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Effects of sub-lethal doses of photo-activated disinfection against *Porphyromonas gingivalis* for pharmaceutical treatment of periodontal-endodontic lesions

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ABSTRACT

Background: Microorganisms treated by photo-activated disinfection (PAD) in combined periodontal-endodontic (perio-endo) lesions would be exposed to sub-lethal doses of PAD (sPAD). This study evaluated the effect of sPAD using toluidine blue O (TBO) in combination with diode laser irradiation on the growth and biofilm-formation ability of *Porphyromonas gingivalis* as an endo-periodontal pathogen.
Methods: The antibacterial and antibiofilm potential of sPAD against *P. gingivalis* was analyzed at sub-lethal doses of TBO and irradiation time of diode laser on a colony-forming unit and crystal violet assays, respectively.
Results: TBO-mediated PAD, using 6.25–100 μg/mL at a fluency of 171.87 J/cm^2 and 12.5–100 μg/mL at a fluency of 137.5 J/cm^2, showed a significant dose-dependent reduction in *P. gingivalis* growth when compared to the control. TBO-mediated PAD showed a significantly inhibitory effect on biofilm formation in *P. gingivalis* than TBO-PAD at sub-lethal levels.
Conclusion: High doses of sPAD revealed antibacterial and antibiofilm potential activity, whereas lower doses of sPAD had conflicting results. Therefore, when PAD is prescribed in combined perio-endo lesions treatment, the dose of PAD used *in vivo* should be taken into account.

1. Introduction

Combined periodontal-endodontic (perio-endo) lesions are characterized by the interrelationship between the periodontal pocket and tooth pulp. Bacterial migration from a periodontal pocket, via dentinal tubules, lateral canals, or apical foramen, into the root canal of the affected tooth leads to pulpal inflammation [1]. Sometimes dental tubules and lateral canals are exposed to bacterial invasion following the removal of cementum due to rigorous scaling, root planning and surgical flap procedures [2]. The prognosis of combined perio-endo lesions depends on successful endodontic therapy proceeding to periodontal treatment [1]. *Porphyromonas gingivalis*, a gram-negative obligate anaerobic bacterium, is one of the etiological agents and keystone pathogens in the initiation of combined perio-endo lesions [3]. The significantly higher *P. gingivalis* counts in subgingival plaque samples, as well as the positive correlation between *P. gingivalis* counts of subgingival and endodontic samples from combined perio-endo lesions, indicate bacterial migration from the periodontal pocket to the root canal [4]. *P. gingivalis* can colonize and survive in the combined perio-endo lesions through a number of persistence factors, especially biofilm formation via fimbriae, which are multifunctional adhesins, and signaling within, microbial biofilms and host tissues [5]. Chronic use of antimicrobial agents against combined perio-endo lesions has led to the emergence of resistance in

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microorganisms and the appearance of drug-resistant strains [6]. Thus, the development of alternative therapies is essential in order to control combined perio-endo infections.

Photo-activated disinfection (PAD) is a minimally invasive new treatment method for the eradication of infectious pathogens using a photosensitizer and light with a specific wavelength [7]. The photosensitizer, e.g., toluidine blue O (TBO), is a cationic (basic) dye with specific features that can absorb light energy and produce cytotoxic reactive oxygen species (ROS) such as H2O2, $O2^{-}$, and HO in the presence of oxygen, causing the destruction of microorganisms [7,8].

The main objective of the treatment of combined perio-endo lesions by PAD is to improve decontamination of endo-periodontal pathogens [9]. If a sub-lethal dose of PAD (sPAD), i.e., a sub-lethal concentration of TBO in combination with a sub-lethal dose of light, reaches the target site, any microorganism, such as *P. gingivalis*, will not only not be eradicated but may also remain viable as a more virulent and more challenging infection than ever before [7]. To the best of our knowledge, there is no report on the effects of sPAD treatment on biofilm formation ability, as a major virulence factor, and on the count of *P. gingivalis*. In the present study, we evaluated whether *P. gingivalis* cells exposed to sPAD exhibited changes in colony-forming unit (CFU) levels and biofilm formation ability.

2. Materials and methods

2.1. Bacterial strain and culture conditions

A clinical strain of *P. gingivalis* was isolated from a patient presenting combined perio-endo lesions and characterized as previously described [10]. The organism was anaerobically grown on a brucella agar plate (Merck, Darmstadt, Germany) supplemented with 5% defibrinated sheep blood, 5 mg/L hemin, and 1 mg/L menadione (sBBA) at 37 °C for 48 h. For experiments requiring cultures in broth, cultures grown in sBBA agar were transferred into brain-heart infusion (BHI) broth (Merck, Darmstadt, Germany), supplemented with 1 mg/mL hemin and 5% horse serum.

