Investigation of photodynamic therapy on streptococcus mutans of oral biofilm

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Received September 17, 2008

We investigated the effect of photodynamic therapy (PDT) with hematoporphyrin monomethyl ether (HMME) on the viability of Streptococcus mutans (S. mutans) cells on biofilms *in vitro*. Streptococcus mutans is the primary etiological agent of human dental caries. Since dental caries are localized infections, such plaque-related diseases would be well suited to PDT. The diode laser used in this study had the wavelength of 635 nm, whose output power was 10 mW and the energy density was 12.74 J/cm^2. HMME was used as photosensitizer. Samples were prepared and divided into five groups: (1) HMME; (2) Laser; (3) HMME+Laser; (4) Control group (+) with chlorhexidine; and (5) Control group (−) with sterile physiological saline. Inoculum of S. mutans incubated with HMME also examined with fluorescence microscopy. PDT exhibited a significantly ($P < 0.05$) increased antimicrobial potential compared with 20 μm/mL HMME only, laser only, 0.05% chlorhexidine, and 0.9% sterile physiological saline, which reduced the S. mutans of the biofilm most effectively. Laser and 0.05% chlorhexidine were caused reduction in the viable counts of S. mutans significantly different ($P < 0.05$) also, but these two test treatments did not statistically differ from each other. HMME group did not statistically differ with negative control group. Fluorescence microscopy indicated that HMME localized primarily in the S. mutans of the biofilm. It was demonstrated that HMME-mediated PDT was efficient at killing S. mutans of biofilms and a useful approach in the treatment of dental plaque-related diseases.

OCIS codes: 170.5180, 170.1530, 170.2520.

doi: 10.3788/COL20080612.0947.

Dental caries is thirdly common diseases in the world followed cardiovascular diseases and cancer. Streptococcus mutans (S. mutans), the primary etiological agent of human dental caries, has developed multiple mechanisms to colonize and form biofilms on the tooth surface.

It is important to control the growth of S. mutans of the oral biofilms for preventing dental caries. Antibacterial agents has been widely used, but problems with general efficacy due to access of topical agents to plaque[1] and the possibility of development of bacterial resistance[2] mean alternative strategies are desirable to control plaque and treat caries.

Photodynamic therapy (PDT) may emerge as a suitable process to combat both biofilm and antimicrobial-related resistance. Since dental caries are localized infections, such plaque-related diseases would be well suited to PDT. The photodynamic approach to kill bacteria is clearly a rapidly emerging alternative to current antimicrobial regimens[3]. Significantly, it is unlikely that bacteria could develop resistance to the photodynamic action of cytotoxic singlet oxygen or free radicals, as has been reported with conventional antimicrobials and antibiotics[4].

The aim of this study was to carry out a preliminary assessment of hematoporphyrin monomethyl ether (HMME)-mediated PDT on biofilms of S. mutans *in vitro*-generated and the cell killing efficacy of PDT with HMME compared with other antimicrobial methods.

25 Freshly extracted human permanent premolars for othodontic reason, free of defects and/or cracks when trans-illuminated, were selected for this study. The roots were resected from enamel-cementum joint and the crowns were splited with handpiece from mesial-distal direction. After ultrasonic cleanout for 5 min to remove debris, each sample was covered with nail polish only exposed 4 × 4 (mm) on the surface of enamel. 50 Enamel pieces were sterilized with 75% ethanol for 72 h.

Unstimulated saliva of human free of dental carries and periodontal disease was collected. Enamel pieces were preconditioned in the filter-sterilized saliva for 37 °C and 48 h to form the achievement membrane.

The microorganism used in this study was S. mutans NCTC 10449 (Capital Medical University School of Stomatology, Beijing, China). To prepare the inoculum, S. mutans was first grown anaerobically on Trypticase Soy Agar(TSA) plates for 48 h. Subsequently, single colonies were inoculated into 10 mL of Trypticase Soy Broth(TSB) and incubated anaerobically at 37 °C for 24 h. The nutrient source in all experiments were TSA and TSB.

Later, 50 enamel pieces were took out from the saliva and immerged into 1ml inoculum of S. mutans (1×10^8) respectively and anaerobically at 37 °C for 48 h.

HMME (Shanghai Fudan, Zhangjiang Bio-Pharmaceutical Co., Ltd. China) was dissolved to obtain a final concentration of 10 mg/mL and was subsequently kept in the dark. The light sources used were a diode laser (Laser Medical Lab of Tianjin Medical University, China), which produces light with the wavelength of 635 nm.

50 enamel pieces were divided into 5 groups with each group of 10 random samples. Each group was disposed as

following: 1) HMME group: 20 μg/mL HMME added as photosensitizer alone. Incubation time was 2 h. 2) Laser group: diode laser irradiation alone. 3) PDT group: after incubated with 20 μg/mL HMME for 2 h and then irradiated with diode laser. The power output of laser was 10 mW. The irradiation times was 90 s and the energy density was 12.74 J/cm^2 (power density was 0.14 W/cm^2). 4) Positive control group:treatment with 1 mL 0.05% chlorhexidine for 90 s served as a positive control. 5) Negative control group:treatment with 1 mL 0.9% of sterile physiological saline solution for 90 s served as negative controls. After antimicrobial treatment, each enamel piece was immerged into 1 mL inoculum of S. mutans (1×10^8) respectively and anaerobically cultivated at 37 °C for another 48 h.

