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Enhancement of erythrosine-mediated photodynamic therapy of *Streptococcus mutans* biofilms by light fractionation

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Objectives: We aimed to increase the bacterial cell killing efficacy of erythrosine-mediated photodynamic therapy (PDT) of *Streptococcus mutans* biofilms by fractionating the delivered light dose into a series of shorter pulses.

Methods: *S. mutans* biofilms of 200 μm thickness were grown in a constant-depth film fermenter (CDFF). Biofilms were incubated with 22 μM erythrosine before being irradiated with white light for increasing periods of time. We also used light dose fractionation to deliver the same overall dose of light in a series of shorter pulses separated by dark periods. Bacterial cell killing as a result of each killing protocol was quantified by colony counting.

Results: A 2 $\log_{10}$ of bacterial cell killing was achieved with 5 min of continuous white light irradiation. For time periods longer than 5 min the amount of killing increased more slowly, which was probably due to photobleaching of the erythrosine. Fractionation of the light dose into $5\times$ 1 min doses separated by dark recovery periods of 5 min increased the amount of bacterial killing by 1 $\log_{10}$ compared with 5 min continuous irradiation. Further fractionation of the light dose into $10\times$ 30 s doses separated by 2 min recovery periods resulted in 3.7 $\log_{10}$ of cell kill, an improvement of 1.7 $\log_{10}$ compared with the continuous irradiation protocol.

Conclusions: Erythrosine-mediated PDT of *S. mutans* biofilms can be further enhanced by fractionation of the applied light dose.

Keywords: plaque, photosensitizers, PDT

Introduction

Antibacterial agents are widely used in the treatment of oral diseases, but problems of development of bacterial resistance[1] mean alternative strategies are required to control bacterial plaque biofilms and treat caries, gingivitis and periodontal disease.

Photodynamic therapy (PDT) is a promising antibacterial treatment.[2] Upon irradiation with light corresponding to an absorption maximum of the photosensitizer, cytotoxic reactive oxygen species are produced that can cause rapid oxidation of cellular constituents and cell death.[3]

Since they are localized infections plaque-related diseases would be well suited to PDT. In addition, local administration of both photosensitizer and light is relatively straightforward in the oral cavity.[4] Antibacterial photosensitizers currently under investigation for use in the mouth include toluidine blue O (TBO)[5] and chlorin *e*6.[6] These agents show great promise but will, of necessity, be subject to lengthy clinical and regulatory

assessment. However, more immediate benefit could be derived from photosensitizers already approved for oral use. One such photosensitizer is erythrosine, currently used clinically as a plaque-disclosing agent. We recently reported the successful use of erythrosine-mediated PDT in the treatment of *Streptococcus mutans* biofilms.[7]

The aim of the present study was to optimize bacterial killing and reduce light irradiation times by fractionating the applied light dose, a technique that has been shown to enhance PDT in cancer treatment.[8,9]

Materials and methods

Bacterial culture

S. mutans (NCTC 10449) was maintained by weekly subculture on Columbia agar (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) horse blood.

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S. mutans biofilms were grown in a constant-depth film fermenter (CDFF) as described previously.[7] Briefly, the CDFF was inoculated with overnight cultures of *S. mutans* in brain heart infusion broth (BHI) (Oxoid). After inoculation, the CDFF was fed with BHI at a rate of 0.7 L per day. Waste was withdrawn from the CDFF by another peristaltic pump. Each of the five plugs in the CDFF pans were recessed by 200 µm and biofilm thickness was maintained at 200 µm by means of a scraper bar. For the treatment of biofilms, pans were aseptically removed through the sampling port in the top plate of the CDFF.

Photodynamic therapy of biofilms

Biofilm-containing pans were removed and incubated with 22 µM erythrosine in ringers saline (RS; Lab M, Bury, UK) for 15 min, in the dark, at room temperature. The biofilm pans were then placed into fresh RS and irradiated using the previously described light system.[7] Average light intensity was 22.7 mW/cm^2 in the region of maximum absorption by erythrosine (500–550 nm). This represented a total light dose of 6.75 J/cm^2 after 5 min of irradiation. Continuous irradiation was carried out for 0, 1, 2, 5, 10, 15 or 30 min. For light fractionation, biofilms were pulsed under the light system for 5× 1 min periods or for 10× 30 s periods. Between light pulses the samples were left in darkness for a 5 min (for 1 min pulses) or 2 min (for 30 s pulses) recovery period at room temperature. Two pans were used per time point, and following irradiation three biofilm-containing plugs (each of 5 mm diameter) were removed from each pan. The biofilms were disrupted from the plugs by vortexing with sterile glass beads (3 mm diameter) for 1 min and were serially diluted in RS. Surviving cells were counted by serial dilution and plating in triplicate on Columbia agar supplemented with 5% (v/v) horse blood. Plates were incubated aerobically in 5% CO$_2$ at 37°C for 48 h. In each case, n = 3–6 and the entire experiment was repeated twice. Statistical analysis was performed using two-sample *t*-tests.

Results

S. mutans biofilms typically contained 10^{10}–10^{11} cfu per plug before treatment. Figure 1 shows that the killing of *S. mutans* biofilm cells with erythrosine-mediated PDT and continuous irradiation occurred in a light dose-dependent manner up to 5 min of irradiation.

