Antimicrobial photodynamic therapy using visible light plus water-filtered infrared-A (wIRA)

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The aim of this study was to investigate the effectiveness of antimicrobial photodynamic therapy (APDT) using visible light together with water-filtered infrared-A (VIS + wIRA) to eradicate single species of planktonic bacteria and micro-organisms during initial oral bacterial colonization in situ. A broadband VIS + wIRA radiator with a water-filtered spectrum in the range 580–1400 nm was used for irradiation. Toluidine blue (TB) was utilized as a photosensitizer at concentrations of 5, 10, 25 and 50 μg ml⁻¹. The unweighted (absolute) irradiance was 200 mW cm⁻² and it was applied for 1 min. Planktonic cultures of Streptococcus mutans and Enterococcus faecalis were treated with APDT. Salivary bacteria harvested by centrifugation of native human saliva were also tested. In addition, initial bacterial colonization of bovine enamel slabs carried in the mouths of six healthy volunteers was treated in the same way. Up to 2 log₁₀ of S. mutans and E. faecalis were killed by APDT. Salivary bacteria were eliminated to a higher extent of 3.7–5 log₁₀. All TB concentrations tested proved to be highly effective. The killing rate of bacteria in the initial oral bacterial colonization was significant (P<0.004) at all tested TB concentrations, despite the interindividual variations found among study participants. This study has shown that APDT in combination with TB and VIS + wIRA is a promising method for killing bacteria during initial oral colonization. Taking the healing effects of wIRA on human tissue into consideration, this technique could be helpful in the treatment of peri-implantitis and periodontitis.

INTRODUCTION

Although photodynamic therapy (PDT) is currently being applied in cancer cell elimination (Allison & Sibata, 2010; Ericson et al., 2008), more attention has been paid to its application in antimicrobial treatment (Wood et al., 2006; Donnelly et al., 2007; Maisch, 2007). Due to the growing number of antibiotic-resistant micro-organisms, research is focusing on PDT as an alternative chemotherapy modality (Garcez et al., 2010; Kharkwal et al., 2011). Since PDT causes damage to different parts of microbial cells and affects different interaction pathways in the micro-organisms, the development of antibiotic resistance against PDT can be excluded (Wainwright et al., 2010).

PDT impresses with its excellent target-cell specificity and high efficiency. The photodynamic effect requires a photosensitizer, suitable light and oxygen. The photosensitizer can be transferred to the high-energy triplet state and reacts with oxygen, producing singlet oxygen and other radical species which themselves cause increased target cell damage (Konopka & Goslinski, 2007). The photosensitizer should be non-toxic and show antimicrobial activity only after activation by illumination. In addition it should not be bio-destructible. Light sources used in PDT should produce low-power visible light at a specific wavelength (or range of wavelengths) which itself does not harm human tissues in the absence of the photosensitizer. Additionally, light sources should be inexpensive, lightweight and highly flexible (Konopka & Goslinski, 2007). Despite the fact that the antimicrobial activity of LED light sources has been intensively investigated in various studies (Omar et al., 2008; Omar, 2010), the efficacy of visible light in combination with water-filtered infrared-A (VIS + wIRA) in killing bacteria has not previously been examined. This combination delivers a broad-band light source which could be used in combination with different photosensitizers. A significant reduction of the total bacterial load was described previously with this combination for the treatment of chronic wounds (von Felbert et al., 2008). Such effects are potentially of great importance in dental...
medicine, since VIS+wIRA is indicated for chronic pain relief and is capable of increasing the tissue oxygen partial pressure and stimulating metabolism (Hartel et al., 2006; von Felbert et al., 2010; Maisch et al., 2007). Additionally, due to its penetration properties, wIRA causes energy transfers into subcutaneous tissue without overheating the skin (Fuchs et al., 2004). Furthermore, it has been shown that wIRA reduces pain in chronic wounds, warts, herpes, scleroderma, gonarthrosis and spondylitis (Fuchs et al., 2004; Hartel et al., 2006). Applying wIRA would enhance the effects of PDT since heating has some synergistic effect when applied together with PDT (Waldow et al., 1983; Kelleher et al., 2003; Yanase et al., 2006). The use of antimicrobial PDT (APDT) could therefore potentially improve the tissue healing process in a number of dental procedures.