2.2. Photosensitizer and light source

A stock solution of high purity grade TBO (Bioworld, Ohio, USA) at 0.2 mg/mL was prepared in sterile 0.9% (wt/vol) NaCl. It was then filter-sterilized by using 0.22-μm-pore-size membrane filters and kept under dark conditions prior to use. A diode laser (Konftec, Taiwan) at a wavelength of 635 nm, with output power of 220 mW, was used with TBO.

2.3. Determination of minimum inhibitory concentration (MIC) of TBO

The MIC of TBO against *P. gingivalis* was determined according to a previous study [11]. In brief, 100 μL of 2× BHI broth was added to each well of a round-bottom 96-well microtiter plate (TPP, Trasadingen, Switzerland) using a multi-channel pipet. 100 μL of 0.4 mg/mL TBO was added to the well in column one and diluted twofold step-wise from column one to column ten. Then, 100 μL/well of bacterial suspension with concentration of 1.0×10^6 CFU/mL, which was verified by spectrophotometry (optical density [OD]600:0.1) [12] was transferred to each well. So, the final bacterial cells concentration in the wells was 5.0×10^5 CFU/mL, and the concentration of TBO was in the range of 100–0.19 μg/mL. One column contained the positive control and another was not inoculated and served as the negative control. After the *P. gingivalis* cells were in contact with TBO for 5 min in an anaerobic atmosphere, 10 μL of each well was plated onto sBBA agar and cultured using the spread technique with an L-shaped bar. The plate was incubated at 37 °C in an anaerobic atmosphere for 48 h.

2.4. Determination of sub-lethal diode laser irradiation time

The sub-lethal diode laser irradiation time against *P. gingivalis* was determined according to a previous study [11]. Briefly, 200 μL of bacterial suspension, at a final concentration of 2.0×10^5 CFU/mL, were transferred to the wells of a round-bottom 96-well microtiter plate and exposed to 1, 2, 3, 4, and 5 min diode laser irradiation times, with energy density of 34.37, 68.75, 103.12, 137.5, and 171.87 J/cm^2, respectively, at wavelength 635 nm at room temperature (25 ± 2 °C). The neighboring test wells were filled with TBO to prevent transmission of light to wells near the test wells, and sheets of black paper were placed under the microplate during diode laser irradiation to prevent the beam reflecting of the tabletop. The sub-lethal diode laser was determined according to the methods mentioned above.

2.5. Determination of sub-lethal PAD

The sPAD was determined according to a previous study [11]. Briefly, 100 μL of 2× BHI broth was added to each well of a flat-bottom 96-well microtiter plate (TPP, Trasadingen, Switzerland), and 100 μL of TBO at 2 × MIC was serially diluted twofold to 1/64 MIC. 100 μL/well of *P. gingivalis* suspension, in concentration of 1.0×10^6 CFU/mL, was then inoculated to each well. The final bacterial cells concentration in the wells was 5.0×10^5 CFU/mL. Instantly, the microplate was incubated in the dark at room temperature in an anaerobic atmosphere for 5 min. The treated bacterial suspension in the well was immediately exposed to sub-lethal diode laser irradiation time. The control group did not have any treatment and all experiments were performed in triplicate. sPAD was determined according to the methods mentioned above.

2.6. Evaluation of biofilm formation ability of treated planktonic P. gingivalis by crystal violet

Biofilm-forming ability and quantitative analysis of the formation assay of biofilm were performed according to a previous study [12]. Briefly, 200 μL aliquots of *P. gingivalis* cells were treated with PAD at sub-lethal dose in a final concentration of 5.0×10^8 CFU/mL, transferred to a flat-bottomed sterile polystyrene microplate, and incubated for 48 h at 37 °C under anaerobic conditions to allow for biofilm formation. After incubation, the resulting biofilm was washed three times with phosphate-buffered saline (PBS) (2 mM NaH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4), stained with 200 μL of 0.1% (wt/vol) crystal violet solution for 15 min at room temperature, washed twice with PBS, and destained with 100 μL of 96% ethanol at room temperature for 10 min to fix the cells. Afterward, the wells were rinsed once with PBS and air-dried. 150 μL of 33% (vol/vol) acetic acid was then added to each well and the absorbance was determined using a microplate reader (Thermo Fisher Scientific, US) at 570 nm. Based on the OD of the treated biofilm and that of the negative control (ODc), the samples were classified as follows: strong ($4 \times ODc < OD$), moderate ($2 \times ODc < OD \leq 4 \times ODc$), weak ($ODc < OD \leq 2 \times ODc$), or non-producer of biofilm ($OD \leq ODc$).

2.7. Statistical analysis

Data from the experiments were expressed as mean values ± standard deviations (mean ± SD) and evaluated using the two-way analysis of variance (ANOVA) followed by Tukey's test. Values of $P < 0.05$ were considered statistically significant.