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Susceptibility of *Streptococcus mutans* biofilms to photodynamic therapy: an *in vitro* study

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***Objectives*: The purpose of this study was to evaluate the antimicrobial effect of toluidine blue O (TBO), in combination with either a helium/neon (HeNe) laser or a light-emitting diode (LED), on the viability and architecture of *Streptococcus mutans* biofilms.**

***Methods*: Biofilms were grown on hydroxyapatite discs in a constant depth film fermentor fed with artificial saliva that was supplemented with 2% sucrose four times a day, thus producing a typical 'Stephan pH curve'. Photodynamic therapy was subsequently carried out on biofilms of various ages with light from either the HeNe laser or LED using energy densities of between 49 and 294 J/cm^2.**

***Results*: Significant decreases in the viability of *S. mutans* biofilms were only observed when biofilms were exposed to both TBO and light, when reductions in viability of up to 99.99% were observed with both light sources. Overall, the results showed that the bactericidal effect was light dose-dependent and that older biofilms were less susceptible to photodynamic therapy. Confocal laser scanning microscopy images suggested that lethal photosensitization occurred predominantly in the outermost layers of the biofilms.**

***Conclusions*: Photodynamic therapy may be a useful approach in the treatment of dental plaque-related diseases.**

Keywords: dental plaque, caries, Stephan curve, light-emitting diode, biofilm structure

Introduction

Dental plaque is the term commonly used for the biofilm that is formed on the tooth surface and consists of a complex microbial community embedded in a matrix of polymers of bacterial and salivary origin.[1] The formation of acid end-products through the metabolism of carbohydrates by acidogenic microorganisms within these biofilms is an important factor in the development of dental caries.[2] The essential process involves demineralization of the tooth structure by high concentrations of organic acids.[3] *Streptococcus mutans* has been implicated as the primary aetiological agent because of its relatively high numbers in plaque prior to the appearance of carious lesions, its ability to degrade carbohydrates rapidly with the formation of abundant acid and its ability to induce a tolerance to low pH environments.[4]

When a community of microorganisms become irreversibly attached to a surface as biofilms the organisms exhibit distinctive phenotypic properties and tend to be far more resistant to antimicrobial agents.[5] Additionally, in view of the growing problem of bacterial resistance to conventional antimicrobials, the use of an alternative approach to which bacteria are unable to gain resistance would be valuable.[6] The current treatment for plaque-related diseases involves the use of traditional antimicrobials in conjunction with the mechanical removal of the biofilm. In the case of caries, a more attractive proposition would be to kill the causative organisms *in situ*.[7]

Photodynamic therapy (PDT) may emerge as a suitable process to combat both biofilm and antimicrobial-related resistance. Using this technique, a photosensitizer, such as haematoporphyrin, phthalocyanine or toluidine blue O (TBO), is activated by irradiation

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with light of a specific wavelength (the maximum absorption of the sensitizer) resulting in the generation of cytotoxic species, including singlet oxygen and free radicals, which are able to exert a bactericidal effect[8] but which are not toxic to host cells.[9,10] Previous studies have shown that PDT is capable of killing oral bacteria in planktonic cultures[10,11] and plaque scrapings,[12] as well as biofilms.[13,14] The purpose of this investigation was to evaluate the antimicrobial effect of PDT, using two different light sources, on the viability and architecture of *S. mutans* biofilms.

Materials and methods

Photosensitizer and light sources

TBO (Sigma, Poole, UK) was dissolved in dH_2O to obtain a final concentration of 100 mg/L and was subsequently kept in the dark. The light sources used were a helium/neon (HeNe) gas laser (Spectra Physics, Mountain View, CA, USA), which produces light with a wavelength of 632.8 nm, and a light-emitting diode (LED; Laserbeam, Rio de Janeiro, Brazil), with a spectrum of emission ranging from 620 to 660 nm and a 638.8 nm predominant wavelength.