The oral biofilm formed on teeth surfaces and soft gingival tissue in the oral cavity acts as a trigger for dental caries, periodontitis, gingivitis and peri-implantitis (Al-Ahmad et al., 2011). The effects of PDT on oral bacteria and oral biofilms have mainly been studied on single bacterial species biofilm generated in vitro. An overview of such studies has been published by Konopka & Goslinski (2007). The differences in the cell wall structure of Gram-positive and Gram-negative bacteria lead to variations in the binding ability of photosensitizing agents to different bacterial surfaces. For example, the affinity of negatively charged photosensitizers to Gram-negative bacteria can be enhanced by modifying the sensitizer by using cationic molecules such as chlorine e6 (Rovaldi et al., 2000). This emphasizes the necessity for the identification of an effective photosensitizer and a suitable light source, which together are capable of eliminating various resistant micro-organisms in the presence of a pronounced bacterial diversity in the oral cavity.

In the oral cavity, bacteria can grow as single cells in human saliva in concentrations up to $10^8$ ml$^{-1}$ (Marsh & Martin, 1999). Oral bacteria can also adhere to the enamel surface or other restorative materials in the oral cavity. After an initial phase of colonization, oral micro-organisms multiply and aggregate to form the dental oral biofilm, which consists of extracellular substances and a diverse range of micro-organisms (Al-Ahmad et al., 2009; Marsh & Martin, 1999). Hence, testing the efficacy of antimicrobial oral therapy should include not only the mature dental biofilm but also the planktonic micro-organisms and the initial bacterial colonization.

The aim of the present study was to assess the effectiveness of APDT in the eradication of oral micro-organisms which initially adhered to bovine enamel slabs in situ. This is believed to be the first time that the effect of VIS+wIRA on initial bacterial oral biofilm has been tested in vivo. The effectiveness of this specific photodynamic therapy was previously examined in vitro using high concentrations of Streptococcus mutans and Enterococcus faecalis. Since the effects of wIRA on wound infections have been shown, it could be expected that PDT using wIRA would have an impact on oral microorganisms and oral initial colonization.

**METHODS**

**Light source and photosensitizer.** A broad-band VIS+wIRA radiator (Hydrosun 750 FS, Hydrosun Medizintechnik) with a 7 mm water cuvette was used in this in situ assay. An orange filter, BTE31, which provides more than a doubled weighted effective integral irradiance regarding the absorption spectrum of protoporphyrin IX compared to the common orange filter BTE395, was fitted to the device.

The continuous water-filtered spectrum covers 570–1400 nm with local minima at 970 nm, 1200 nm and 1430 nm due to the absorption of water molecules (Piazena & Kelleher, 2010). The unweighted (absolute) irradiance was 200 mW cm$^{-2}$ VIS+wIRA, including approximately 48 mW cm$^{-2}$ VIS and 152 mW cm$^{-2}$ wIRA, and the application time was 1 min.

The photosensitizing agent applied was toluidine blue (TB) (Sigma-Aldrich). TB was dissolved in 0.9 % saline solution to obtain concentrations of 5, 10, 25 and 50 µg ml$^{-1}$. TB solutions were freshly prepared and stored in a dark place prior to use. In saline solution, TB has absorption maxima at 590 nm, 620 nm and 637 nm. Absorption minima are also seen at 570 nm and 650 nm. The broad-band light source used in this study allows optimal light absorption by TB.

**Planktonic bacterial strains and culture conditions.** The bacterial strains used to test the adequacy of the APDT, Streptococcus mutans DSM 20523 and Enterococcus faecalis T9, were obtained from Professor Dr. J. Hübner, Department of Medical Infectiology, University of Freiburg, Germany. In addition, unstimulated human saliva from a healthy 45-year-old volunteer who had not used antibacterial mouthwashes and/or antibiotics for 6 months prior to the start of the experiment was investigated. Streptococcus mutans, Enterococcus faecalis and saliva were cultivated on Columbia blood agar plates at 37°C in an aerobic atmosphere with 5% CO$_2$. The overnight cultures of bacterial strains were prepared in tryptic soy broth (Merck). Exponential-phase cells were used for testing in the study. The unstimulated saliva and 8 ml cell suspensions of each organism were centrifuged at 4000 g for 10 min. The supernatants were then removed, the pellets washed in sterile 0.9% saline solution, and the centrifugation step repeated. After discarding the supernatant, 8 ml 0.9% saline solution was finally added.

