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In Vitro Study of Photodynamic Therapy for Treatment of Bacteremia in Whole Blood

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Abstract: Antibiotic resistance has been compromised hospitalized patients with serious infections. The main cases of bacteremia can be caused by antibiotic resistant pathogens. Photodynamic therapy (PDT) has been shown as an alternative for inactivation of microorganisms in blood. In this therapy photochemical mechanisms occur that may prevent the development of bacteria. This study aims optimization of PDT parameters for blood decontamination. Concentration of photosensitizer (PS), light dose (LD) and incubation time (IT) were studied for hemolysis and cell toxic effects. It was observed that PDT can be used for microbial inactivation in total blood reducing 0.85 $\log_{10}$ CFU/mL of *S. aureus* at 15 J/cm^2 and 50 μg/mL of Photogem®.

Key words: *Staphylococcus aureus*, photodynamic therapy, bacteremia, blood.

1. Introduction

Bacteremia is defined by presence of viable bacteria in the blood. Multiple organ failure is cause of death in septic patients, a clinical syndrome of systemic inflammatory [1]. Bloodstream infections may be complications of infections as pneumonia or meningitis or even during surgery [2].

Bacteremia in adults is caused from 1 to 30 Colony Forming Units per milliliter (CFU/mL). In children, especially neonates, the number may exceed a little more than 1,000 CFU/mL [3].

Infected patient's blood is diagnosed through pH: a glucose measurement or polymerase chain reaction (PCR) technique that detect the presence of bacterial DNA [4]. Current treatments consist of antibiotic therapy. However, use of irregular and excessive of antibiotics can result in selection of antibiotic resistant bacteria that remain the biggest concern for hospitals [5]. Among the main bacteria responsible for blood infections is *Staphylococcus aureus*, which may be antibiotic resistant as methicillin and vancomycin [6].

In search of alternatives for antibacterial therapy that does not cause selection of antibiotic resistant microorganisms, the photodynamic therapy (PDT) has been considered an efficient treatment for infections caused by bacteria, fungi, protozoa and viruses [7]. PDT requires an interaction of light source, molecular oxygen (O_2) and photosensitizer (PS). Cytotoxic effects occur when light source and PS are used together [8].

The PDT mechanism occurs through absorption of a photon by PS that promotes an energy electron of ground state to excited singlet state, in which there is a high probability of transit to excited triplet states. The interaction of PS and O_2 can occur through two reactions called type I and type II [9]. In type I reaction, the PS reacts with organic substances of cellular components by transfer of electrons forming reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion radical and hydroxyl. The PS, in the state T1, transfers energy directly to the molecular oxygen in type II reaction that excites for a

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highly reactive singlet state [10]. Singlet oxygen reacts with amino acids, proteins, unsaturated lipids and nucleic acids in order to promote tissue death via necrosis or apoptosis [11].

Considering that inactivating microorganisms in blood is still a serious challenge and that studies of literature proposed criteria of microbial inactivation in the blood components separately, this study proposes to investigate some strategies to improve protocols of PDT in whole blood.

2. Materials and Methods

2.1 Bacteria

A strain *Staphylococcus aureus* (ATCC 25923) was cultured in BHI medium (Brain Heart Infusion) and stove at 37 °C. Microbial growth after overnight incubation was performed. Bacterial samples were centrifuged at 1,000 g for 15 min at 25 °C and the cells were resuspended in phosphate buffered saline (PBS) and subsequently diluted bacterial inoculum to obtain a concentration of 10^8 CFU/mL. In the standardized concentration the bacterium was resuspended in human blood.

2.2 Photosensitizer

The stock solution was prepared by diluting 5 mg Photogem® (Moscow, Russia) to a final volume of 1 mL saline solution (0.9%, w/v, NaCl). From this solution new dilutes were carried out on obtained concentrations of 8, 25, 50, 75 and 92 μg/mL for microbiological assays.