Inoculum and media

The microorganism used in this study was *S. mutans* NCTC 10449. To prepare the inoculum, *S. mutans* was first grown anaerobically on brain–heart infusion (BHI; Oxoid, Basingstoke, UK) agar plates for 3 days. Subsequently, single colonies were inoculated into 10 mL of BHI broth and incubated anaerobically at 37°C overnight. The nutrient source in all experiments was mucin-containing artificial saliva, the composition of which has been described previously.[15]

Production of biofilms

A constant depth film fermentor (CDFF; University of Wales, Cardiff, UK) *in vitro* model was used for the production of biofilms.[16] The CDFF consists of a rotating turntable that holds 15 polytetrafluoroethylene (PTFE) pans, which rotates beneath two PTFE scraper blades, spreading the incoming media over the pans and maintaining the biofilms at a constant depth. Each pan contains five cylindrical holes (5.0 mm in diameter) containing PTFE plugs. Hydroxyapatite (HA) discs of the same diameter were placed on top of the PTFE plugs and recessed to a depth of 300 μm. Artificial saliva (100 mL) was pumped into the CDFF for 3.5 h to simulate the formation of a salivary pellicle. Subsequently, 10 mL of an overnight culture of *S. mutans* was added to 750 mL of artificial saliva, mixed and pumped into the CDFF for 24 h. After this period, the inoculum flask was disconnected and the CDFF fed from a medium reservoir of sterile artificial saliva.[17] The artificial saliva was delivered by a peristaltic pump (Watson-Marlow, Falmouth, UK) at a rate of 0.5 mL/min, similar to the unstimulated salivary flow rate in healthy individuals.[15] Additionally, an aqueous solution of 2% (w/v) sucrose was also pumped over the biofilms for periods of 30 min at the same speed via a second peristaltic pump.[18] The sucrose pulsing was carried out four times a day and during this period the artificial saliva supply was maintained. On days 3, 7 and 10 intact biofilms were removed aseptically for testing.

pH measurements

On days 7 and 9 the pH of the biofilm effluent was determined using a pH meter (pH-boy; Camlab, Cambridge, UK). The instrument was recalibrated before each sample and its accuracy was ±0.1 pH units. The pH measurements were taken at 15, 30, 45 min, 4.5 h and 5.5 h after sucrose pulsing.[18]

Photodynamic therapy

HA discs containing the biofilms were removed from the CDFF on days 3, 7 and 10, and 25 μL of TBO (100 mg/L) was placed onto each biofilm and subsequently left in the dark for 5 min (pre-irradiation time). Following this time, the biofilms were exposed for 5, 15 or 30 min to HeNe laser or LED light. The power output of both light sources was 32 mW. The energy density for the different irradiation times was 49, 147 and 294 J/cm^2, respectively. The biofilms were then placed into 1 mL of phosphate-buffered saline (Oxoid) and vortexed for 60 s in order to disperse the biofilms. Ten-fold serial dilutions were carried out and aliquots plated onto BHI agar, which were then incubated anaerobically at 37°C for 3 days before the number of viable organisms were enumerated. In order to determine the effect of the light alone on bacterial viability, biofilms were processed in the same way excluding treatment with TBO (S^-L^+). Additional controls consisted of biofilms treated with TBO, but not exposed to light sources (S^+L^-) and biofilms that were not sensitized with TBO or exposed to light (S^-L^-).

Confocal laser scanning microscopy

The HA discs were placed into a Petri dish (5 cm in diameter), biofilms upwards, and 10 mL of saline solution containing 2 μL of live/dead stain (Molecular Probes, Eugene, OR, USA) carefully added without disturbing the samples.[19] After incubation in the dark for 15 min, the biofilms were examined with a Radiance 3000 confocal laser-scan head at wavelengths of 488 and 543 nm (Bio-Rad GmbH, Jena, Germany) in conjunction with a BX51 stereomicroscope (Olympus UK Ltd, Southall, UK) equipped with a 40× HCX water immersion dipping lens. The laser power settings used for the scan was 2–9% for 488 nm and 10–25% for 543 nm. The resulting collections of confocal optical sections were collected by Bio-Rad Lasersharp software as stacks of images. The images were subsequently analysed using ImageJ (National Institutes of Health, Bethesda, MD, USA) to produce *xy* projections (the sum total of pixel brightness in the *z*-axis) and partial sagittal projections (~6 μm thick projections at a point along the *x*-axis).

Statistical analysis

The dependent variables were sensitizer and light source (LED or HeNe). First, the data were evaluated to check the equality of variances and normal distribution of errors. To determine the significance of the irradiation alone, the presence of sensitizer alone and the combination of sensitizer and light, the data were analysed by a variance analysis (ANOVA) model using the factorial (2×2) design. The Tukey test was chosen for evaluating the significance of all pairwise comparisons with a significance limit of 5%.

