of life-threatening nosocomial infections[4].

In this setting, the development of novel animicrobial strategies is required. Photodynamic therapy (PDT) involves the killing of organisms by light in the presence of a non-toxic photoactivable dye or photosensitizer (PS). Excitation of the PS by absorption of light of appropriate wavelenght in the presence of oxygen converts the PS to its photoactive triplet state, which will then generate reactive oxygen species, such as singlet oxygen and superoxide, resulting in cell death[5].

PDT has been suggested as an alternative approach for treating local infections since it has been shown that a wide range of microrganisms including bacteria, viruses and yeasts can be killed by photodynamic effect[6,7]. The eradication of wound infecting bacteria, e.g. *P.aeruginosa* using lethal photosensitization has been reported in the literature[8,9,10].

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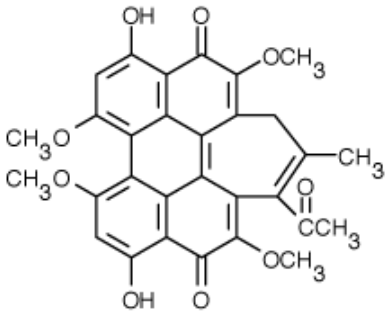
PDT has advantages over conventional antibiotic therapy. As the mechanism of killing is non-specific, with reactive oxygen species causing damage to many bacterial components, resistance is unlikely to develop from repeated use[11,12]. Secondly, both the PS and the light are applied locally to the target tissue; therefore reducing the risk of adverse systemic effects[13]. Treating localized infections by PDT could be an useful alternative to systemic medications, thus avoiding the development of microbial resistance to systemic drugs.

Hypocrellin B (HB) is a photosensitizer extracted from the fungus *Hypocrella bambusae,* a native pigment frequently found in Asian forests, mainly in countries like China and Sri Lanka. This compound has been target of study in last two decades due to properties such as high singlet oxygen generation quantum yield and absorption in the range of 460, 546 and 584 nm[14,15]. Properties like strong absorption bands, easy preparation, fast elimination from the body besides high singlet oxygen generation quantum yield are found in perylenequinonoid pigments, such as hypocrellins and hypericin. HB associated to lanthanide ions (HB:La^{+3}), as showed in previous study[16], was responsible for a remarkable redshift of 30 nm (from 584 to 614 nm) in the HB absorption band, as revealed by absorption spectroscopy. In addition, HB:La^{+3} in ethanol provided an enhancement of the singlet oxygen generation quantum yield of HB, from 0.47 to 0.62 (32%)[16]. So, hypocrellin B complexes with metal ions possess even more notable optical and photodynamic properties. It has been showed that lanthanide ions change the HB molecular structure and displacements in peaks are observed [16]. Figure 1 shows the chemical structure of Hypocrellin B and Hypocrellin B associated to lanthanide ions. Figure 2 shows the optical absorption spectra for HB and HB:La^{+3} solutions. It can be seen that HB:La^{+3} presents a very large absorption band in the visible, ranging from about 400 to 650 nm.

Recent study showed the potentiality of the HB:La^{+3} in eliminate *Candida albicans in vitro*. The bacterial suspension was irradiated with a λ=470±20 nm LED and a λ=660 nm laser, both with 330 mW/cm^2 irradiance. Ten-μM of HB:La^{+3} was sufficient to reduce 100% *C.albicans* colonies following only 30s of irradiation[17].

Other study showed that HB:La^{+3} associated to blue LED was effective in diminishing antibiotic resistant strain of *P. aeruginosa* in burn wounds. Mice PDT-treated showed 2 logs of bacterial reduction in burn tissues compared to control group. Moreover, mice that received only HB:La^{+3} or HB:La^{+3} associated to λ=460±20 nm LED, but without infection did not die within 7 days, so HB:La^{+3} did not show any lethal effect in this model[18].