2.3 Light

Biotable is a device developed by Laboratory of Technical Support—LAT (São Carlos Institute of Physics, Brazil). It is composed of LED lamps at wavelength 630 nm and intensity 30 mW/cm^2. The Photogem® (PS) absorbs light in a spectrum range from 500 to 630 nm. Light dose (LD), determined by exposure time, and ranged from 1.8 to 31.8 J/cm^2. LD was calculated using Eq. (1) below:

$$LD = I \cdot t \quad (1)$$

where LD = light dose (J/cm^2), I = intensity of device (mW/cm^2) and t = lighting time.

2.4 Photodynamic therapy

A procedure to evaluate the efficiency of PDT in blood contaminated with *S. aureus* was performed by colony forming unit (CFU) with analysis of viable cell numbers in CFU/mL before and after treatment. In each experiment, there were three control groups and one treatment group: (1) control (blood bacterial inoculum); (2) light control (blood bacterial inoculum + PBS + illumination); (3) PS control (blood bacterial inoculum + PS) and (4) PDT (blood bacterial inoculum + PS + lighting). Samples from each group were distributed in 24-well plates, remaining 2.4; 15; 30; 45; 62.4 minutes of incubation period at dark and 37 °C, before being submitted at lighting by Biotable.

Bacterial cells were grown in culture Petri plates (Brain Heart Infusion Agar) at 37 °C for 24 hours and colony-forming unit count in each group was observed.

The absorbance of hemoglobin is released by erythrocyte membrane rupture, which was measured in a spectrophotometer (413 nm). The results were expressed as percentage of hemolysis based on absorbance obtained from lysed cell in distilled water (100% hemolysis).

2.5 Experimental Design

Experimental design guarantees advantages such as reduction of time and costs of experiments. Response contour surface technique is based on experimental design allowing combining optimal parameters in microbial response. The determination of number of experiments is performed according to number of variables studied and levels stipulated for these variables. A central composite planning (PCC) was performed by software STATISTICA 13. A PCC was composed at two levels with three variables: two replicates at central point and 6 experiments at axial points (α), totaling 16 experiments [12]. An

experimental design at two-level was defined represented by +1, -1, +alfa and -alfa for variables PS (photosensitizer (μg/mL)), IT (incubation time (min)) and LD (light dose (J/cm^2)).

2.6 Optical Microscopy

Experiments were performed to analyze qualitatively the whole blood after treatments with Photogem® at concentration 50 μg/mL and light dose (630 nm) at 15 J/cm^2. For qualitative evaluation and morphological analysis of the cells, blood smear blades stained with May Grünwald-Giemsa were made at the end of treatments. The blades were observed by optical microscopy.

2.7 Fluorescence Confocal Microscopy

Whole blood was diluted in saline solution (0.9%, w/v, NaCl) to make the images on confocal microscopy (20 μL of whole blood in 3 mL of saline solution). Photogem® was used at a concentration of 50 μg/mL. Confocal microscopy was performed by the transmitted light with two fluorescence channels with excitation at 405 nm (channel 1: 425 to 600 nm corresponding the auto fluorescence in green; channel 2: 600 at 760 nm corresponding the Photogem® fluorescence).

3. Results and Discussion

3.1 Antimicrobial Assays

Photogem® and light at 630 nm wavelength did not present toxicity in *S. aureus* at the fluids tested. The results indicated the potential of the use of PDT in the inactivation of the *S. aureus* microorganism in whole blood with best condition Photogem® at 50 μg/mL, LD at 15 J/cm^2 and IT of 30 minutes. Table 1 shows the logarithmic bacterial reduction and percentage of hemolysis of *S. aureus* in each experiment.

Table 1 shows that Photogem® associated with light at 630 nm was able to produce deleterious effects on *S. aureus* under the conditions studied. The variations of PS, IT and LD resulted in a reduction from 0 to 0.85 Log (CFU/mL). Nine of the 16 trials had a deviation of less than 20% (experiments 2, 3, 4, 6, 7, 8, 10, 12 and 16). A hemolysis rate was close to the acceptance criteria of 0.8%. However, although the technique causes damage to blood cells, its use may be viable. The experiments 15 and 16 of central point confirm the best condition for microbial inactivation in whole blood, besides being one condition with lower potential

Table 1 PDT and hemolysis results for each test of experimental design.