Results

Figure 1 illustrates the pH curve measured from the biofilm effluent under sucrose supplementation conditions. At both days there was a drop in the pH of the effluent from near neutral to around pH 4.2 after 75 min post-pulsing. After this time the pH recovered to the same values as those seen prior to pulsing. The number of microorganisms increased according to the age of biofilms reaching 2.49×10^8 cfu in 3 days, 5.07×10^8 cfu in 7 days and 1.44×10^9 after 10 days of growth. Significant differences between cfu from biofilms of different ages were observed only after 10 days of growth ($P = 0.01$).

Controls were carried out for all exposure times and all ages of biofilm. Neither irradiation of the biofilms in the absence of TBO (S^-L^+) nor incubation with TBO alone (S^+L^-) had a significant effect on the viability of *S. mutans* biofilms at any stage. Indeed,

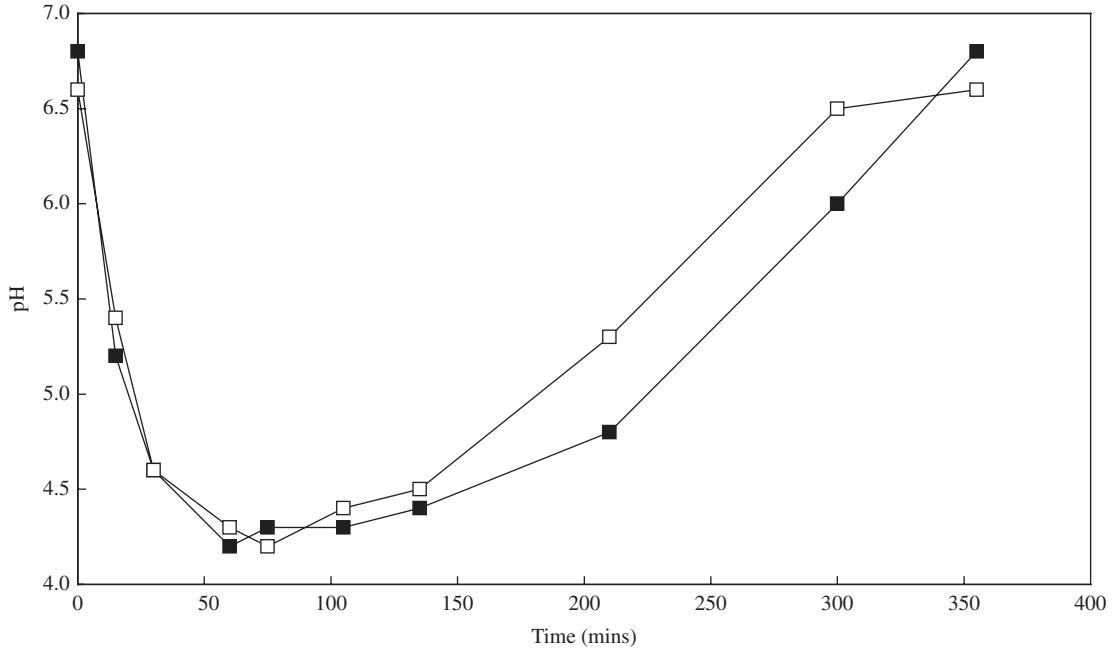

Figure 1. pH of biofilm effluent after the addition of sucrose to the system (time = 0). Filled squares indicate samples taken after 7 days of biofilm growth and open squares after 9 days of biofilm growth (n = 2).

