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# Dual-light photodynamic therapy administered daily provides a sustained antibacterial effect on biofilm and prevents Streptococcus mutans adaptation

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ABSTRACT

Antibacterial photodynamic therapy (aPDT) and antibacterial blue light (aBL) are emerging treatment methods auxiliary to mechanical debridement for periodontitis. APDT provided with near-infrared (NIR) light in conjunction with an indocyanine green (ICG) photosensitizer has shown efficacy in several dental in-office-treatment protocols. In this study, we tested *Streptococcus mutans* biofilm sensitivity to either aPDT, aBL or their combination dual-light aPDT (simultaneous aPDT and aBL) exposure. Biofilm was cultured by pipetting diluted *Streptococcus mutans* suspension with growth medium on the bottom of well plates. Either aPDT (810-nm) or aBL (405nm) or a dual-light aPDT (simultaneous 810nm aPDT and 405nm aBL) was applied with an ICG photosensitizer in cases of aPDT or dual-light while keeping the total light energy constant at 100J/cm$^2$. Single-dose light exposures were given after one-day or four-day biofilm incubations. Also, a model of daily treatment was provided by repeating the same light dose daily on four-day and fourteen-day biofilm incubations. Finally, the antibacterial action of the dual-light aPDT with different energy ratios of 810 nm and 405 nm of light were examined on the single-day and four-day biofilm protocols. At the end of each experiment, the bacterial viability was assessed by the colony-forming unit method. Separate samples were prepared for confocal 3D biofilm imaging. On a one-day biofilm, the dual-light aPDT was significantly more efficient than aBL or aPDT, although all modalities were bactericidal. On a four-day biofilm, a single exposure of aPDT or dual-light aPDT was more efficient than aBL, resulting in a four logarithmic scale reduction in bacterial counts. Surprisingly, when the same amount of aPDT was repeated daily on a four-day or a fourteen-day biofilm, bacterial viability improved significantly. A similar improvement in bacterial viability was observed after repetitive aBL application. This viability improvement was eliminated when dual-light aPDT was applied. By changing the relative amount of light irradiance of 810nm and 405nm in dual-light aPDT, the increase in aBL improved the antibacterial action when the biofilm was older. In conclusion, when aPDT is administered repeatedly to *S. mutans* biofilm, a single wavelength-based aBL or aPDT leads to a significant biofilm adaptation and increased *S. mutans* viability. The combined use of aBL light in synchrony with aPDT arrests the adaptation and provides significantly improved and sustained antibacterial efficacy.

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FORK NOTE

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SAFETY WARNINGS

The safety issue concerning S. mutans can be found at ATCC: Streptococcus mutans (ATCC® 25175™)

BEFORE STARTING

Make sure you have taken every possible precaution regarding sample contamination.

## Grow the planctonic bacteria and provide a standard bacterial concentration in suspension

1 *Grow Streptococcus mutans* (ATCC 25175) bacteria for 18 h in an incubator (NuAire DH autoflow 5500, NuAire inc, US), at +36 degrees C, 5% $CO_2$in BHI broth (Bio-Rad 3564014, Bio-Rad Laboratories, Inc, US).

1.1 Dilute the resulting bacterial suspension with a 0.9% NaCl solution until an optical density (OD) of 0.46 is reached. The optical density can be measured by a spectrophotometer (Varian Cary 100 Bio UV-VIS, Agilent Technologies, Inc, US), and reassured with a Den 1 McFarland Densitometer (Biosan, Riga, Latvia).

## Grow the biofilm from the suspension in 96 well plates

2 Grow biofilms in flat-bottom 96-well plates (Thermo Fisher Scientific Inc, US) by placing 100 µl of 0.46 OD *S. mutans* suspension in each well, with 100 µl of BHI-broth growth medium. The well plates must be then incubated in a growth chamber (36°C, 5% $CO^2$). The BHI-broth medium is changed daily to supply fresh growth medium and to wash away the debris. The change of the medium in each well is performed by removing 100 µl of the medium by carefully pipetting and replacing it with a similar amount of fresh BHI broth.