Exp.	PS	IT	LD	PDT		Hemolysis	
	(μ/mL)	(min)	(J/cm²)	(Log_{10} CFU/mL)		(%)	
1	25	15	5	0.2	± 0.2	4	± 2
2	75	15	5	0.35	± 0.03	6	± 1
3	25	45	5	0.43	± 0.01	4	± 2
4	75	45	5	0.31	± 0.06	4.5	± 0.6
5	25	15	25	0.13	± 0.06	7	± 9
6	75	15	25	0.14	± 0.03	24	± 7
7	25	45	25	0.16	± 0.01	7.4	± 0.2
8	75	45	25	0.46	± 0.04	22	± 6
9	8	30	15	0.00	± 0.06	0.9	± 0.1
10	92	30	15	0.31	± 0.09	13	± 3
11	50	2.4	15	0.3	± 0.2	1.49	± 0.04
12	50	62.4	15	0.15	± 0.01	7.2	± 0.1
13	50	30	1.8	0.00	± 0.01	9	± 10
14	50	30	31.8	0.3	± 0.1	30	± 13
15	50	30	15	0.7	± 0.2	3	± 3
16	50	30	15	0.85	± 0.04	1	± 2

for hemolysis. These groups have most viable parameters for the use of the technique. Isolated terms, interactions and quadratics of the three variables studied of PDT were analyzed by STATISTICA 13. The mathematical model for representing data based on multiple regression was represented below (Eq. (2)):

$$PDT = 0.03325497*PS - 0.000309559475*PS^2 - 0.000804890236*IT^2 - 0.00193474672*LD^2 \quad (2)$$

where PDT = photodynamic therapy: PS = photosensitizer (μg/mL), IT = incubation time (min) and LD = light dose (J/cm^2).

The response surface methodology aims to create a model of relevant variables of the photodynamic therapy from the regression equation. The response surface determines the influence of the interaction of the parameters studied for each experiment. Negative signals in quadratic terms show that the response surface has the optimal point in the regression equation. The correlation coefficient (R^2) 0.86 indicates an adequate adjustment of experimental data in the inactivation of *S. aureus*, showing that 86% of the variability of data were explained by the empirical equation. The calculated F (Fc) was lower than the tabulated F at a significance level of 5%. This comparison can be interpreted through a hypothesis test. The null hypothesis (H0) is the difference between the groups. In order to obtain a better visualization of the effect of the independent variables on PDT, response surface was constructed (Figs. 1a-1d).

Fig. 1a shows that the interaction between a higher PS and higher IT results in increase of microbial death. In Fig. 1b it was observed that PS was more relevant to the result when compared to LD. The interaction of LD and IT showed the importance of LD in treatment (Fig. 1c). Confocal microscopy shows PS in erythrocytes and bacteria (Fig. 1d), proving the interaction of PS with cells. The factors that can intervene in the PDT response in whole blood are: erythrocytes are the target of PS interaction, presence of biomolecules carriers of PS in blood and high optical absorption of blood in the visible spectrum.

Blood proteins can promote the aggregation of molecules to the PSs by damaging the photodynamic reaction. It can reduce the capacity of PS to generate EROs.

Although the PDT inactivated only 0.85 $\log_{10}$ CFU/mL of S. aureus, too often, a bacterial infection in blood does not respond to treatment with antibiotics. PDT may be a possibility of adjuvant therapy to antibiotic for reduction of blood microorganisms. The amount of bacteria present in the blood of adult patients is in the range of 1 to 30 CFU/mL blood [3].

PDT induces the release of pro-inflammatory molecules and stimulates the innate and adaptive response of the immune system [12, 13]. PDT can act to stimulate the immune response in organisms, recruiting specific cells that help in eliminating the infectious focus [13, 14]. It may thus be considered an auxiliary treatment to the primary treatment of infections caused in the blood. PDT is applied with a fluence capable of causing both the death of bacteria and the accumulation of neutrophils in the infected area [15].