In this study we have investigated whether PDT using HB:La^{+3} associated to λ=460±20 nm LED or λ=645±10 nm LED can avoid bacteremia and consequently sepsis through *P. aeruginosa* reduction in burn wounds.



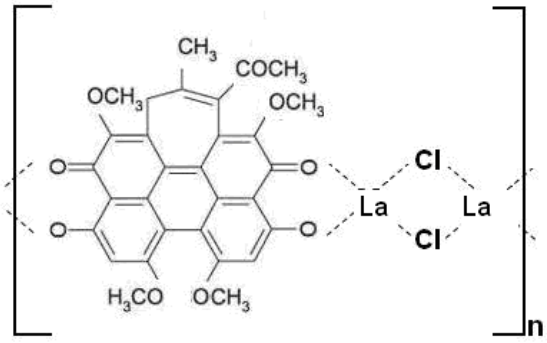


Fig.1: Hypocrellin B *($C_{30}H_{24}O_9$)* HB:La^{+3}

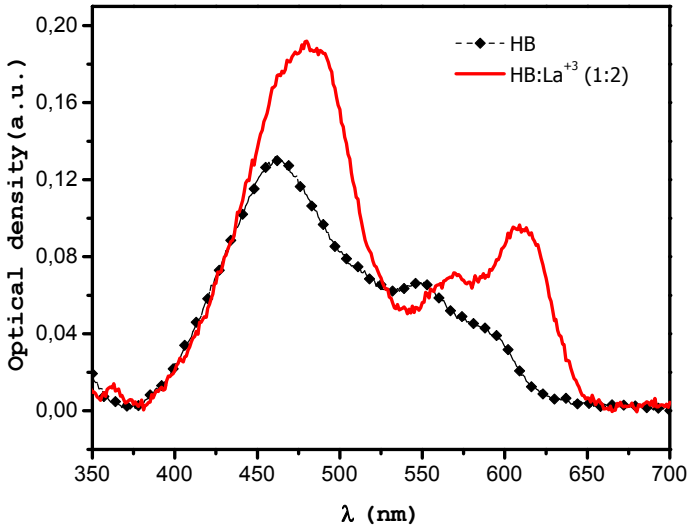


Fig.2: HB and HB:La^{+3} optical absorption spectra.

2. MATERIALS AND METHODS

Mice

Thirty 6-8 weeks female Balb/c (20-25 g) mice were used. All experimental procedure performed in this study was approved by the Ethic Committee on Research Animal of IPEN-CNEN (São Paulo/Brazil).

The animals were housed individually in ventilated cages in a central animal research facility. The facility maintains an environment of controlled temperature and relative humidity, with a 12-hour light-dark cicle. The mice were supplied with sterile bedding, standard chow, and water *ad libitum*.

Thermal injury model

Mice were anesthetized by intraperitoneal injection of ketamine (90mg/kg) and xylasine (10mg/kg), and their back hair was shaved using a shaving blade. Butorfanol (2mg/kg) was used as a postburn analgesic. Thermal injury was induced by pressing a pre-heated steel device (6cm^2 area) against the back of the animal for 10s. The steel device was pre-heated in boiling water to about 95°C and produced a third degree burn, confirmed by histological analysis, of 6-6.5cm^2 area that corresponds to 9-9.5% of body surface area calculated according to Meeh's formula[19]. All mice exposed to the burn injury survived when they were not infected.

Bacterial infection

The wild-type strain of *P. aeruginosa* was isolated from a haemodynamic catheter of a patient with septicemia (Emílio Ribas Hospital/ Sao Paulo-Brazil) and it is resistant to thirteen types of antibiotic.

P. aeruginosa was grown in triptic soy agar (TSA) for 24h at 37°C. The infecting bacterial inoculum was prepared in sterile sodium phosphate buffer (PBS), transmittance of 16% at 620 nm (1x10^9 ufc/mL). The number of infecting bacteria was verified by plating serial dilutions of the injected inocula onto TSA plates.

The inoculum of 100μL of the bacterial suspension was injected under the burn immediately after burning.