**Treatment of bacterial strains and salivary bacteria.** The bacterial concentration tested was up to $10^8$ c.f.u. ml$^{-1}$. The bacterial suspensions were tested with four different TB concentrations (5, 10, 25 and 50 µg ml$^{-1}$) as mentioned above. For irradiation, 1 ml of the bacterial solution containing a given photosensitizer concentration was placed into multwell plates (24-well plate, Greiner bio-one) in triplicate. Irradiation was applied at 37°C for 1 min. All experiments were conducted twice. After irradiation, a dilution series of the treated bacterial solution was prepared and each dilution was streaked onto Columbia blood agar and cultured at 37°C in an aerobic atmosphere with 5% CO$_2$. To quantify the c.f.u. a gel documentation system (ChemidocXRSi, Bio-Rad) was used. The surviving c.f.u. were compared with those of the untreated control.

Salivary bacteria, which served as controls, were washed and diluted 1:10 in 0.9% sterile saline prior to the application of APDT as described above. Four different TB concentrations were also utilized following the same procedure as previously mentioned for the bacterial strains.
Subjects and specimens for oral bacterial colonization. Six healthy volunteers participated in the study. Prior to the experiments, a clinical oral examination was performed. The subjects showed no signs of gingivitis or caries. Informed written consent had been given by the participants in the study. The study design was reviewed and approved by the Ethics Committee of the University of Freiburg (EK 63/07). Cylindrical bovine enamel slabs (BES) were prepared as described in detail previously (Al-Ahmad et al., 2009). In brief, bovine incisors were obtained from BSE-free and freshly slaughtered 2-year-old cattle for preparation of the BES. First, the teeth were separated from their roots and prepared using a grinding unit (Exakt-Mikroschleifsystem, Exakt-Apparatebau). Cylindrical enamel slabs (diameter 5 mm, height 1.5 mm, surface area 19.63 mm²) were prepared from the labial surfaces of the bovine incisors. The final grinding of the bovine enamel was carried out using a grinding machine (Knuth-Rotor-3, Streuers) using sandpaper of 1200, 2400 and 4000 grids in decreasing order of grain size. The surface of the enamel specimen was controlled by a light microscope (Leica Wild M3Z).

Six enamel slabs were fixed on individual upper jaw splints with the aid of a polysiloxane impression material (Aquasil Ultra, Dentsply DeFrey), as previously described (Hannig et al., 2007). This ensured that only the surface of the slabs was exposed to the oral cavity, as most of the margins were completely covered by the impression material (Fig. 1). The specimens were attached to the buccal surfaces of the upper premolars and the first molar and held in the oral cavity for 2 h. The volunteers carried the splint systems twice to deliver a sufficient number of BES for the PDT tests that followed.

Treatment of adhered bacteria. Each volunteer carried an individual upper jaw splint to which six BES were attached for 2 h. This procedure was carried out twice in each volunteer. After exposure in the oral cavity, each specimen was rinsed with 0.9% sterile saline solution for 30 s. Two specimens of a total of six BES per participant served as a control and the remaining four BES were treated with APDT. Four different TB concentrations (5, 10, 25 and 50 µg ml⁻¹) were tested on each of the two control specimens which were initially colonized by oral bacteria. A total of 12 control BES was examined for each TB concentration. For APDT treatment the BES were placed into multiwell plates (24-well plate, Greiner bio-one) in duplicate. The radiation was applied for 1 min at 37 °C. The adherent bacteria were subsequently quantified by determination of c.f.u. as described elsewhere in detail (Hannig et al., 2007). Briefly, the back side of the slabs consisting of dentine as well as their margins were brushed off with sterile small swab pellets and the BES were placed into sterile tubes with 1 ml 0.9% saline, vortexed and then treated in an ultrasonic bath on ice for 30 s. The suspension from the untreated BES (control) was then serially diluted up to 1 : 10⁵ in 0.9% saline; the preparation of a similar dilution series for the treated BES was not necessary. The c.f.u. were then determined.

Statistical analysis. For statistical analysis the Mann–Whitney U Test (IBM SPSS Statistics 19) was used to detect significant differences between the tested TB concentrations and the control adhesion targets with respect to the c.f.u. values of the initial bacterial colonization.

RESULTS

As shown in Fig. 2, 98% of S. mutans cells were killed by each of the TB concentrations ranging from 5 to 50 µg l⁻¹. Only 1% of E. faecalis cells survived after APDT, irrespective of the TB concentration. This high bacterial eradication rate of the APDT corresponds to 1.7–2 log₁₀ steps of the original treated bacterial culture.

