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PDI using nebulized indocyanine green for pneumonia treatment

Mariana C. Geralde*, Giulia Kassab, Natalia M. Inada, Cristina Kurachi and Vanderlei S. Bagnato
São Carlos Institute of Physics, University of São Paulo, PO Box 369, 13560-970, São Carlos, SP, Brazil

ABSTRACT

Infectious pneumonia is a major cause of morbidity/mortality, mainly due to the increasing rate of microorganisms resistant to antibiotics. Photodynamic Inactivation (PDI) is emerging as a promising treatment option, which effects are based on oxidative stress, targeting several biomolecules and probably preventing potential resistant strains. In previous studies, the *in vitro* inactivation of *Streptococcus pneumoniae* using indocyanine green (ICG) and infrared (IR) light source (780 nm) was successful, and achieving satisfactory reduction of colony-forming units (CFU/mL). In the present study, a proof-of-principle protocol was designed to treat lung infections by PDI using extracorporeal irradiation with a 780 nm laser device and nebulized ICG as photosensitizer. Balb/c mice were infected with *S. pneumoniae* and PDI was performed two days after infection using 800 μM of nebulized ICG and extracorporeal irradiation. Our results indicate that IR-extracorporeal PDI using nebulized ICG may be considered a potential pneumonia treatment, and pulmonary decontamination with PDI may be used as a single therapy or as an adjuvant for antibiotics.

Keywords: Photodynamic Inactivation, Photosensitizer, Pulmonary Diseases, Extracorporeal Irradiation, Infrared light.

*mcgeralde@outlook.com; +55 16 33739810; cepof.ifsc.usp.br

1. INTRODUCTION

Community-acquired pneumonia (CAP) is the major cause of morbidity in children, responsible for 20% of child deaths worldwide [1]. According to the World Health Organization (WHO), the incidence of pneumonia is of around 450 million cases every year, with approximately 4 million deaths. It is accountable for the deaths of about 1 million children under five years of age yearly [2]. In the USA, the estimated annual number of CAP episodes in adults is of approximately 5.2 million, with the highest incidence among those aged 65 years and older [3]. These numbers describe a situation that is prone to get worse rather than better due to the aging profile of the population in Western countries, and to the higher incidence of single- and multi-drug resistance infectious pathogens. As a result, there is a need for alternative or adjunct treatments to reduce morbidity and mortality rates.

Nosocomial infections are usually caused by resistant pathogens and occur in hospitals worldwide, with high rates of morbidity, mortality and hospital costs [4]. Microorganisms can infect the lower respiratory tract, which is considered largely sterile in healthy individuals, by four different mechanisms: a) aspiration of secretions containing pathogens from oropharynx, gastric cavity or nasal cavities; b) spread of bacteria from a nearby area, such as pleura; c) aspiration through respiratory therapy devices and inhalation of contaminated aerosols; d) hematogeneous translocation into lungs from systemic infection sites. In hospitals, intubation and mechanical ventilation are commonly followed by infection ("ventilator-associated pneumonia"), which is considered the main complication for ventilated patients [5].

In developing countries, the main etiological agents causing pneumonia are bacteria, mostly *Streptococcus pneumoniae* (30-50%). The second most common agent is *Haemophilus influenzae* type b (Hib), followed by *Staphylococcus aureus* and *Klebsiella pneumoniae* [6].

Photodynamic therapy (PDT) consists in a reaction between a photosensitizer (PS), a light at a specific wavelength, and molecular oxygen. A resulting reaction can occur by the transfer of either hydrogen or electrons, leading to the formation of free radicals, reactive oxygen species (ROS) (type-I reaction) or by energy transfer to molecular oxygen (type-II reaction), producing mainly singlet oxygen. Both reactions can kill cells/microorganism almost immediately by oxidative damage [7,8].

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PDT targeting microorganisms is called photodynamic inactivation (PDI) and depends on a higher concentration of PS at the target cells, for example, bacteria, and a lower threshold dose, when compared to the surrounding host tissues. This is essential to promote a relatively selective toxic effect on the microorganisms and being safe to the host parenchyma [9].

Notably, PDI is already used clinically as treatment for several different infections disease, such as skin ulcers, oral candidiasis, periodontal diseases, acne vulgaris and many others [10–14].

Indocyanine green (ICG) is a tricarbocyanine, an organic dye that has been used clinically for diagnostic purposes since 1956 in several applications [15–17], approved by the USA Food and Drug Administration in 1959. It is soluble in inorganic solvents such as DMSO and methanol, as well as in aqueous media, including PBS and cell culture medium. The absorption peak of ICG is at 805 nm (near-infrared) where penetration into biological tissues is higher due to low absorption by hemoglobin, melanin, and water, which makes ICG an ideal photosensitizer for deep-tissue PDT [18,19] applications, which require light penetration through the parenchyma.

In two previous studies of our group, we have successfully demonstrated the efficacy of PDI with ICG killing *S. pneumoniae in vitro* where alveolar macrophage viability was preserved, and *in vivo* using instilled ICG. These findings are presented elsewhere [20,21]. However, the PS delivery in the animal model was by instillation, which is not an appropriate method for humans.