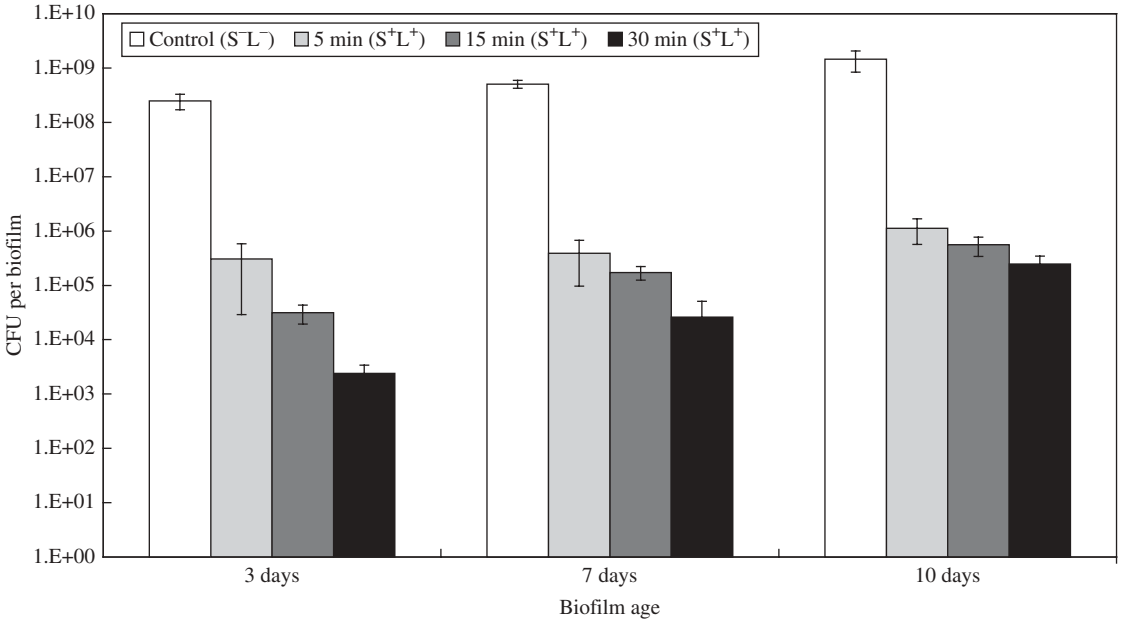

Figure 2. Effect of negative control (S^-L^-) and the association of TBO and HeNe laser (S^+L^+) at different biofilm ages. Data represent mean values (n = 4) and error bars represent standard deviations.

the 95% confidence intervals for all the controls at the three time points were 2.49×10^8 to 4.62×10^8 (3 days), 5.07×10^8 to 5.07×10^8 (7 days) and 1.06×10^9 to 2.11×10^9 (10 days).

Significant decreases in the viability of *S. mutans* biofilms were only observed when biofilms were exposed to both TBO and light. There was a significant relationship between the dye, light source and irradiation time ($P < 0.001$). The antimicrobial effect of photodynamic therapy using different energy doses of HeNe and LED laser light on the viability of *S. mutans* biofilms after 3, 7 and 10 days growth is shown in Figures 2 and 3. The biofilms were sensitized with 100 mg/L TBO and irradiated either with a HeNe laser or an LED light with an energy density of 49 J/cm^2 (5 min), 147 J/cm^2 (15 min) or 294 J/cm^2 (30 min). When 3 day biofilms were submitted to photodynamic therapy there was a considerable reduction in the median viable counts from 2.45×10^8 (control) to 3.06×10^5, 3.13×10^4 and 2.41×10^3 after 5, 15 and 30 min of irradiation with an LED light and 2.29×10^5, 1.73×10^5, 1.06×10^5 with HeNe laser light, respectively. These values correspond to

