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ORIGINAL ARTICLE

The impact of antimicrobial photodynamic therapy in an artificial biofilm model

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Abstract The susceptibility of bacterial cultures in biofilm formations is important for a variety of clinical treatment procedures. Therefore, the aim of the study was to assess the impact of laser-induced antimicrobial photodynamic therapy on the viability of *Streptococcus mutans* cells employing an artificial biofilm model. Using sterile chambered coverglasses, a salivary pellicle layer was formed in 40 chambers. *Streptococcus mutans* cells were inoculated in a sterile culture medium. Employing a live/dead bacterial viability kit, bacteria with intact cell membranes stained fluorescent green. Each pellicle-coated test chamber was filled with 0.7 ml of the bacterial suspension and analysed using a confocal laser scanning microscope within a layer of 10 μm at intervals of 1 μm from the pellicle layer. Phenothiazine chloride was used as a photosensitizer in all 40 test chambers. A diode laser (wavelength 660 nm, output power 100 mW) was used to irradiated 20 chambers for 2 min. Fluorescence values in the test chambers after laser irradiation (median 2.1 U, range 0.4–3.4 U) were significantly lower than baseline values after adding the photosensitizer (median 3.6 U, range 1.1–9.0; $p<0.05$). The non-irradiated control chambers showed no change in fluorescence at the end of an additional photosensitizer residence time of 2 min without laser irradiation (median 1.9 U, range 0.7–3.6; median 1.9 U, range 0.8–6.0, respectively; $p>0.05$). The present study indicated that laser irradiation is an essential part of antimicrobial photodynamic therapy to reduce bacteria within a layer of 10 μm. Further studies are needed to evaluate the maximum biofilm thickness that still allows a toxic effect on microorganisms.

Keywords Antimicrobial photodynamic therapy · *Streptococcus mutans* · Artificial biofilm · Pellicle formation · Confocal laser scanning microscopy

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Introduction

Antimicrobial photodynamic therapy (aPDT) is a minimally invasive antimicrobial treatment. The therapy utilizes a photosensitizing agent, which is irradiated with a light source tuned to a wavelength that matches the absorption spectrum of the agent [1]. The subsequent photochemical reaction results in oxygen-mediated destruction of pathogenic bacteria. The main cytotoxic agent in aPDT is widely accepted to be singlet oxygen [2], a highly reactive oxygen species that oxidizes biological substrates. Critical sites of action for singlet oxygen in aPDT include mitochondria, DNA and lipid membranes [3, 4]. Sustained exposure of the treated tissue results in breakdown of cellular microstructures and cell death. Several photosensitizers have been shown to be effective against target microorganisms without inducing damage to the host tissues [5].

An aPDT regimen can be beneficial for elimination of microorganisms during systematic therapies of bacterial infections [6, 7]. With respect to periodontal infections,

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many microbial species are present in an oral biofilm that forms on periodontal root surfaces. The removal of this biofilm [8] and mineralized deposits from the root surface are fundamental aspects of periodontal therapy [9]. However, bacterial reservoirs can remain on the tooth surface and affect periodontal healing following treatment. Adjunctive procedures such as locally delivered [10] and systemic antibiotics [11] have been evaluated. With respect to the possible development of bacterial resistance to antibiotics, the use of these agents should be restricted to specific groups of periodontal patients, for example those with highly active disease or a specific microbiological profile [12]. aPDT could be an adjunctive antimicrobial approach for preventive and therapeutic periodontal regimens [13] without the disadvantages of antibiotic agents. Development of bacterial resistance has not yet been shown and could not be generated in an experimental study protocol [14]. aPDT was shown to be able to eliminate the specific periodontopathogenic bacterium *Aggregatibacter actinomycetemcomitans* in a planktonic biofilm model [15], and to effectively eradicate planktonic and biofilm cultures of the opportunistic pathogen *Pseudomonas aeruginosa* [16]. Moreover, multispecies microbial biofilms and suspensions of microorganisms were developed from dental plaque samples and treated photodynamically. Bacteria in biofilms have been shown to be less affected by a photodynamic procedure than bacteria in the planktonic phase [17]. However, more scientific evidence concerning the impact of aPDT on microorganisms in dental plaque biofilms is needed before the clinical implementation of reliable photodynamic treatment procedures in dentistry.

