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Immunomodulatory effect of photodynamic therapy in *Galleria mellonella* infected with *Porphyromonas gingivalis*

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ABSTRACT

Porphyromonas gingivalis is an important pathogen in the development of periodontal disease. Our study investigated if the treatment with antimicrobial photodynamic therapy (aPDT) that employs a nontoxic dye, followed by irradiation with harmless visible light can attenuate the experimental infection of *P. gingivalis* in *Galleria mellonella*. Firstly, different concentrations of *P. gingivalis* ranging from 10^2 to 10^6 cells/larva were injected into the animal to obtain a lethal concentration. Next, the following groups of *G. mellonella* infected with *P. gingivalis* were evaluated: inoculation of the photosensitizer and application of laser (P + L+), inoculation of physiologic solution and application of laser (P-L+), inoculation the photosensitizer without laser (P + L-) and inoculation of physiologic solution without Laser (P-L-). The effects of aPDT on infection by *P. gingivalis* were evaluated by survival curve analysis and hemocytes count. A lethal concentration of 10^6 cells/larva was adopted for evaluating the effects of aPDT on experimental infection with *P. gingivalis*. We found that after 120 s of PDT application, the death of *G. mellonella* was significantly lower compared to the control groups ($p = 0.0010$). Moreover, the hemocyte density in the P+L+ group was increased by 9.6×10^6 cells/mL (2.62-fold increase) compared to the infected larvae with no treatment (L-P- group) ($p = 0.0175$). Finally, we verified that the aPDT led to a significant reduction of the number of *P. gingivalis* cells in *G. mellonella* hemolymph. In conclusion, PDT application was effective against *P. gingivalis* infection by increasing the survival of *G. mellonella* and was able to increase the circulating hemocytes indicating that PDT activates the *G. mellonella* immune system.

1. Introduction

Hundreds of bacterial species that colonize the oral cavity maintain the balance of the ecosystem protecting against the invasion by others pathogenic species. Although some of these species can become pathogenic in response to the host genotype, stress, diet or behavior (e.g. smoking) [1–5]. Periodontal diseases are among the most common chronic inflammatory diseases in humans and are associated with the presence of *Porphyromonas gingivalis*. This oral bacterial specie is an anaerobic Gram-negative coccobacillus, which belongs to the Bacteroidaceae family, present colonies black-colored in blood agar and requires anaerobic conditions for its growth. This microorganism obtains its metabolic energy through the fermentation of aminoacids, an important property for its survival in deep periodontal pockets, where carbohydrates are scarce [6,7].

P. gingivalis is described as a “keystone pathogen” in chronic periodontitis. In the oral cavity, this species is able to deregulate the host immune response to favor the biofilm formation, thereby interrupting homeostasis with the host causing dysbiosis and local disease [8]. The pathogenicity of *P. gingivalis* is attributed to a number of virulence factors associated with its surface that include cysteine proteinases (gingipains), fimbriae, haem-binding proteins, and outer membrane transport proteins. Specially, cysteine endoproteinases, Arg-specific gingipains (RgpA and RgpB) and Lys specific gingipain (Kgp) have multiple effects on both innate and adaptive immune responses in the host [9,10]. Virulence factors, such as fimbriae and gingipans, are important for the identification, in typing methods, of strains associated with the disease [11].

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The conventional treatment for periodontitis involves the mechanical removal of dental plaque and mineralized deposits from tooth surfaces, but Antimicrobial photodynamic therapy (aPDT) has been proposed as an adjunctive strategy for periodontitis treatments [12–14]. One of the main advantages of laser is its ability to kill subgingival bacteria in seconds, which minimizes the need for maintaining high concentration of antimicrobial agents within the lesion for a long time [15]. Many oral bacteria are susceptible to the application of laser in the presence of photosensitizers, such as toluidine blue O, methylene blue and malachite green. These findings suggest that aPDT could be potentially advantageous in periodontal therapy [16].

In the last decades, several invertebrate models have been used in the study of the microorganism pathogenicity, host-pathogen interaction and as screening for testing new therapies [17]. The main invertebrate models of experimental infection are a *Galleria mellonella*, *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* [18–20]. *G. mellonella* presents numerous advantages over other invertebrate models as a sufficient size for the injection and the ability to survive at temperatures ranging from 25 to 37 °C, simulating the natural environment of mammalian hosts. Moreover, this insect have an immune system exhibits both humoral and cellular components; Hemocytes are the major mediator of cellular defenses, which perform similar functions to human macrophages and neutrophils [21–23].

Since *in vivo* studies are crucial for the evaluation of the microorganism pathogenicity and development of alternative treatments, the aim of this study was to verify the effectiveness of aPDT against experimental infection by *P. gingivalis* in *G. mellonella* model.