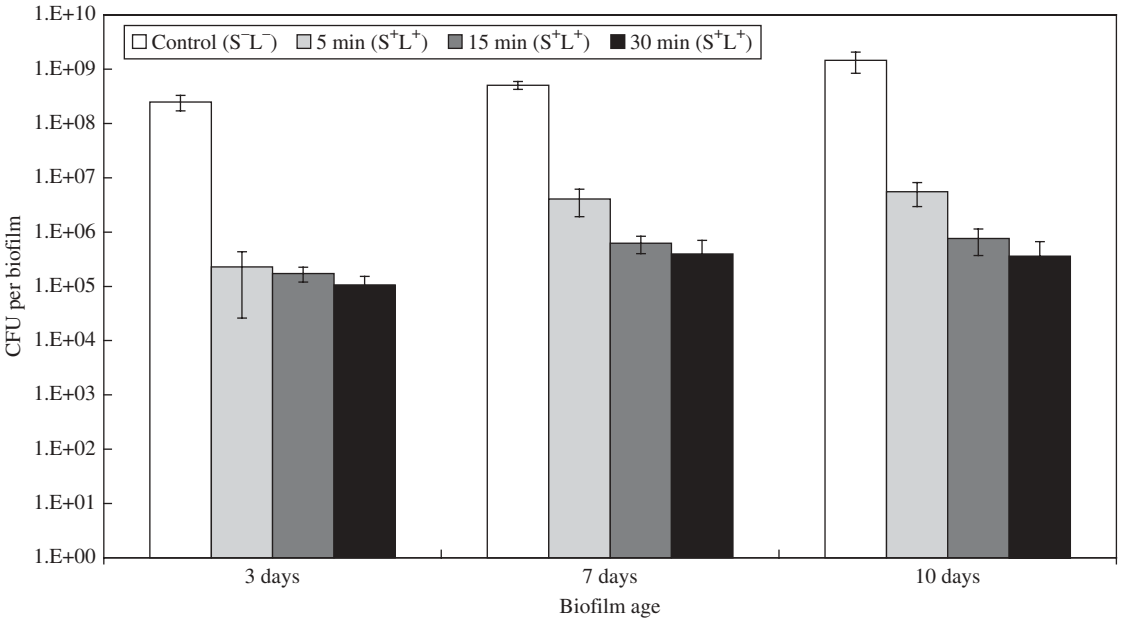

Figure 3. Effect of negative control (S^-L^-) and the association of TBO and LED light (S^+L^+) at different ages. Data represent mean values ($n = 4$) and error bars represent standard deviations.

percentage reductions ranging from 99.87% to 99.99%. Similar results were obtained with 7-day-old biofilms with bacterial counting reduced from 5.07×10^8 (control group) to 3.88×10^5, 1.73×10^5 and 2.59×10^4 after 5, 15 and 30 min, respectively, of irradiation with an LED light and 4.04×10^6, 6.19×10^5 and 3.94×10^5 with HeNe laser light. These values correspond to percentage reductions ranging from 99.20% to 99.99%. Again, after 10 days growth percentage reductions ranging from 99.61% to 99.98% were observed. Overall, the results showed that bactericidal effect was light dose-dependent and that older biofilms were less susceptible to photodynamic therapy.

Comparing the two light sources used, the association of TBO and LED was more effective than TBO and HeNe treatment when 3-day-old biofilms were exposed to 147 J/cm^2 ($P = 0.0103$) or 294 J/cm^2 ($P < 0.001$) energy densities. Also, TBO and LED killing was higher when 7-day-old biofilms were exposed to an energy density of 49 J/cm^2 ($P < 0.001$) or 294 J/cm^2 ($P < 0.001$). There was no significant difference between HeNe laser and LED light when photodynamic therapy was used to kill 10-day-old biofilms using 15 or 30 min of irradiation.

In addition to viability studies, confocal microscopy was also carried out. Representative confocal laser scanning microscopy images of biofilms prior to and after photodynamic therapy are shown in Figure 4. The arrows indicate the position of the sagittal section (6 µm thick) and the total area of the images was 300 × 300 µm. Figure 4(a and b) refer to biofilms neither sensitized with TBO nor exposed to light with 3 and 10 days of growth, respectively. Dead stained areas (in blue) can be observed in older biofilms even when not submitted to photodynamic therapy, especially in deeper regions. Biofilms exposed to both 100 mg/L TBO and 49 J/cm^2 energy density can be observed in Figure 4(c) (HeNe laser) and Figure 4(e) (LED light). Although one cannot quantitatively compare the efficacy of the two treatments by confocal laser scanning microscopy images, dead stained areas can be observed, after irradiation with both light sources, at the external surface of biofilms. Biofilms submitted to photodynamic therapy using a 294 J/cm^2 energy density are illustrated in Figure 4(d) (HeNe laser) and Figure 4(f) (LED light). Large kill proportions can be observed after 30 min of irradiation, characterized by a shift from live (green) to dead (blue) stained cells.