Thus, the aim of the present study was to assess the impact of laser-induced aPDT employing a representative oral microorganism in order to generate an artificial oral biofilm model. The characteristic property of *Streptococcus mutans* is its pronounced relevance in the generation of carious lesions and its ability to produce extracellular polysaccharides [18] to form a structured oral biofilm. Therefore, the hypothesis that aPDT is able to eradicate bacterial cultures and the hypothesis that laser irradiation is necessary to eradicate bacteria during the aPDT procedure needed be tested.

Materials and methods

Sterile chambered coverglasses (Lab-Tek no. 1; Nunc, Naperville, IL) were used to enable analysis by confocal laser scanning microscopy. A salivary pellicle layer was formed in 40 chambers. Saliva from four periodontally healthy subjects was collected and sterilized by filtration (FP 30/0.45 CA-S; Schleicher and Schuell, Dassel, Germany). Each chamber was coated with 1 ml of the filtrate and placed in an incubator (Memmert, Schwabach, Germany) for 4 h at 37°C. Then, the surplus saliva was removed, leaving a pellicle layer on the surface the chamber. Cells of *S. mutans* (Clarke 1924; German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), representing one of the typical microorganisms in the oral cavity, were inoculated in a sterile culture medium (CM287; Oxoid, Basingstoke, UK) for 6 h. The respective extinction of the bacterial cultures was between 0.8 and 1.1 U representing the density of cells measured with a spectrophotometer at 600 nm (WPA CO 8000; Biochrom, Cambridge, UK).

For the analysis of the microorganisms in the chambers by confocal laser scanning microscopy, a specific staining procedure was employed. A live/dead bacterial viability kit (LIVE/DEAD BacLight L7012; Invitrogen, Carlsbad, CA) was used in which bacteria with intact cell membranes were stained fluorescent green. The viability kit used two nucleic acid stains: the green fluorescent SYTO 9 stain and the red fluorescent propidium iodide stain. When used alone, SYTO 9 stain labels both live and dead bacteria. In contrast, propidium iodide penetrates only bacteria with damaged membranes, thus reducing SYTO 9 fluorescence. Thus, live bacteria with intact membranes fluoresce green. In the present study, we did not evaluate any red fluorescence. Thus, the amount of green fluorescence was used to measure the relative number of living *S. mutans* cells. The bacterial culture was added to the pellicle-containing chambers (0.7 ml per chamber) to allow attachment of planktonic aggregate to the preconditioned glass surface which was expected to start minutes after adding the culture according to the findings of a similar study [19]. The biofilm was allowed to grow for 4 h before the photodynamic treatment procedure was performed, as a previous study had demonstrated that considerable growth and attachment with no initial lag phase could be expected between 1 and 3 h with only a slight increase in the mean total area covered by aggregate cells in a 24-h growth period [19].

Chambers were evaluated by confocal laser scanning microscopy (LSM 710; Carl Zeiss MicroImaging, Jena, Germany) within a layer of 10 μm at intervals of 1 μm from the pellicle layer upwards. After measuring the baseline fluorescence of each untreated specimen in a central area of 134.69×134.69 μm, phenothiazine chloride (Helbo, Wels, Austria) was added as the photosensitizer to all 40 chambers. The photosensitizer dye contains 3,7-bis (dimethylamino)phenothiazin-5-ium chloride (methylene blue) at a concentration of 1% buffered to pH 3.5 with a citrate buffer, isotonized and viscosity-modified with 1% hydroxypropyl methylcellulose. The photosensitizer is a near-infrared fluorescent agent with absorption peaks at 668 and 609 nm (Merck Index). For the laser scanning microscopy, excitation wavelengths of 488 nm and 514 nm were used. Thus, no major impact on the fluorescence detection of the

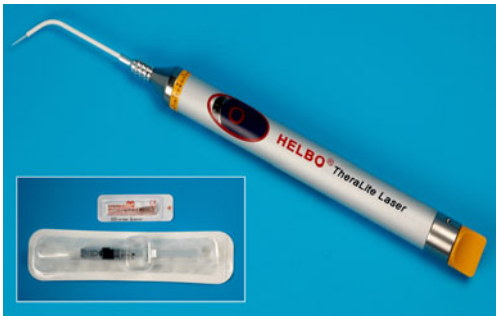

Fig. 1 Diode laser (660 nm, 100 mW; class 2 M) of the aPDT system, and the respective photosensitizer (inset)