To harvest S. mutans of biofilm, each enamel pieces was transferred to a sterile 5-mL polypropylene tube containing sterile physiological saline (1 mL, room temperature) and vortexed vigorously for 2 min. The suspensions were then serial dilutions (from 10^{-2} to 10^{-5}) in sterile physiological saline and inoculated on TSA incubated anaerobically at 37 °C. After 48 h, the number of colony-forming units (CFUs) was counted with the aid of a stereomicroscope.

To determine the significance of the presence of sensitizer alone, the laser irradiation alone and the combination of sensitizer and laser, the data was 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%.

Inoculum of S. mutans incubated with HMME for 2 h were placed into a Petri dish (5 cm in diameter), and examined with a BX51 fluorescence microscopy (Olympus Jappen, INC).

To determine the antimicrobial activity of PDT, the number of CFU obtained from different treatment methods groups were compared with negative control group (sterile physiological saline). The results are summarized in Table 1.

PDT group, with 20 μg/mL HMME and diode laser irradiated, effectively eliminated bacteria ($P < 0.05$). Laser group and 0.05% chlorhexidine group were caused reduction in the viable counts of S. mutans significantly different ($P < 0.05$) also, but these two test treatments did not statistically differ from each other. HMME group did not statistically differ with negative control group.

PDT group exhibited a significantly ($P < 0.05$) increased antimicrobial potential compared with 20 μg/mL HMME only, laser only, 0.05% chlorhexidine, and 0.9% sterile physiological saline, which reduced the S. mutans of the biofilm most effectively.

Table 1. CFU/mL of S. Mutans after Different Treatment Methods

Groups	Antimicrobial Treatment Methods	CFU/mL ($x \pm$ SD)
HMME	HMME	316 ± 28
Laser	Laser	$126 \pm 22^*$
PDT	HMME+Laser	$16 \pm 8^*$
Positive Control	Chlorhexidine	$145 \pm 18^*$
Negative Control	Sterile Physiological Saline	341 ± 35

*Represent values that are significantly different ($P < 0.05$) compared with the negative control group.

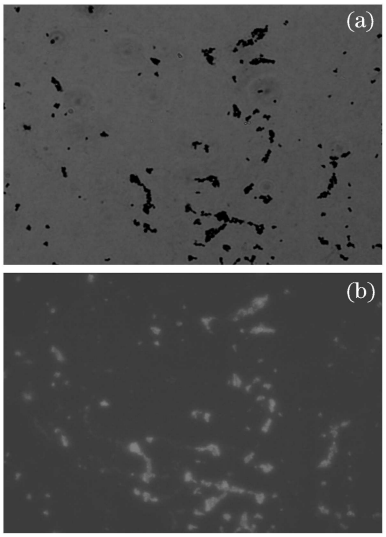

Fig. 1. Fluorescence microscope imaging shown uptake of HMME into S. mutans of biofilms. (a) Reflective S. mutans cellular is located using fluorescence microscope reflection-mode imaging and (b) the red areas of HMME localization is detected in fluorescence mode.

S. mutans biofilm, imaged on the fluorescence microscope in reflected-light mode, is shown in Fig. 1(a). Poorly reflective regions correspond to regions of S. mutans cellular, while highly reflecting areas are the channels and voids typical of biofilm architecture.

By viewing the same image in fluorescence mode, the red areas of HMME localization are revealed in Fig. 1(b). The two images are superimposable, indicating that HMME localizes primarily in the biomass of the biofilm, although whether the photosensitizer is associated with the bacterial cells, the extracellular matrix or a combination of the two is not known at present.

PDT is a medical treatment that utilizes light to activate a photosensitizing agent (photosensitizer) in the presence of oxygen. The exposure of the photosensitizer to light results in the formation of oxygen species, such as singlet oxygen and free radicals, causing localized photodamage and cell death.

Several studies have shown that oral bacteria are susceptible to PDT when they are grown as planktonic cultures[5,6]. However, the causative agents of caries and other oral diseases are present as organized biofilms. 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[7].

Bacteria in biofilms display increased resistance to antimicrobial agents[8]. PDT represents an alternative antibiotic treatment for drug-resistant organisms[9]. It is unlikely that bacteria would develop resistance to the cytotoxic action of singlet oxygen or free radicals. Bacteria that grow in biofilms, implicated in diseases like dental caries is susceptible to PDT[10].

The results of this study showed that PDT was

effective in significantly reducing the viability of S. mutans biofilms, whereas laser and chlorhexidine caused significant, but limited, reductions in the viable counts, and HMME failed to demonstrate a significant antimicrobial efficacy. Fluorescence microscopy images of biofilms after exposure to diode laser in the presence of HMME showed uptake of HMME into S. mutans of biofilms.

In conclusion, the results of this study showed that S. mutans biofilms were susceptible to diode laser in the presence of HMME, suggesting that this approach may be useful in the treatment of dental plaque-related diseases. We report the use of the dental plaque-disclosing agent HMME in the PDT of oral biofilm bacteria. We have demonstrated HMME to be an effective photosensitizer for the killing of the cariogenic bacterium S. mutans, which highlights the excellent clinical potential of HMME-mediated PDT in the control and treatment of dental plaque biofilm bacteria. Further work is now required to evaluate the clinically effect of antibiotic treatment of HMME-mediated PDT. We will further investigate the mechanism of HMME-mediated PDT for the treatment of dental caries.

This work was supported by the National Natural Science Foundation of China (No. 60678047) and the Science Foundation of Tianjin (No. 05YFJZJC02300). Y. Li is the author to whom the correspondence should be addressed, his e-mail address is yingxinli@tijmu.edu.cn or yingxinli@yahoo.com.

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