Photosensitizer

Hypocrelin B ($C_{30}H_{24}O_9$) with lanthanide ions (HB:La^{+3}) was obtained from Optical Spectroscopy Laboratory (CLA/IPEN-CNEN/SP, Brazil). Hypocrellin B (HB) was purchased from Shaanxi Tianze Bio-Technology CO., LTD. HB concentration for complexes in ethanol was 1mM. Stock solutions of 1mM were dissolved in PBS to a final concentration of 10μM.

Light source
The illuminations were carried out with a light-emitting diode (LED) (Eccofibras/Sao Carlos-Brazil) emitting at λ=460nm±20nm, 225mW, 200 mW/cm^2, and a LED (MMOptics/ São Carlos-Brazil) emitting at λ=645 nm±10nm, 225 mW, 200mW/cm^2.

***P. aeruginosa* photodynamic inactivation in burn wounds and bacterial counting in bloodstream**
Thirty animals were submmitted to thermal injury and bacterial infection as described above, and PDT was performed using 10μM of HB:La^{+3} with a pre-irradiation time (PIT) of 5 minutes and irradiation time of 120 s (28 J/cm^2). The groups were divided as follows:

- B+I+: mice with infected burns that received no treatment at all;
- B+I+HB+: mice with infected burns treated with HB:La^{+3} but kept in the dark;
- B+I+HB-B+: mice with infected burns that were illuminated with blue LED in the absence of HB:La^{+3};
- B+I+HB-R+: mice with infected burns that were illuminated with red LED in the absence of HB:La^{+3};
- B+I+HB+B+: mice with infected burns that received HB:La^{+3} and were irradiated with blue LED;
- B+I+HB+R+: mice with infected burns that received HB:La^{+3} and were irradiated with red LED.

HB:La^{+3} was injected under the burn (100μL) three hours after bacterial inoculation and burns were illuminated directly. In all experiments the light source was placed vertically in contact with the animal skin, which was protected with sterile plastic film.
In order to measure the quantity of bacteria in bloodstream, blood samples were collected 7, 10, 15, 18, and 22h pos-infection in all groups. Numbers of *P. aeruginosa*/mL of blood were determined by serial dilution plate-count in triplicate on triptic soy agar (TSA).
Two hundred-μL of blood were collected and placed into 1.8 mL of triptic soy broth (TSB) with sodium sulfonate (SPS). After serial dilution from 10^{-1} a 10^{-4} times the original concentration, ten-μL aliquots of each dilution were streaked onto an agar plate in triplicate and incubated to 37°C for 12h to allow colony growth[20]. All the experiments were performed in triplicate.

Statistics
The results obtained were expressed as means ± standard deviation and were analysed using one-way analysis of variance (ANOVA). Mean comparisons were carried out using Tukey's test and differences were considered significant at $p<0.05$.

3. RESULTS AND DISCUSSION

Bacteremia after burn wound infection
Bacteremia was evident in the non treated infected burn group (B+I+), as well as PS alone (B+I+HB+) and light alone groups (B+I+HB-B+ and B+I+HB-R+) after 7 hours of bacterial inoculation. These groups presented 4 x10^4 cfu/mL in blood samples while mice PDT-treated (B+I+HB+B+ e B+I+HB+R+) did not present any bacteria at this time (Table 1).
After 10 hours of bacterial inoculation, control group presented 4 x10^4 cfu/mL while PDT-treated groups presented 1x 10^2 cfu/mL. After 15 hours of bacterial inoculation, control group presented 1x10^5 cfu/mL while PDT-treated group presented 1x 10^2 cfu/mL. After 18 hours of bacterial inoculation, control group presented 1x10^5 cfu/mL while PDT-treated group presented 1x 10^3 cfu/mL. After 22 hours of bacterial inoculation, control group presented 1x10^5 cfu/mL while PDT-treated group presented 1x 10^3 cfu/mL.
There was no significant difference ($p<0.05$) in the numbers of cfu/mL when untreated, PS alone and light alone were compared, but there was significant difference when group PDT-treated, was compared with groups untreated, PS alone and light alone. There was no difference when groups PDT blue and PDT red were compared between them ($p<0.05$).