stained bacterial membranes was observed. One drop of the photosensitizing agent was placed in each corner of the chamber using the syringe provided by the manufacturer (Helbo). After a residence time of 3 min measured with the time controller provided (Helbo), the photosensitizing agent was uniformly distributed in every single specimen. Subsequently, the fluorescence of the specimens was measured in the same area as used for the baseline measurements as the laser scanning device allowed exactly the same scanning position to be reproduced. After fluorescence measurement, 20 chambers (test chambers) were irradiated with a diode laser at a wavelength: of 660 nm and output power of 100 mW/cm^2 (Helbo) for 2 min each before laser scanning microscopy (Fig. 1). The optical fibre tip was ground flat at the end and shone forward. An additionally mounted spacing piece provided a constant distance from the irradiated surface and thus the correct power density for the treatment. The remaining 20 (control) chambers were evaluated at the end of an additional photosensitizer residence time of 2 min without irradiation.

The normality of the distribution of the values was assessed with the Shapiro-Wilk test. Since not all data were normally distributed, values were analysed with the nonparametric Wilcoxon two-sample paired signed ranks test using the SPSS software (SPSS, Chicago, IL). Differences were considered as statistically significant at $p<0.05$. Box plots show the median, first and third quartiles, and minimum and maximum values (whiskers). Values of more than 1.5 to 3 times the interquartile range were specified as outliers and are represented by circles. Values more than 3 times the interquartile range were specified as far outliers and are represented by asterisks.

Results

aPDT with respect to biofilm layers

Each of the 11 biofilm layers analysed showed green fluorescence. Fluorescence values depended on the treatment procedure performed. The highest fluorescence values were found at baseline in the lowermost (pellicle) layer before addition of the photosensitizer (median 20.03 U, range 6.26–37.86). The lowest fluorescence values were found in the topmost (10 μm) layer after laser irradiation (median 0.69 U, range 0–1.39; Fig. 2). Overall, after adding the photosensitizer dilution caused decreases in the values in each layer ($p<0.05$). Additional decreases in fluorescence were found after laser irradiation in every layer within a total range of 10 μm ($p<0.05$; Fig. 3).

Impact of laser irradiation

The effect of laser irradiation could be determined by comparing the overall values for the biofilm layers between the two treatment procedures. Laser irradiation caused a decrease in bacteria, whereas the presence of the photosensitizer without laser irradiation did not cause a change in the amount of fluorescent bacteria. Fluorescence values in the test chambers after laser irradiation (median 2.1 U, range 0.4–3.4 U) were significantly lower than baseline values after adding the photosensitizer (median 3.6 U, range 1.1–9.0; $p<0.05$; Fig. 4). The non-irradiated control chambers showed no change in fluorescence at the end of the additional photosensitizer residence time of 2 min without laser irradiation (median 1.9 U, range 0.7–3.6; median 1.9 U, range 0.8–6.0, respectively; $p>0.05$; Fig. 5).

Discussion

The present study demonstrated that laser irradiation during aPDT reduced the numbers of live *S. mutans* cells within a

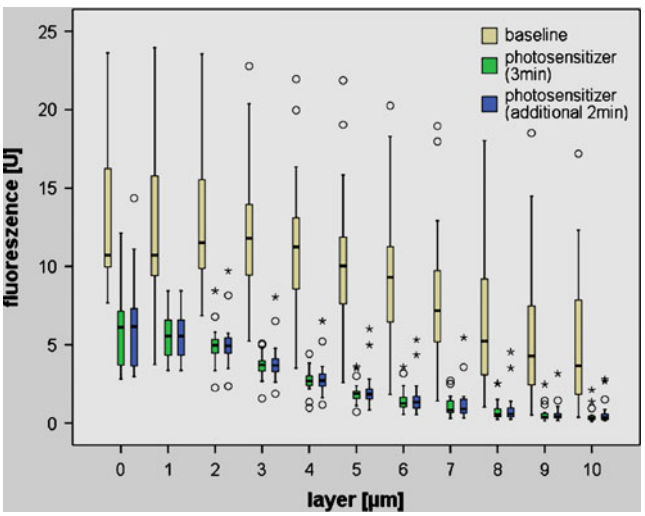

Fig. 2 Fluorescence at baseline, after adding the photosensitizer and after an additional 2-min residence time at intervals of 1 μm from the pellicle layer in the 20 control chambers