3.2 Hemolysis Assays

To ensure that PDT may be an alternative technique for controlling microorganisms in blood, hemolysis assays were performed that determined the percentage of damage caused to erythrocytes. The same analysis was performed for the hemolysis results through multiple regression and it obtained Eq. (3):

$$Hemolysis = 15.6844382 + LD*(-1.95257802 + 0.0597663721*LD) + 0.01455*PS*LD \quad (3)$$

The response surface obtained for the hemolysis analyses demonstrates the parameters that provide the most statistically significant response. The light dose was the most significant parameter followed by the combination of photosensitizer and light dose which correspond to the most effective variable of PDT. The incubation time did not show influence the hemolysis response. The correlation coefficient (R^2) of 0.97 indicates an adequate fit of the experimental data of

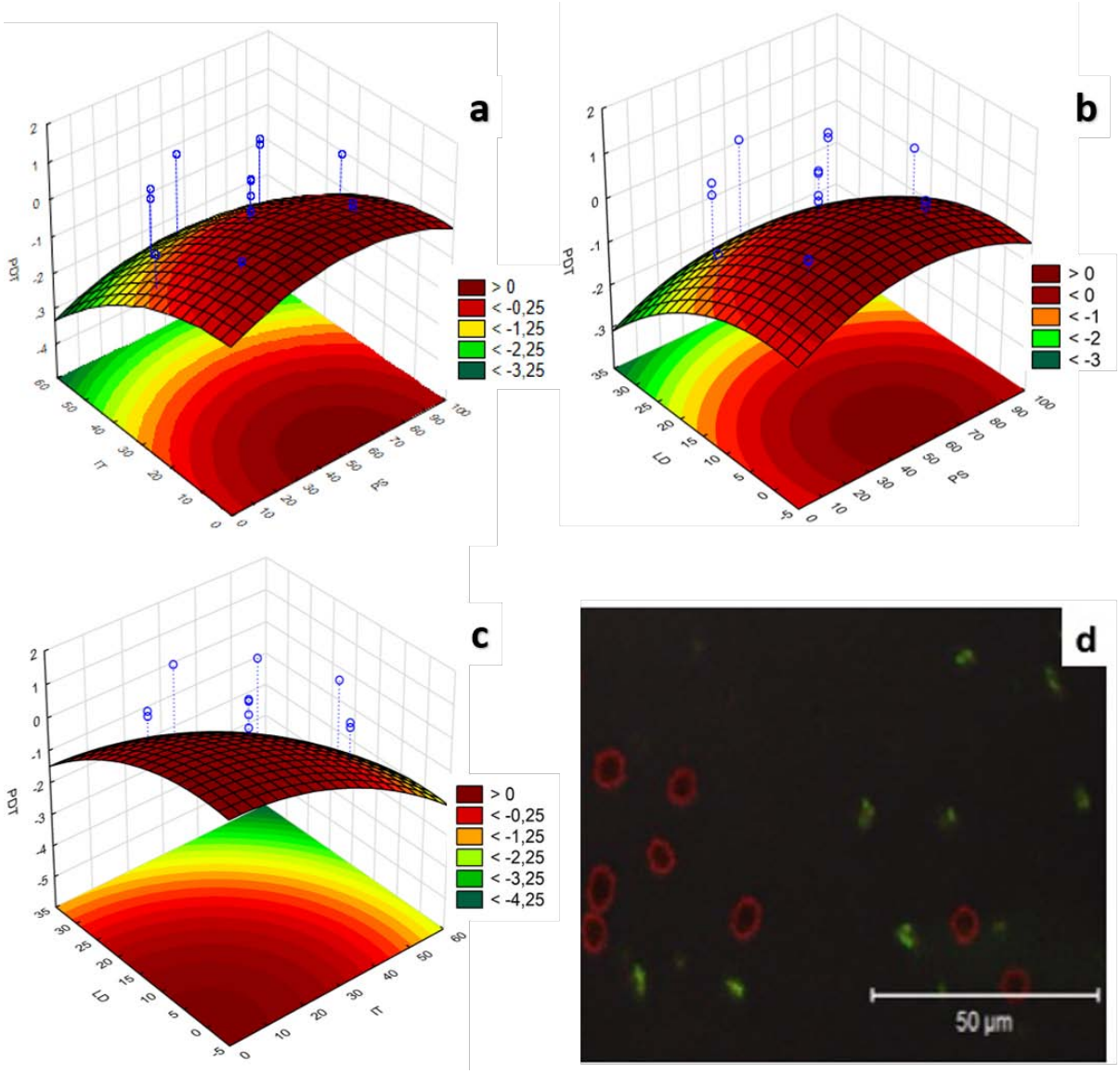

Fig. 1 Response surface of *S. aureus* inactivation in function of (a) PS (µg/mL) and IT (min), (b) PS (µg/mL) and LD (J/cm^2), (c) IT (min) and LD (J/cm^2), and (d) confocal microscopy of PS in erythrocytes and bacteria.