Salivary bacteria were eliminated to a higher extent of 3.7–5 log₁₀, which corresponds to a reduction rate higher than 99.99% of the original salivary bacterial counts (8.6 log₁₀). All TB concentrations used were highly effective (Fig. 3).

Considering the initially adhered oral bacteria (Fig. 4), the untreated control revealed a log₁₀ c.f.u. value of 4.3 (median 4.2). The log₁₀ c.f.u. of the adhered bacteria after APDT was 1.8 ± 1.2 (median 1.7) after treatment with 50 µg ml⁻¹ TB, 1.4 ± 1.5 (median 1.1) with 25 µg ml⁻¹ TB, 1.2 ± 1.2 (median 1.2) with 10 µg ml⁻¹ TB and 3.1 ± 0.5

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**Fig. 1.** Front view of an individual upper jaw splint with six BES attached to the buccal surfaces of both premolars and first molar. The specimens were fixed in small cavities with silicone impression material.

**Fig. 2.** Eradication rates of (a) S. mutans and (b) E. faecalis after the application of APDT using VIS + wLR at four TB concentrations, plus a negative control (C). Data shown are means ± sd (n=6).
(median 3.3) with 5 µg ml⁻¹ TB. The differences between all tested TB concentrations and the untreated control were significant (P=0.004) despite the interindividual variations among the volunteers involved in the study as shown by the high standard deviations of the c.f.u. numbers obtained. This antimicrobial activity of all tested TB concentrations is of great clinical importance, since it corresponds to an elimination rate of more than 90%.

**DISCUSSION**

APDT has become an interesting research topic in the field of medical microbiology, not only due to the increasing antibiotic resistance of various micro-organisms but also because of its healing properties on sensitive human tissues. Up until now, light sources using light-emitting diodes have been frequently utilized in APDT. The encouraging results showing pain reduction in patients with multiple actinic keratoses when VIS+wIRA was applied (von Felbert et al., 2010) inspired the present study. The effectiveness of this specific light source was investigated for its efficacy against *Streptococcus mutans* and *Enterococcus faecalis in vitro* as well as against initial oral bacterial colonization in vivo. *S. mutans* was chosen since it is considered to be a principal causative agent of dental caries (Beighton et al., 1991; Al-Ahmad et al., 2006; Yoo et al., 2011). *E. faecalis* is frequently isolated in secondary endodontic infections and is considered to be capable of withstanding unfavourable environmental conditions in obturated root canals (Schirrmeister et al., 2009; Zhu et al., 2010). Apart from testing the effects of APDT on planktonic bacteria it is necessary to study the effectiveness of new antimicrobial therapeutic modalities against adherent bacteria in biofilms, due to the higher antibiotic resistance of micro-organisms embedded within biofilms (Costerton et al., 1999). Moreover, a study in which the presence of a high bacterial diversity is investigated would deliver more realistic results concerning the clinical adequacy of APDT. To date however, most studies using APDT have been conducted either on single species in planktonic culture or on single-species biofilm generated *in vitro*. In the present study, the effectiveness of APDT using VIS+wIRA on multispecies biofilm in the initial oral bacterial colonization was tested, we believe for the first time. Furthermore, this appears to be the first time that VIS+wIRA has been assessed as a light source for APDT. The APDT had a much higher effect on salivary bacteria than on the planktonic cultures. A possible explanation for this could be the antimicrobial ingredients of human saliva, which adhere to bacterial cells and could cause a synergistic effect with the photosensitizers. However, some new photosensitizers should also be tested in future studies.

