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METHODS AND PROTOCOLS

Bactericidal effects of hematoporphyrin monomethyl ether-mediated photosensitization against pathogenic communities from supragingival plaque

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Abstract Photodynamic antimicrobial chemotherapy (PACT) is proposed as a potential candidate to inactivate pathogens in localized infections due to the rapid evolution of bacterial resistance. The treatment modality utilizes nontoxic agents called photosensitizers and harmless visible light to generate reactive oxygen species which result in microbial cells' killing. Hematoporphyrin monomethyl ether (HMME) as a novel and affordable photosensitizer has been used in treating various clinical diseases for years, but few applications in infection. In this report, we studied the bactericidal effects of the HMME-mediated photodynamic reaction on the pathogenic microbes in supragingival plaque which can lead to many oral infectious diseases such as caries, gingivitis, and so on. Our findings demonstrated that HMME promoted an effective action in bacterial reduction with the application of laser energy. Moreover, the antimicrobial activities were dramatically enhanced as the HMME concentration and exposure time were increased, but reached a plateau when matched the appropriate agent concentration and illumination. It was found that the survival fraction of microorganisms is exponentially dependent on the product of HMME concentration and irradiation time. These promising results suggest the HMME may be an excellently cost-effective photosensitizing agent for mediating PACT in the treatment of supragingival plaque-related diseases. An optimized HMME concentration and irradiation time has been found to achieve the best results under our experimental conditions. The high HMME concentration matching short curative time, or vice versa, can achieve the similar therapeutic effect, which may provide more flexible treatment plans according to specific conditions.

Keywords HMME · PACT · Supragingival plaque · Oral pathogens · Diode laser

Yi Sun and Defeng Xing contributed equally to this work.

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Introduction

Supragingival plaque is an oral biofilm formed on the surface of dental cervix and at the gingival margin. It is composed of complex microbial communities embedded in an exocellular polymeric matrix of human salivary glycoproteins and polysaccharides (Hojo et al. 2009). The microbial

communities keep dynamic balance in normal state as the microbes undergo a process of metabolism continually. However, if the delicate balance ever fails, it will result in microflora disorders. Then, the disordered microbial communities are called pathogenic communities which can cause prevalent oral infectious diseases (Wen et al. 2010). The bacteria on the enamel produce acid substances through the metabolism of carbohydrates, demineralizing the tooth structure, and are responsible for dental caries (Tong et al. 2010). Microbial plaque lying in close proximity to gingival tissue is a very important ecological factor in triggering and promoting the gingival inflammation and, in some cases, subsequent losing the periodontal tissue (Aimetti et al. 2007). Therefore, it is very crucial to kill the pathogenic community in order to prevent or treat the plaque-related diseases. Furthermore, the inherent conditions of oral environment, such as temperature and humidity, are suitable for bacterial colonization and growth, so the supragingival plaque is easy to form but very hard to get rid of, which brings about that these oral diseases reoccur repeatedly within a short time even after being thoroughly eradicated from dental cleaning. From the above, the current difficult problem requires that the therapeutic method is not only effective but also economical, repeatable, and with fewer side effects.

Conventional methods for the control of supragingival plaque involve application of antibiotics and mechanical removal, such as scaling and root planning. Although traditional therapies are available in most cases, treatment effects are usually not satisfactory and might give rise to many severe complications. Mechanical therapy is laboursome to completely eliminate pathogens and prevent frequent reinfection (Jia et al. 2009), and trauma caused by operation may even result in bacteremia (Castillo et al. 2011). The use of antibiotics also has many problems such as loss of taste, subjective dryness of the oral cavity, allergic reactions and specially bacterial resistance, a matter of increasing concern in medicine (Walker 1996). Therefore, it is essential to explore new antimicrobial strategies to overcome these deficiencies.

Photodynamic antimicrobial chemotherapy (PACT) showed promising potential as an alternative methodology to inactivate microbe, and it has produced an efficient treatment outcome (Braham et al. 2009; Lim et al. 2012; Pereira Gonzales and Maisch 2012; Rolim et al. 2012). Using this technique, a nontoxic photosensitizer agent is excited by irradiation with relatively low intensity harmless light of a suitable wavelength in the presence of oxygen, resulting in the generation of reactive oxygen species, such as singlet oxygen ($^1O_2$) and free radicals, which can kill target cells (Maisch 2009). The PACT has many attractive advantages over traditional methods, such as free of toxins, selective target, easy repeatability, no drug resistance, and safe (Dai et al. 2009) which can go far towards solving the clinical difficulties. However, this modality is progressing slowly in the clinical practice. There are several reasons for this situation including high cost of photosensitizer, no specified guidelines for the procedure, and lack of reliable instrument.

Hematoporphyrin monomethyl ether (HMME), first developed in China by the early 1990s, is a promising second-generation photosensitizer. HMME is a porphyrin-related agent, consists of two monomer porphyrins, 3-(1-methyloxyethyl)-8-(1-hydroxyethyl) deuteroporphyrin IX and 8-(1-methyloxyethyl)-3-(1-hydroxyethyl) IX (Fig. 1). Compared with the first-generation photosensitizers, HMME is advantageous in terms of physicochemical properties and reliability in performance, such as higher target selectivity, lower toxicity, stronger photodynamic effects, and shorter skin photosensitivity (Lei et al. 2012). Clinical practice has also demonstrated that HMME is not only effective but also very safe in mediated photodynamic therapy to cure many diseases, such as portwine stains (Gu et al. 2007), corneal neovascularization (Wang and Liu 2005), and neoplastic diseases (Song et al. 2007; Song et al. 2011). Moreover, HMME is quite cheap in price, making it a good candidate for PACT applications.