	7h cfu/mL (log_{10})	10h cfu /mL (log_{10})	15h cfu/mL (log_{10})	18h cfu/mL (log_{10})	22h cfu/mL (log_{10})
B+I+	4.38±0.05	4.84±0.01	5.08±0.02	5.18±0.01	5.58±0.02
B+I+HB+	4.37±0.12	4.84±0.01	5.06±0.03	5.18±0.01	5.60±0.01
B+I+HB-B+	4.36±0.03	4.85±0.04	5.07±0.08	5.16±0.09	5.59±0.02
B+I+HB-R+	4.34±0.06	4.84±0.04	5.09±0.04	5.19±0.06	5.63±0.02
B+I+HB+B+	0	2.01±0.35	2.71±0.23	3.35±0.28	3.66±0.40
B+I+HB+R+	0	2.19±0.46	2.91±0.32	3.33±0.07	3.79±0.75

Table 1: Mean values ± SD of cfu/mL of P. aeruginosa in bloodstream at 7, 10, 15, 18 and 22h pos-infection. B+I+: mice with infected burns that received no treatment at all; B+I+HB+: mice with infected burns that received HB:La^{+3} but were kept in the dark; B+I+HB-B+: mice with infected burns that were illuminated with blue LED in the absence of HB:La^{+3} ; B+I+HB-R+: mice with infected burns that were illuminated with red LED in the absence of HB:La^{+3} ; B+I+HB+B+: mice with infected burns that received HB:La^{+3} and were irradiated with blue LED; B+I+HB+R+: mice with infected burns that received HB:La^{+3} and were irradiated with red LED.

Despite of PDT has been suggested as an alternative approach for treating local infections[8,9,10], there are few accounts about the role and systemic benefits away. Hamblin *et al*., 2003, used an excisional wound and $5x10^6$ cfu/mL of *P. aeruginosa* bioluminescent to induce infection. *P. aeruginosa* was invasive in that mouse model, and all 3 groups of control mice died within 5 days; in contrast, 90% of PDT-treated mice survived. In our model, we used a burn wound and it was only possible to induce death with $1x10^9$ cfu/mL of MR *P. aeruginosa* innoculated under the burn. Differences in results can be attributed to wound model and differences in bacterial strains; a burn wound does not expose subjunctive tissue like an excisional wound and a multi-resistant strain can have different behavior from an ATCC strain.

In our study, mice PDT-treated presented lower bacterial levels in bloodstream compared to untreated group. Blood samples analysis showed that PDT performed in infected burns can delay bacteremia and keep bacterial levels 2 logs lower compared to control group. In addition, mice PDT-treated survived 24h more than untreated group. Clinically, it could be important, since that delay in bacteremia occurence, as well as lower bacterial levels, can give enough conditions for that other therapies can be established, in order to avoid septicemia and consequently death.

PDT can be an important alternative of treatment in antibiotics resistance era. However, further studies, mainly *in vivo*, must be done for that photodynamic therapy could be introduced in clinical trials.

4. CONCLUSIONS

Our study suggests that HB:La^{+3} associated to blue LED or red LED can delay and diminish MR *P.aeruginosa* bloodstream invasion in third-degree-burned mice. Moreover, mice PDT-treated survived 24h more than untreated mice.

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REFERENCES

[1] Edward-Jones, V. and Greenwood, J.E. “What’s new in burn microbiology?” James Laing memorial prize essay 2000. Burns 29, 15-24 (2003).

[2] Sheng, Z. “Prevention of multiple organ dysfunction syndrome in patients with extensive deep burns.” Chin J Traumatol 5(4), 195-199 (2002).