Discussion

A number of studies have shown that oral bacteria are susceptible to photodynamic therapy when they are grown as planktonic cultures.[10,11,20] However, the causative agents of caries and other oral diseases are present as organized biofilms. We have used a biofilm model to grow simple single-species *S. mutans* under similar environmental conditions that we would expect *in vivo*. By modelling such a complex system as the oral cavity there are inevitable compromises between the reality of the *in vivo* ecosystem and the simplification and controllability necessary to gain meaningful, useful results.[1] It has been known that biofilm-grown cells differ from their planktonic counterparts in a number of respects including the presence of a extracellular polymeric substances (EPS), cell wall composition, growth rate, metabolic activity and gene expression.[21]

The results of this study show that photodynamic therapy was effective in significantly reducing the viability of *S. mutans* biofilms grown under conditions reflecting those found *in vivo*. Indeed, the addition of 2% sucrose into the CDFF resulted in an acidic system with a pH drop to 4.3 after sucrose pulsing before returning to pH 6.8 before the beginning of the next cycle, typical of the Stephan curve.[22] Similar results have been shown in previous studies using the CDFF to grow oral biofilms supplemented with sucrose.[17,23] Additionally, pH levels as low as 4.3 are similar to the pH of approximal plaque following a sucrose rinse *in vivo*.[24] The inner regions of plaque biofilms can become inaccessible to saliva exchanges and can remain at these low pH values for long