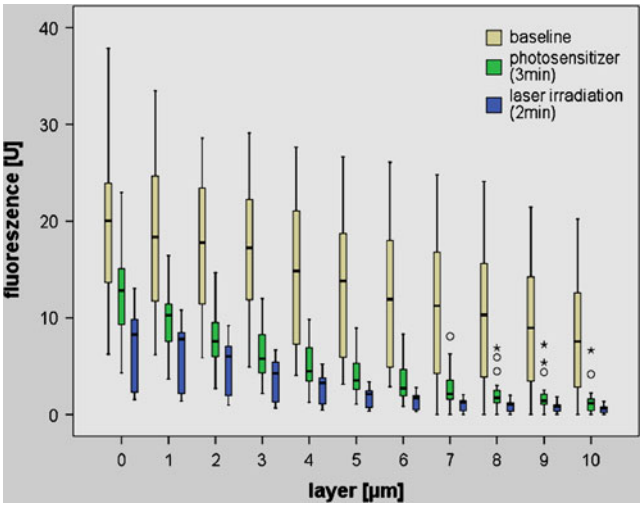

Fig. 3 Fluorescence at baseline, after adding the photosensitizer and after laser irradiation for 2 min at intervals of 1 µm from the pellicle layer in the 20 test chambers

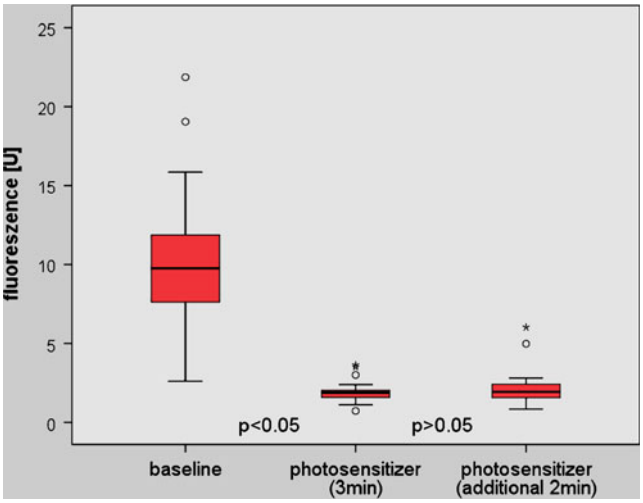

Fig. 5 Overall values of fluorescence in the 20 control chambers at baseline, after adding the photosensitizer and after an additional residence time of 2 min. The addition of photosensitizer was followed by a residence time of 3 min and then by an additional residence time of 2 min without laser irradiation

layer of 10 µm in an artificial biofilm model. In contrast, the presence of photosensitizer without laser irradiation did not result in a change in the numbers of live bacteria. Thus, these findings suggest that laser irradiation is essential to obtain an antimicrobial effect with an aPDT procedure.

The study design comprised cultures of *S. mutans* in an artificial biofilm model. A previous study demonstrate the impact of aPDT using toluidine blue as the photosensitizer and a light-emitting diode on a planktonic *S. mutans* culture [20]. The study also showed that the treatment efficiently killed microorganisms and prevented the formation of a biofilm. Another study evaluated the susceptibility of *S. mutans* biofilms to aPDT employing toluidine blue in combination with either a helium/neon laser or a light-emitting diode [21]. A significant decrease in the viability of bacteria was only observed when the biofilms were exposed to both photosensitizer and light. This result is in accordance with the results of the present study, even though phenothiazine chloride was used as the photosensitizing agent instead of toluidine blue. Lethal photosensitization occurred predominantly in the outermost layers of the biofilms as shown by confocal laser scanning microscopy. This result is also in accordance with those of the present study, as the reduction in fluorescent microorganisms was not equal in all biofilm layers.