2. Materials and methods

2.1. Microbial strain and culture conditions

Porphyromonas gingivalis (ATCC 33277) strain used in this study was stored at −80 °C and cultured on blood agar plates with Fastidious anaerobic agar base (FAA, Acumedia, Michigan, USA) enriched with 1% menadione (Sigma-Aldrich, São Paulo, Brazil) and hemin (Sigma-Aldrich, São Paulo, Brazil) and incubated in anaerobic jars (Permution, Paraná, Brazil) for 5–7 days at 37 °C.

2.2. Preparation of the invertebrate model of Galleria mellonella

G. mellonella created in the laboratory of Microbiology and Immunology of the Institute of Science and Technology of São José dos Campos - UNESP, in the final instar larval stage were used in this study. Sixteen randomly chosen *G. mellonella* larvae with similar weight and size (250–350 mg) were used per group in all assays. Two control groups were included in the assays that form part of this study: one group were inoculated with PBS to enable us to observe the demise of the larvae due to physical trauma, and the other received no injection as a control for general viability. A 10 μl Hamilton syringe (Hamilton Inc, EUA) was used to inject 10 μl inoculum aliquots into the hemocoel of each larvae via the last left proleg. After injection, larvae were incubated at 37 °C in plastic containers.

2.3. Survival curve analysis

For analysis of virulence in *G. mellonella* and determination of the concentration that was used in the experiments of photodynamic therapy, standardized suspensions was adjusted in physiological solution (PBS) between 10^2 to 10^6 cells/larva by spectrophotometer (B582, Micronal, São Paulo, Brazil) at the wavelength of 660 nm. After injection, larvae were stored in plastic containers at 37 °C and observed every 24 h for a period of 7 days, and considered dead when they displayed no movement in response to touch. All experiments were repeated at least twice, and representative experiments are presented. For the survival curve analysis, 16 randomly chosen larvae were used per group in a total of 208 larvae.

2.4. Photodynamic therapy

For this study, the methodology described by Chibebe et al. [24] was used with some modifications. The phenothiazinium salt methylene blue (MB, Sigma Aldrich, São Paulo, Brazil) was used as the photosensitizer in this study. MB solutions at a final working concentration of 600 mM were prepared by dissolving the dye in physiological solution. A new photosensitizer solution was prepared on the same day of each experiment. After the photosensitizer injection, larvae were maintained in the dark until the time of light irradiation.

G. mellonella larvae were irradiated in a 24-well culture plate (Costar Corning, New York, NY, USA) as shown in Fig. 1. Light source of Gallium and Aluminum Arsenide laser with wavelength of 660 nm (visible red), power of 50 mW, with energy density of 15 J/cm^2 was used for light delivery. The experiments were performed as follows: *G. mellonella* received the photosensitizer injection (10 μL) 90 min after the bacterial infection. We waited for at least 30 additional min after the photosensitizer injection to allow a good dispersion of the photosensitizer into the insect body, prior to the light irradiation.

2.5. Quantification of G. mellonella hemocyte

Larvae were infected with *P. gingivalis* (10^6 cells/larvae) by injecting the bacteria at the last left proleg and submitted to PDT as described above. After the larvae were incubated at 37 °C for 4 h. At each time point, the larvae were cut in the cephalocaudal direction with a scalpel blade and squeezed to remove the hemolymph, which was transferred to an Eppendorf tube. The tubes contained cold, sterile insect physiologic saline (IPS) (150 mM sodium chloride; 5 mM potassium chloride; 100 mM Tris-hydrochloride, pH 6.9

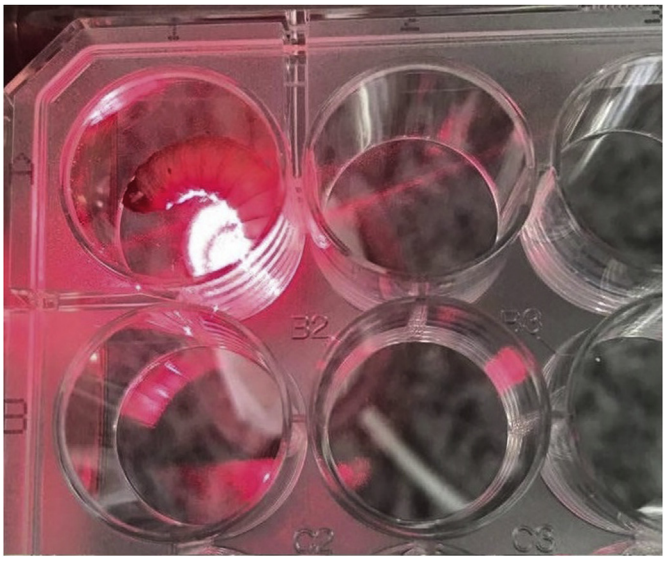

Fig. 1. *In vivo* photosensitization. *G. mellonella* being subjected to light irradiation. Each larvae was irradiated in a separate well of a 24-well culture plate.