TB is known to be an effective photosensitizer which is capable of damaging cell membranes during APDT, thus leading to the eradication of bacterial cells (Wakayama et al., 1980). Zanin et al. (2006) used TB and irradiation of 85.7 J cm⁻² to kill *in vitro*-generated single-species biofilm of *S. mutans* and other oral streptococci. The elimination rates of the bacteria were found to vary between 95 and 99.9%. In a similar *in vitro* study of Bevilacqua et al. (2007), *S. mutans* was completely eliminated and biofilm formation was prevented by APDT using 100 µg TB ml⁻¹ and LED light at 2.18 J cm⁻². However, favourable antimicrobial effects can be achieved at a much lower TB concentration of 5 µg ml⁻¹ according to the outcome of our research. In a recent project, Souza et al. (2010) succeeded in killing *E. faecalis in vitro* using APDT with methylene blue and TB as photosensitizers. The authors failed however to reveal whether they had found any significant differences concerning the efficacy of the two photosensitizers. This suggests that methylene blue could probably also be combined with VIS+wIRA as the light source. In addition, Sharma et al. (2008) applied APDT on an *in vitro*-formed biofilm of *Staphylococcus aureus* and *Staphylococcus epidermidis*, while also choosing TB as the photosensitizer and a diode laser (25 J cm⁻²) as the light source. Among the main results, APDT accomplished a significant inactivation of the biofilm of these clinically relevant staphylococci, a finding that was confirmed by the broad-spectrum efficacy of TB demonstrated in the present
study. Furthermore, Kömerik et al. (2003) showed that TB-mediated lethal photosensitization of Porphyromonas gingivalis, which results in decreased bone loss, is possible in vivo. These findings suggest that APDT, in particular using VIS+wIRA, may result in fewer undesired side effects and a strong stimulation of the healing of human tissue and may be useful as an alternative approach for the antimicrobial treatment of periodontitis.

APDT could also play a key role as an alternative antimicrobial modality in endodontic therapy, as suggested by Silva Garcez et al. (2006). The authors compared the capability of 0.5% NaOCl versus PDT with azulene to eradicate E. faecalis recovered from infected root canals in vitro. It was reported that APDT with azulene achieved a significantly higher reduction of the resistant E. faecalis population when compared to NaOCl. Although azulene was not tested in our study, it should be considered as a potential effective photosensitizer in combination with VIS+wIRA.

Microbial biofilm, which mainly contains bacteria up to 500 times more resistant to antimicrobials than their planktonic counterparts, is a challenging target for the most modern antibacterial agents (Costerton et al., 1999). The application of APDT using methylene blue to eradicate dental plaque bacteria revealed that micro-organisms in oral biofilms are less affected by APDT than bacteria in the planktonic phase (Fontana et al., 2009). However, the authors stated that the antibacterial effect of APDT on oral biofilm bacteria was stronger than that found after antibiotic treatment under similar conditions. Such a limitation of antimicrobial agents in the elimination of biofilms was illustrated in one of our earlier studies, where chlorhexidine failed to eradicate an in vitro-formed biofilm of S. mutans (Al-Ahmad et al., 2008). This emphasizes the need for improved antimicrobial treatments such as APDT. Other research groups have applied APDT using erythrosine as the photosensitizer, which is already available for use in the mouth, and has been used to eradicate an in vitro-generated S. mutans biofilm (Wood et al., 2006). This combination proved to be effective against the in vitro biofilm of S. mutans. The latter reports are quite promising, since they suggest that the effective application of non-toxic and biocompatible photosensitizers in future APDT studies could be possible. Moreover, chlorine e6 proved to be a more effective photosensitizer than TB when combined with VIS+wIRA as a light source in order to kill E. faecalis according to the results of our recent project (data not shown). Further studies are needed to confirm the high elimination rates of APDT for salivary bacteria and the initial oral bacterial colonization as well. It has been already shown that APDT using a diode laser as light source and phenothiazine as photosensitizer effectively reduced Streptococcus mutans within a layer of 10 μm in an in vitro biofilm (Schneider et al., 2012). Furthermore, Nastri et al. (2010) reported the elimination of different periopathogens and the photoinactivation of an in vitro Aggregatibacter actinomycetemcomitans biofilm using APDT with TB and a diode laser. Hence, an impact of APDT using wIRA and TB could be assumed, but has to be further studied.

Although the present study highlighted the effectiveness of APDT in eliminating the initial oral bacterial colonization, an impact on mature oral biofilm formed in vivo is also a necessity if this procedure is to be applied clinically. Hence, the applicability of wIRA within the oral cavity has to be established in order to conduct further in vivo clinical studies. Nevertheless, the limited ability of different antimicrobial agents, including antibiotics and disinfectants, to eradicate biofilm microbiota will encourage microbiologists to pay more attention to the application of APDT in this field of research with the aim of developing more effective treatments.

In conclusion, this study has shown that APDT using the combination of TB and VIS+wIRA is an effective method which is capable of killing bacteria within the initial oral bacterial colonization. Taking the healing effects of wIRA on human tissue into consideration as well, this technique could prove to be helpful in the treatment of peri-implantitis and periodontitis, although this would need to be evaluated within the framework of more clinical trials. Moreover, the broad-band light source consisting of VIS+wIRA has a high potential for use in combination with other photosensitizers.

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