As ~98% of the cell killing occurred in the first 5 min of irradiation, we decided to fractionate a 5 min irradiation period. This also prevented significant photobleaching, which we have shown occurs at irradiation times of longer than 15 min.[7]

The results for the light fractionation regimes compared with continuous irradiation are shown in Figure 2. Continuous irradiation for 5 min resulted in 2 ± 0.2 log$_{10}$ cell kill. The dose–response curve was biphasic, plateauing after 3 min of irradiation. Five times 1 min light pulses of the erythrosine-treated biofilms, with 5 min recovery periods between pulses, resulted in a 3.0 ± 0.3 log$_{10}$ kill of biofilm bacteria. Ten times 30 s light pulses, with 2 min recovery periods, again improved cell killing to 3.7 ± 0.3 log$_{10}$ (although this was not significantly different to the cell killing seen with 5× 1 min pulses). Both of the light fractionation regimes caused statistically significantly more cell killing (P < 0.005) when compared with the 5 min continuous irradiation regime.

The two light fractionation regimes were also superior to longer continuous irradiation times of up to 30 min. The
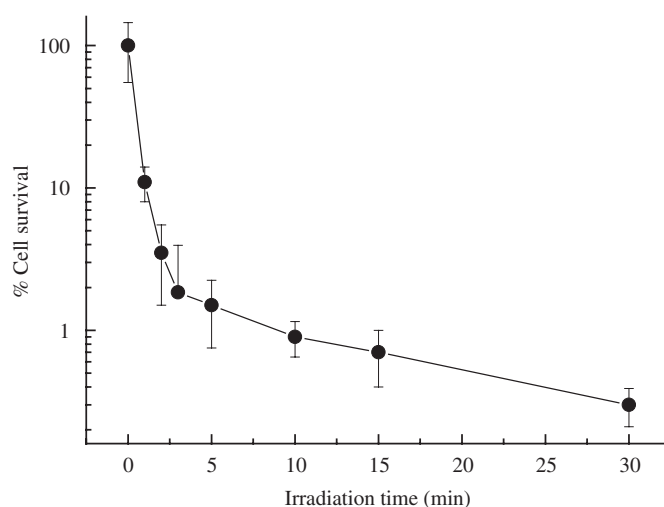

Figure 1. Effect of continuous irradiation time on biofilm cell survival. Seven day biofilms were incubated with 22 µM erythrosine for 15 min and then irradiated with white light for 0–30 min. Error bars indicate the SD of 6–12 determinations.
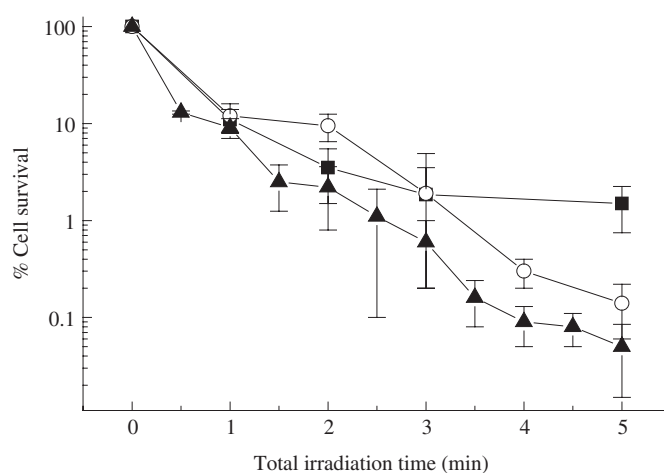

Figure 2. A comparison of continuous irradiation and light fractionation in the erythrosine-mediated PDT of *S. mutans* biofilm cells. Biofilms were incubated with 22 µM erythrosine for 15 min and then: irradiated with 0, 1, 2, 3 or 5 min of continuous white light (filled squares); subjected to 5 × 1 min white light pulses separated by dark recovery periods of 5 min (open circles); or subjected to 10× 30 s white light pulses separated by dark recovery periods of 2 min (filled triangles). Error bars indicate the SD of 6–12 determinations.

10 × 30 s light pulse regime (3.7 ± 0.3 log$_{10}$ kill) gave almost a 10-fold increase in cell killing compared with 30 min continuous irradiation (2.8 ± 0.1 log$_{10}$). This increase was statistically significant (P < 0.005).

Discussion

The present study has shown that the fractionation of white light during the erythrosine-mediated PDT of *S. mutans* biofilms grown *in vitro* results in increased cell killing compared with

continuous irradiation. This may be due to the replenishment, during dark periods, of target molecules (such as oxygen) for the excited photosensitizer. The general replenishment or redistribution of the photosensitizer itself during dark periods, in both aerobic and anaerobic cases, is also a possibility. The photodynamic process also leads to diminished erythrosine levels due to photobleaching,[7] so any photosensitizer concentration gradient might be equilibrated during dark periods.

Overall, this work further highlights the clinical potential of erythrosine-mediated PDT. In conjunction with light dose fractionation, irradiation times can be reduced to clinically acceptable levels. Further work is now being undertaken to determine the efficacy of erythrosine-PDT on natural oral biofilms formed *in vivo*.

Acknowledgements

Transparency declarations

None to declare.

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