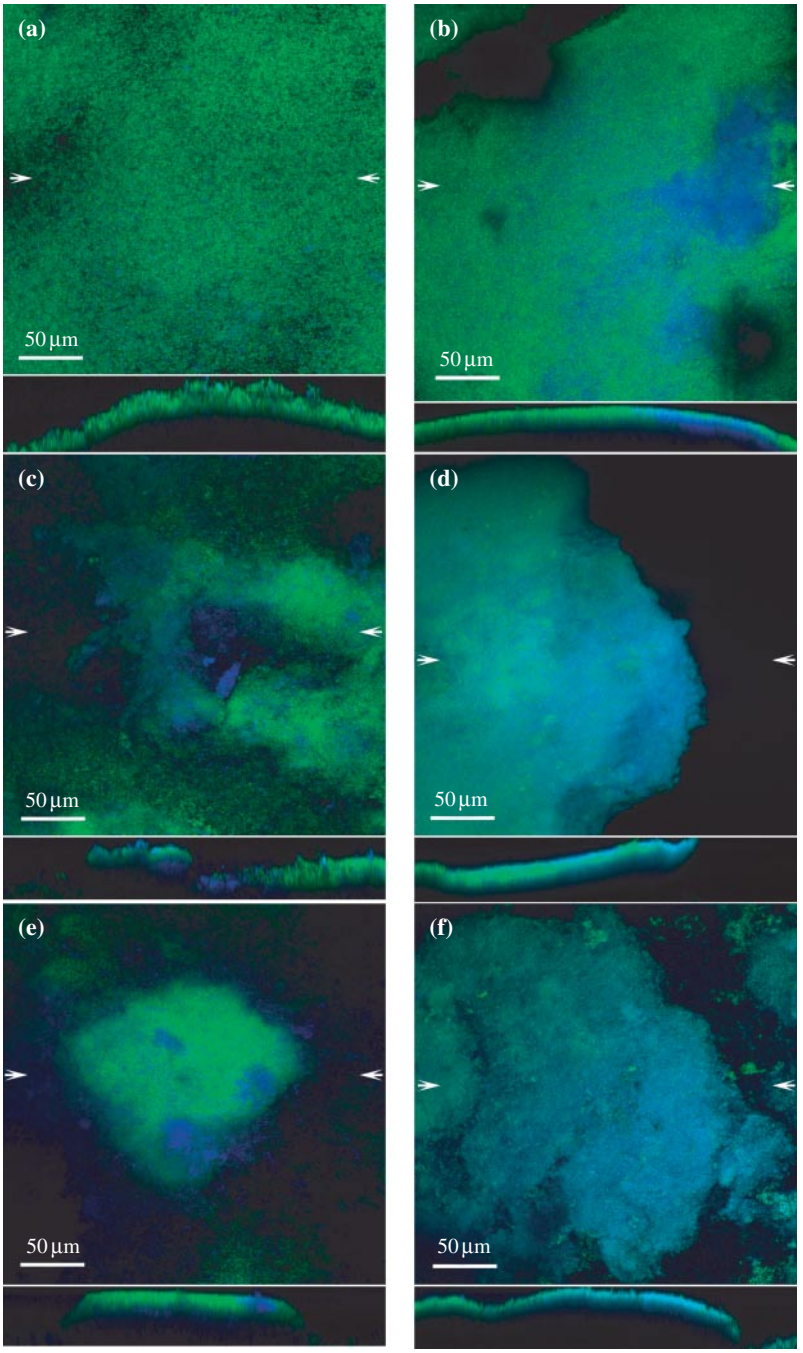

Figure 4. Three-dimensional confocal laser scanning microscopy reconstruction of control and PDT-treated biofilms based on a series of *xy* projections. The arrows indicate the position of the sagittal section (6 µm thick). Images (a) and (b) refer to S^-L^- treatment for HeNe and LED laser light. Images (c) (HeNe laser) and (e) (LED light) indicate S^+L^+ treatment with 49 J/cm^2 energy densities, and (d) and (f) for 294 J/cm^2 energy densities for HeNe and LED laser light, respectively.

periods, thereby allowing enamel demineralization to take place.[25] In the biofilm model used, the 300 µm thick biofilms would be similar to approximal areas, thus allowing a low pH to exist within the biofilm.[17]

In a study analysing bacteria in supragingival plaque scrapings, Wilson *et al.*[12] found that substantial kills could be achieved by laser light in the presence of an appropriate photosensitizer. It has also been shown previously that the viability of single-species *Streptococcus sanguis* biofilms can be reduced by PDT.[13] In a study involving multi-species biofilms, Wood *et al.*[14] reported that widespread killing occurred when oral biofilms formed *in situ* were treated with a cationic Zn(II) phthalocyanine photosensitizer and exposed for 30 min to a 400 W tungsten filament lamp (although this was determined by confocal and transmission electron microscopy and the extent of killing was not quantified). Recently, Soukos *et al.*[26] studied the association of PDT and the use of photomechanical waves (PW) on the viability of periodontal bacteria, and concluded that PW may be a potential tool for killing such bacteria when associated with PDT as it may improve the penetration of the sensitizer within biofilms.

The results of the present study have shown that a large number of bacteria present in *S. mutans* biofilms can be killed when treated with TBO and irradiated with either an HeNe laser or an LED. Interestingly, similar results were obtained for the two light sources. This represents an advantage when one considers that the best results described in the scientific literature have been obtained using conventional lasers to perform therapy. This would mean by using LED as a light source, the technology could be simplified and a lower cost of treatment in comparison to the complex laser systems.

Confocal laser scanning microscopy images of biofilms after exposure to HeNe laser or LED light in the presence of TBO suggest that lethal photosensitization occurred predominantly in the outer layers of the biofilms, leaving some of the innermost bacteria alive, which may be due to the inability of the photosensitizer to diffuse through into these inner regions.[27] Interestingly, Gad *et al*.[28] have demonstrated that lethal photosensitization can be affected by the presence of EPS. However, at the same concentration, they observed that absolute uptakes of photosensitizer by cells were 10-fold higher when a cationic photosensitizer (pL-$_{ce6}$) was used compared with their anionic counterparts (free-$_{ce6}$), which did not always correlate to higher kills, suggesting that other factors are involved in PDT action. This may be explained by the EPS 'trapping' the photosensitizer on the outside of the cell owing to ionic or hydrophobic interactions and therefore reducing the amount of photosensitizer that was able to penetrate to the plasma membrane, which is thought to be one of the important sites of PDT-mediated damage. Although the overall ionic charge of EPS has not been studied, the authors suggest that characteristics of EPS may play a significant role in the determining the binding and intracellular penetration of photosensitizers that vary in charge and hydrophobicity.

Owing to the emergence of antibiotic resistance, photodynamic therapy has become a viable alternative antibacterial therapy for biofilm-related diseases such as dental caries. The advantages of photodynamic therapy over conventional antimicrobial agents are first, rapid killing of target organism depending mainly on the light energy dose delivered and therefore the power output of the light source used. Hence, resistance development would be unlikely as killing is mediated by singlet oxygen and free radicals and high concentrations of photosensitizer do not need to be maintained in the disease site for more than a few minutes, in contrast with hours or even days necessary in the case of conventional antimicrobial agents. Finally, antimicrobial effects can be confined to the site of the lesion by careful topical application of photosensitizer and the area of irradiation can be restricted further by using an optical fibre.[7]

In conclusion, the results of this study showed that *S. mutans* biofilms were susceptible to either HeNe laser or LED light in the presence of TBO, suggesting that this approach may be useful in the treatment of dental plaque-related diseases.

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