[3] Vostrugina, K., Gudaviciene, D. and Vitkauskiene, A. “Bacteremia in patients with severe burn trauma.” Med (Kaunas) 42(7), 576-579 (2006).

[4] Cunha, B.A. “Nosocomial pneumonia. Diagnostic and therapeutic considerations.”Med Clin North Am 85, 79-114 (2001).

[5] Castano, A.P.T., Demidova, T.N. and Hamblin, M.R. “Mechanisms in photodynamic therapy I. Photosensitizers, photochemistry and cellular localization.” Photodiagn Photodyn Ther 1, 279-293 (2004).

[6] Wilson, M., Dobson, J. and Harvey, W. "Sensitization of oral bacteria to killing by low-power laser radiation." Curr. Microbiol 25, 77–81 (1992).
[7] Zeina, B., Greenman, J., Purcell, W.M. and Das, B. "Killing of cutaneous microbial species by photodynamic therapy." British J Dermatol 144, 274-278 (2001).
[8] Reszka, K.J., Denning, G.M. and Britigan, B.E. "Photosensitized oxidation and inactivation of pyocyanin, a virulence factor of *Pseudomonas aeruginosa*." Photochem Photobiol 82, 466-473 (2006).
[9] Omar, G.S., Wilson, M. and Nair, S.P. "Lethal photosensitization of wound-associated microbes using indocyanine green and near-infrared light." BMC Microbiology 8, 111-121 (2008).
[10] Hamblin, M.R., Zahra, T., Contag, C.H., McManus, A.T. and Hasan, T. "Optical monitoring and treatment of potentially lethal wound infections *in vivo*." J Infect Dis 187, 1717-1725 (2003).
[11] Jori, G., Fabris, C., Soncin, M., Ferro, S., Coppellotti, O., Dei, D., Fantetti, L., Chiti, G.and Roncucci G. "Photodynamic therapy in the treatment of microbial infections: basic principles and perspective applications." Lasers Surg Med 38, 468-481 (2006).
[12] Lambrechts, S.A., Demidova, T.N., Aalders, M.C., Hasan, T. and Hamblin, M.R. "Photodynamic therapy for *Staphylococcus aureus* infected burn wounds in mice." Photochem Photobiol Sci 4, 503-509 (2005).
[13] Hamblin, M.R. and Hasan, T. "Photodynamic therapy: a new antimicrobial approach to infectious disease?" Photochem Photobiol Sci 3, 436-450 (2004).
[14] Ma, J., Zhao, J. and Jiang, L. "The aluminium (III) complex of hypocrellin B as a PDT photosensitizer." New J. Chem 25**,** 847–52 (2001).
[15] Diwu, Z. and Lown, J.W. "Hypocrellins and their use in photosensitization." Photochem Photobiol 52, 609-616 (1990).
[16] Toffoli, D.J., Gomes, L., Vieira Jr., N.D. and Courrol, L.C. "Photodynamic potentiality of hypocrellin B and its lanthanide complexes." J Opt A: Pure Appl Opt 10,104026 (8pp) (2008).
[17] Toffoli, D.J., Prates, R.A., Ribeiro, M.S., Hashimoto, M.C.E. and Courrol, L.C. "Effectiveness in total reduction of *Candida albicans* promoted by PDT with hypocrellin B:Lanthanum." Procc SPIE 7380, 60 (2009).
[18] Hashimoto, M.C.E., Toffoli, D.J., Prates, R.A., Courrol, L.C. and Ribeiro, M.S. "Photodynamic inactivation of antibiotic resistant strain of *Pseudomonas aeruginosa in vivo."* Procc SPIE 7380, 73803F-1 (2009).
[19] Gilpin, D.A. "Calculation of a new Meeh constant and experimental determination of burn size." Burns 22, 607-611 (1996).
[20] Jett, D.B., Hatter, K.L., Huycke, M.M. and Gilmore, M.S. "Simplified agar plate method for quantifying viable bacteria." Bio Techniques 23, 648-650 (1997).