3

## Light exposure/ PDT treatment

4 Before the light exposure takes place, the growth medium must be removed by pipetting and subsequently replaced with an equal amount of indocyanine green solution (Verdye, Diagnostic Green, GmBH), tittered to a concentration of 250 µg/ml. The indocyanine green is left to incubate at room temperature and in the dark for 10 minutes. After this incubation period, the biofilm is washed with a 0.9% NaCl solution, by pipetting the ICG solution away and replacing it with NaCl 0.9%, and repeating the procedure for 3 times. Then, the 0.9% solution of NaCl is added to each well to reach a total volume of 200 µl.

5 Light exposure can be performed by using appropriate light sources. We performed this by using specific, custom-made LED light sources (Lumichip Oy, Espoo, Finland). The exposure time was calculated from the determined light amount and known irradiances, which had been previously measured with a light energy meter (Thorlabs PM 100D with S121C sensor head, Thorlabs Inc, US) and a spectroradiometer (BTS256, Gigahertz-Optik GmbH, Germany), respectively.



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6 After the light exposure, the NaCl 0.9% solution is removed and replaces by BHI broth of the same amount. The plates are then placed in the incubator, or, if the light exposure is final, the biofilm must be removed for CFU counting, as described later. Excitation lights were applied with two single-wave LED light sources with peak intensities at 810 nm or at 405 nm, and with a dual-wave LED light chip simultaneously producing two separate peak intensities at 405 nm and at 810 nm.

7 Antibacterial photodynamic therapy light exposure must be administered at an 810-nm peak wavelength LED array on top of the well plate. The resulting light irradiance in this experiment was 100mW/cm$^2$, and the provided light energy was accordingly 100J/cm$^2$. Antibacterial blue light is administered at a 405-nm peak wavelength LED array, with a resulting irradiance of 80mW/cm2, and resulting light energy of 100J/cm$^2$. The dual light is administered with two light peaks identically placed and providing LED arrays on top of the well plate, producing a synchronous irradiance of 50mW/cm$^2$for the 405-nm light, and 50mW/cm$^2$ for the 810-nm light. The light energies produced will be 50J/cm$^2$ (405 nm) and 50J/cm$^2$ (810 nm), respectively.

8 To rule out the sample heating and subsequent effect on bacterial viability, temperature controls are measured (Omega HH41 Digital Thermometer, Omega Engineering, US) in the biofilm wells to confirm temperature levels below 35 degrees during the treatment, with a 100mW/cm$^2$ radiant flux

9 To test the antibacterial efficacy of dual-light treatment in terms of the relative amounts of 810-nm and 405-nm light irradiance, different light combinations can be employed, with simultaneous use of the single-peak-emitting light sources. Firstly, a 1:1 irradiance ratio of aBL to aPDT applies to 70 mW/cm$^2$ irradiance for the 405-nm light and 70 mW/cm$^2$ for the 810-nm light, the light energy emitted being at 50J/cm$^2$ and at 50J/cm$^2$, respectively. Secondly, a 3:1 irradiance ratio of aBL to aPDT applies to 130 mW/cm$^2$ irradiance for the 405nm light and 40mW/cm$^2$ for the 810nm light, the light energy provided being at 75J/cm$^2$ and at 25J/cm$^2$, respectively. Thirdly, a 1:3 irradiance ratio of aBL to aPDT applies to 40mW/cm$^2$ irradiance for the 405-nm light and 130mW/cm$^2$ for the 810nm light, the light energy applied being at 25J/cm$^2$and at 75J/cm$^2$, respectively.

## CFU counting

10 After the final light exposure, the entire biofilm from each well is collected and placed into a 1-ml test tube, forming 200 µl of suspension. After meticulous vortexing (Vortex-Genie, Scientific Industries Inc, US), a serial dilution assay ranging from 1:1 to 1:100 000 is performed, using sterile ART filter tips (Thermo Scientific, Waltham, US).