Other authors are indicating the use of ICG for treatment of several cancer types, acne vulgaris, in occlusion of choroidal neovascularization, and in eradicating microorganisms present in wounds and burns [22–27].

Based on the dire need for novel therapies for pneumonia, and the promising results obtained with PDI in other infectious diseases, we hypothesized that PDI could be an alternative or adjunct therapy for pneumonia treatment. To this end, we evaluated the efficacy of PDI with extracorporeal illumination using an infrared light source and nebulized ICG as PS for the treatment of pneumonia in an experimental mouse model.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Xylazine and Ketamine were purchased from Sespo-Ceva Santé Animale (Paulínia, SP, Brazil). Indocyanine green – ICG was obtained from Ophthalmos (São Paulo, SP, Brazil). Sodium chloride, potassium chloride, dibasic sodium phosphate, monobasic potassium phosphate were obtained from Sigma-Aldrich (St. Louis, Missouri, United States).

2.2 Animal model and treatments

Animal experiments were approved by the Animal Ethics Committee of São Carlos Institute of Physics, IFSC/USP – University of São Paulo (number 12/2016, approved on October 17th, 2016). Eight-week-old male Balb/c mice (n=12) were used. For induction of the lung infection, animals were anesthetized by intraperitoneal administration of Xylazine (5 mg/kg) and Ketamine (75 mg/kg) and then, the total amount of 10^9 CFU of *Streptococcus pneumoniae* (ATCC 49619) bacteria were suspended in phosphate-buffered saline (PBS) and instilled into the nostrils. The total volume of the instilled inoculum was 30 µL per animal (15 µL per nostril). Animals were randomly divided into 2 experimental groups: control and photodynamic inactivation (PDI) with ICG and extracorporeal irradiation.

Before any protocol, animals were anesthetized again using the same protocol as the one described for the infection, and the dorsal fur was removed by a hair clipper and a hair removal cream. For the PDI group, the ICG was dissolved in water for injection, and diluted to a final concentration of 800 µM. The animals received 10 minutes of nebulized ICG using an Omron NE-C801 air-jet nebulizer, being 5 minutes before and 5 minutes during the irradiation process.

For the irradiation, each animal was positioned inside a custom-made laser device containing 18 laser diodes emitting monochromatic light at 780 nm. An irradiance of 60 mW/cm^2 and a total dose of 120 J/cm^2 were delivered onto the animal dorsum.

For the control group, animals received nebulized PBS for 10 minutes, but were not exposed to irradiation. The PS only and the Light only groups were not performed once both does not cause any effect upon *S. pneumoniae* inactivation [21].

3. PRELIMINARY RESULTS

For the control group (no treatment), CFU recovery ranged between 10^6 and 10^7, and two animals died 3 days after the infection. For the PDI group, however, CFU recovery ranged between 10^4 and 10^7, and only one animal died only 7 days after the infection (Panel A, Figure 1). Although statistical analysis did not show any difference between the groups, it is important to consider the animals' death, as well as the fact that the infection induced was very aggressive, indicating that a single PDI session is not effective to control the pneumonia.