inactivation response of *S. aureus*, showing that 97% of the variability of the data were explained by the proposed empirical equation. The result of calculated F was higher than the tabulated F for a significance level of 5%. The effect of the independent variables on hemolysis was observed in Fig. 2.

Fig. 2a shows that the interaction between higher LD with PS results in a higher percentage of hemolysis. However, the most significant parameters studied for effective PDT in blood are at the threshold of acceptable hemolysis. Fig. 2b shows optical microscopy of whole blood after PDT with normal aspect of cells in the highlighted area it was possible to observe few modified cells. Hemolysis is considered a normal and intrinsic process of the organism. About 0.8% to 1% of the total erythrocytes of an individual were hemolysis daily and produced new erythrocytes. Hemolysis in large proportions can cause serious problems that can usually occur due to reactions to some drugs, or due to autoimmune to the presence of abnormal hemoglobin.

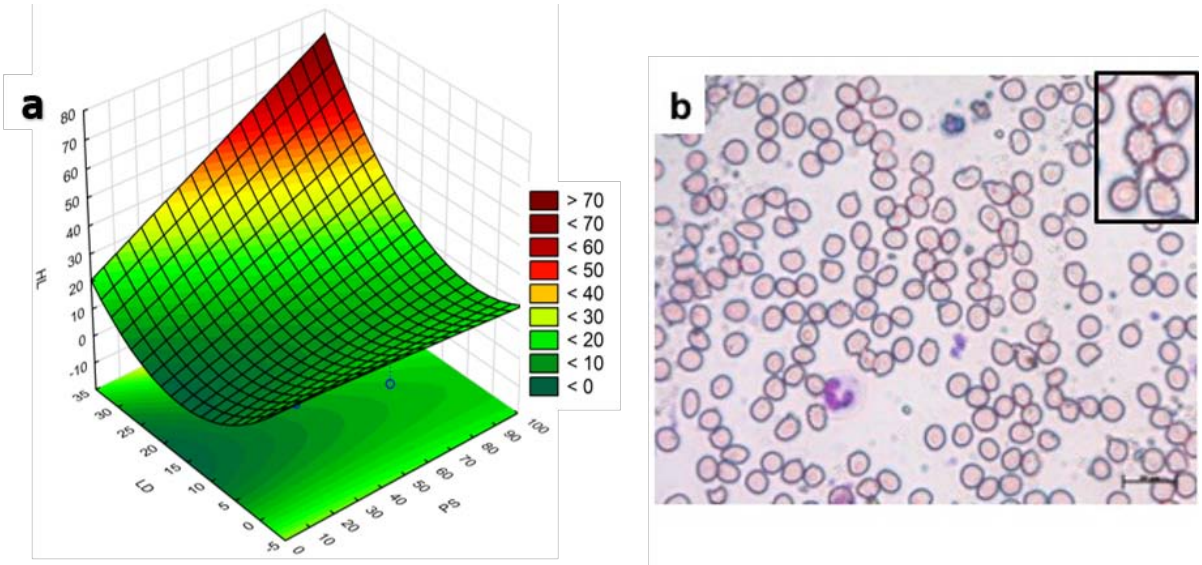

Fig. 2 (a) Response surface for the hemolysis response in IT and LD function and (b) optical microscopy of whole blood after PDT.

4. Conclusions

According to the experimental design it was possible to optimize the PDT parameters to inactivate *S. aureus* in whole blood with a hemolysis rate acceptable to clinical standards. However, it is possible to adjust the conditions of the PDT by associating nanoparticles, antibodies that direct PS to the desired target or even modifying formulations for FS delivery.

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