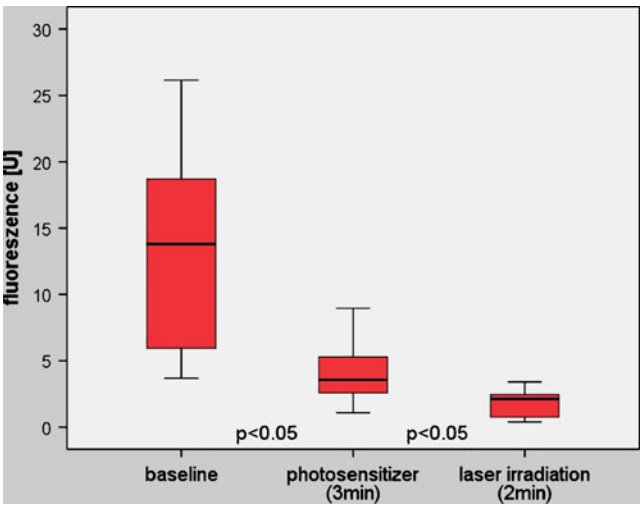

Fig. 4 Overall values of fluorescence in the 20 test chambers at baseline, after adding the photosensitizer and after subsequent laser irradiation for 2 min. The addition of photosensitizer dye was followed by a residence time of 3 min before the 2-min laser irradiation

The limited access of topical agents to oral plaque biofilms and the development of antibiotic resistance have led to the necessity for alternative strategies to control biofilms and to treat periodontal diseases [2]. aPDT is mediated by singlet oxygen which has a direct effect on extracellular molecules. Thus, the polysaccharides present in the extracellular matrix of the polymers of a bacterial biofilm are also susceptible to photodamage [2]. Such dual activity is not exhibited by antibiotics and may represent a significant advantage of aPDT. Moreover, development of resistance to the cytotoxic action of singlet oxygen or free radicals seems unlikely. aPDT is effective against both antibiotic-resistant and antibiotic-susceptible bacteria. Moreover, repeated photosensitization procedures have not induced the selection of resistant strains [22], and bacterial resistance could not be generated in an experimental study protocol [14].

In the present study, bacterial fluorescence was measured at baseline, after adding the photosensitizer and after laser

irradiation. The baseline values were significantly higher than those obtained after adding the photosensitizer. This result is in accordance with a study employing toluidine blue as photosensitizer [23]. The authors observed a reduction in bacteria with the photosensitizer dye alone. However, a similar observation was only made after adding the photosensitizer to the test chambers in the present study. Thus, it is possible that the photosensitizer only diluted the bacterial suspension and thus decreased the fluorescence. The fluorescence values after adding the photosensitizer dye in the control chambers were different from those in the test chambers. This observation could be because the biofilm specimens in the test and control chambers were not generated on the same day. Thus, with baseline extinction values of 0.8 and 1.1 U, the test and control chambers contained different numbers of bacteria. As a consequence, no comparisons were performed between the groups but only within each group. The overall percentage fluorescence after adding the photosensitizer decreased by 66% in the test chambers and 72% in the control chambers ($p>0.05$). However, the laser irradiation procedure was only performed in the test chambers. Consequently, only the changes in fluorescence after the 2 min irradiation period in the test chambers and the additional residence time of 2 min in the control chambers were compared to assess the impact of laser irradiation. The amount of fluorescence did not change after an additional residence time of 2 min without laser irradiation. As a consequence, it is not possible to ascribe the reduction in the number of live bacteria to the antimicrobial properties of the photosensitizer. Even if the photosensitizer alone had shown toxicity outside the limits of this study, laser irradiation would have contributed to the overall antimicrobial effect. This reduction in the number of live *S. mutans* bacteria following the photodynamic procedure using phenothiazine chloride as photosensitizer is in accordance with similar results obtained using toluidine blue and methylene blue in *Candida albicans* [24].

With respect to the reduction in the numbers of live bacteria in the present study, the "photobleaching" phenomenon has to be discussed. Photobleaching is described as a loss of fluorescence caused by repeated stimulation of fluorescence emitting agents [25, 26]. In the present study this stimulation could have been caused by the repeated use of laser scanning microscopy. However, it is not appropriate to ascribe the whole loss of fluorescence to the photobleaching effect since different results were obtained with and without laser irradiation. Any photobleaching effect would have occurred in all groups to a similar extent. Also lethal photosensitization in biofilms using a confocal scanning laser as the excitation source has been observed [27]. This effect cannot be excluded in the present study, but would have occurred in both test and control chambers. Thus, it was possible to perform an intra-experimental comparison between the control and test chambers.

The present study indicates that laser irradiation is an essential part of aPDT to reduce bacteria within a layer of 10 μm in an artificial biofilm model. A photodynamic treatment protocol comprising photosensitization and laser irradiation should be followed carefully to obtain an antimicrobial effect.

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