11 To enumerate the viable cells in each well, 100 µl of resulting biofilm dilutions are then evenly spread over an entire BHI agar plate, using a sterile L-shape rod.

12 As a tip: performing all the serial dilution assays is very time-consuming and after you have gained experience, the estimation of the biofilm mass and the given treatments can help to waive the unnecessary dilutions. As an example, treated biofilms can be most usually serially diluted from 1:1 to 1:10$^4$, and controls usually from10$^5$to 10$^6$, with single plating from each dilution. Typically, a dilution where CFU count on the plate was between 30 to 800, was considered the most reliable and selected for analysis.

13 The plates are then assembled into the incubator, the bacteria were grown for 48 h at the 5% carbon dioxide environment described above.

14 After the incubation, the plates are photographed (Canon D5 DSLR camera with Canon EF 24-70 mm f/4L lens, Canon, Japan) on a light table (Artgraph Light Pad Revolution 80, Artograph Inc, US). The entire surface of each plate is included in the image.

15 Use Image J software (National Institute of Health, US) to count the CFUs from the photograph.



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## Confocal scanning laser microscope imaging

16 The structural organization of the biofilm can be examined with confocal fluorescence imaging with a Leica TCS CARS SP 8X microscope (Leica Microsystems, Wetzlar, Germany), using HC PL APO CS2 20X/0.75 numerical-aperture multi immersion and HX PL APO CS2 63X/1.2 numerical-aperture water immersion objectives.

17 We used live/dead BacLight bacterial viability kit (Molecular Probes. Invitrogen, Eugene, Oregon, USA to stain the bacteria/biofilms. The stains are prepared according to manufacturer directions and must to incubate at room temperature and in the dark for 15 min prior to examination under the confocal scanning laser microscope (CSLM).

18 Light excitation is performed with a two-laser system, a 488-nm Argon laser, and a 561-nm DPSS laser, the emission windows configured to exclude the excitation wavelength of the two lasers and to meet the emission wavelength of the live/dead fluorescence marker. The emission window for the 488-nm laser must be set at 500 nm - 530 nm, and for the 561-nm laser, at 620 nm - 640 nm.

## Absorption spectroscopy assessment of ICG within a Streptococcus mutans pellet

19 Add one ml of *S. mutans* suspension with 0.46 OD, corresponding approximately to $100x10^6$ CFUs to an Eppendorf tube, centrifuge for 5 minutes at 8000 rpm (Heraeus Megafuge 1.0, Thermo Scientific, Waltham, US) to form a 10-µl pellet to the bottom of a 2-ml Eppendorf tube.

20 Remove the supernatant and replace by a 1-mg/ml ICG solution to establish a total volume of 1 ml. Then mix the pellet into the solution by vortexing for 60 seconds and left to incubate for 10 minutes. Then wash twice by centrifuging the bacteria into a pellet and by replacing and vortexing the supernatant into a 0.9% NaCl solution. Re-centrifuge at 8000 rpm for 20 minutes.

21 Vortex the *S. mutans*-formed pellet into a fresh 0.9% NaCl solution to form 0.46 OD for an absorption spectroscopy analysis with a Gary 100 Bio UV-visible spectrophotometer (Varian Inc., Palo Alto, CA). For comparison purposes, an ICG 4-µg/ml NaCl 0.9% solution is used. The *S. mutans* 0.46 OD 0.9% NaCl solution suspension is used as a reference sample for the ICG/*S. mutans* suspension and 0.9% NaCl for the ICG 4 µg/ml NaCl 0.9% solution.

22 For antibacterial effectivity assessment, the 200ul of washed and resuspended ICG incubated bacteria solution can be divided into 5 wells of Nunclon Delta well plates (Thermo Fisher Scientific Inc, US) followed by excitation with 810nm NIR LED light. Light intensity was 100mW/cm^2 and the total delivered light dose was 100J/cm^2. Control samples are prepared identically to treated samples excluding the ICG incubation step.



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