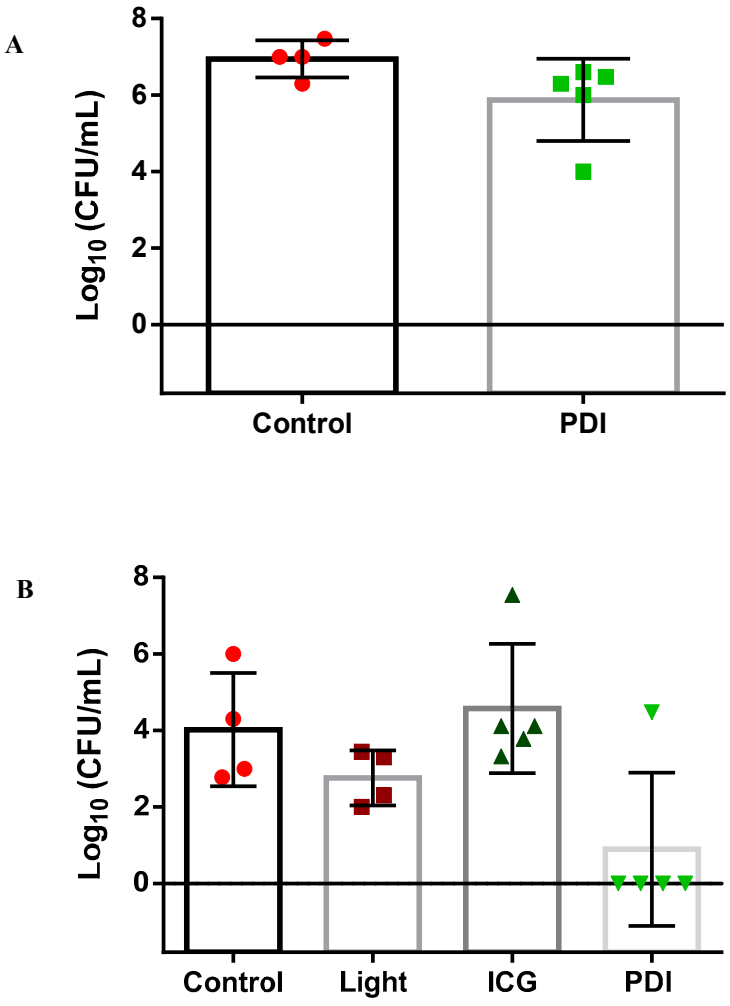

Figure 1. Panel A: Scatter plot and mean±SD of CFU recovery for mice infected with 10^9 *S. pneumoniae* CFU and treated with only PBS nebulization (Control) or 800 µM of nebulized indocyanine green and 120 J/cm^2 of light dose (PDI). $p<0.0873$ vs. Control, Mann-Whitney test, n=6 each. Two animals of control group died on the 3rd day after infection, and one animal of PDI group died on the 7th day after infection, and therefore it was not possible to recover the CFU count for them. Panel B: previous results obtained with instilled ICG [21].

Comparing to the results using instillation, nebulized ICG was not so successful and perhaps, due to the ICG molecule dynamics, is necessary more than one successive PDI sessions to achieve similar or better results than with instilled one. Moreover, the strain used in the experiments of nebulized ICG was obtained lyophilized directly from the ATCC sample

bank, while the strain used in the tests of instilled ICG was acquired as a donation, initially already hydrated and stored in a biofreezer at -80 ºC. The difference between the strains may imply variations in the virulence of the bacteria and in the aggressiveness of the infection. Thus, induction of infection from a more virulent strain results in an aggressive infection. It is noted that, in the experiment presented in the present study, animals in the control group were recovered at around 10^6 - 10^7 CFU/animal, and in the experiments performed with instilled ICG were recovered around 10^3 - 10^4 UCF/animal, in both experiments recovery was done seven days after infection. A more severe infection may require multiple PDI sessions for significant efficacy, since it is more difficult to obtain a complete treatment with only one application.

A study of penicillin G-resistant strains of *S. pneumoniae* showed that in order to inactivate 2 $\log_{10}$ bacteria in the lungs of mice infected with resistant strains, subcutaneous administration of 30 mg/kg penicillin G was required. Inactivating 3 $\log_{10}$ of sensitive *S. penumoniae* required doses of 0.6 mg/kg penicillin G administered six times at 1 h intervals between one application and another. Also in this study, the efficacy of the antibiotic Imipenem, which has a higher spectrum among beta-lactams and is highly resistant to beta-lactamases, has been evaluated and is therefore widely used for polymicrobial and hospital infections. To reduce from 10^7 CFU/animal to 10^5 CFU/animal, a dose of 0.6 mg/kg Imipenem was required [28]. It is important to emphasize that in the present study a single session of PDI was applied and, still, a bacterial reduction similar to the reduction observed with the use of antibiotics was obtained. Moreover, the photodynamic response has a more nonspecific action of cell death, that is, when with sufficient amounts of PS and fluency, they act indifferently on the type of microorganism.

Still on the difference in the number of CFUs recovered in the different experiments, it is necessary to consider the difference of the sexual gender of the animals used. In the results shown with instilled ICG were used female SKH-1 mice, and in the results presented in this study with nebulized ICG were used male BALB/c mice.

Sabino *et al.* (2016), evaluated light parameters in skin samples from BALB/c and C57BL/6 albino mice (males and females), and in their discussion raise the fact that the skin of female mice has a more abundant adipose layer compared to males, which, on the other hand, have a thicker dermis and a greater amount of subcutaneous tissue (especially BALB/c). Although the epidermis and muscle layer thickness were similar between the groups, it was observed that the skin of BALB/c male mice presented lower transmittance and higher reflectance compared to females, especially between 600 and 1300 nm. BALB/c males exhibit significantly thicker skin tissue due to a greater amount of connective tissue in the dermis [29]. Variations in light transmittance in the skin may interfere with the activation of PS within the lungs, thereby decreasing the effectiveness of PDI.

An important limitation of instilled model is the respiratory system which would be a problem to treat bedridden patients in the future. Because mice are much smaller, the droplets generated by the nebulizer are too big to distribute evenly in their lungs. However, it was still possible to detect ICG in the murine lung via fluorescence (data not published), indicating that there is a need to optimize the nebulization and PDI parameters.

Future studies may include multiple PDI sessions and a smaller CFU count for the infection.

4. CONCLUSION

This study presented a proposal for PDI as a technique for the control of pulmonary infections caused by *Streptococcus pneumoniae*, using nebulized ICG and extracorporeal irradiation. Although more experiments should be conducted to increase the PDI efficacy, for example applying multiple PDI sessions, this study opens the way for the advancement in pulmonary infections treatments, presenting a promising protocol for bacterial inactivation.

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