The purpose of this study is to test whether the low-cost photosensitizer HMME could performance excellently in photoinactivating pathogenic communities in supragingival plaque so that to evaluate its clinical perspective. To optimize experimental parameters, drug concentration and illumination time, is also the highlight.

Materials and methods

Supragingival plaque samples collection

We randomly screened 15 voluntary patients who were consistent with the clinical type of periodontopathy. The research on human subjects followed the Declaration of Helsinki and institutional policies. The patients signed the

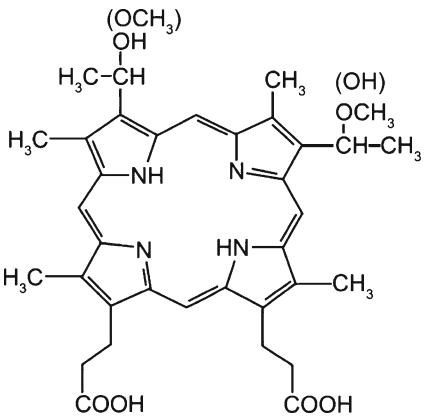

Fig. 1 Chemical structure of HMME

informed consent for the study, and the microbiology committee of Harbin Medical University approved the use of bacterial samples. The bacteria samples were taken from the tooth plaques near infected supragingival tissue using a sickle. Under aseptic conditions, the scraped samples were placed into 1 mL sterile 0.90 % (*w*/*v*) sodium chloride solution and transported to the laboratory immediately.

Supragingival plaque samples pyrosequencing

We selected three individuals at random from all the supragingival plaque samples for pyrosequencing. A 0.5-ml sample suspension was centrifuged at 5,000×*g* for 1 min to remove supernatant. Total genomic DNA was extracted from samples using a Bacteria genomic DNA Isolation Kit (Watson Biotechnologies Inc., Shanghai, China) according to the manufacturer's instructions. The quantity and quality of the extracted DNA were checked by measuring its absorbance at 260 and 280 nm using a Beckman DU800 spectrophotometer.

In order to construct amplicon libraries for 454 pyrosequencing, the V1–V3 region of bacterial 16S rRNA genes was amplified by using the universal primers 8 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 533R (5′-TTACCGCGGCTGCTGGCAC-3′). There was a ten-nucleotide barcode inserted between the Life Sciences primer A and the 8 F primer in the fused forward primer. After being purified and quantified, a mixture of amplicons was performed for pyrosequencing on a Roche massively parallel 454 GS-FLX. Low-quality sequences were removed to minimize the effects of random sequencing errors, such as those that without a recognizable reverse primer, without an exact match to the forward primer, length shorter than 200 nucleotides, and contained any ambiguous base calls (Ns). We trimmed the barcodes and primers from the resulting sequences. Finally, pyrosequencing produced relevant high-quality V1–V3 tags of the 16S rRNA-gene with an average length of 455 bp.

We clustered sequences into operational taxonomic units (OTUs) by setting a 0.03 distance limit (equivalent to 97 % similarity) using the MOTHUR program (http://www.mothur.org/wiki/Main_Page). Rarefaction curves, richness estimator were generated for each sample. After phylogenetic allocation of the sequences down to the phylum, class, and genus level, relative abundance of a given phylogenetic group was set as the number of sequences affiliated with that group divided by the total number of sequences per sample. A Venn diagram with shared and unique OTUs was used to depict the similarity and difference between the three samples.

Bacterial culture conditions

In this study, we chose Brain Heart Infusion (BHI; Oxoid Ltd., Basingstoke, UK), a highly nutritious infusion medium recommended for the cultivation of *Streptococci*, *Prevotella*, and other fastidious organisms. The medium consisted of the following: brain infusion solids (12.5 g), beef heart infusion solids (5 g), proteose peptone (10 g), glucose (2 g), sodium chloride (5 g), and disodium phosphate (2.5 g) per liter of distilled water. The pH was adjusted to 7.5 and the broth autoclaved at 121 °C for 15 min. The BHI broth was supplemented with 5 % (*w*/*v*) sterile defidrinated sheep blood (Harbin Veterinary Research Institute, Harbin, China), and added extra hemin and menadione (Sigma Ltd., Poole, UK) prior to use so that the final concentrations were 5 and 0.5 μg/ml, respectively.

For experimental purposes, the sample suspensions were inoculated into the liquid culture and incubated overnight at 37 °C in a mixed gas atmosphere (85 % N_2, 10 % H_2, and 5 % CO_2). Bacteria were used for experiments in mid-log growth phase, with a concentration of 1×10^8 cells/ml, which was assessed with a spectrophotometer (Mini 1240, Shimadzu) corresponding to an optical density value of 1 at 600 nm.

Photosensitizer and light source

HMME was provided by the Laboratory for Antimalarial Drug Research of the Second Military Medical University (Shanghai, China). HMME solution was made at a concentration of 10 mg/ml and kept in the dark at −20 °C When used, the stock solution was appropriately diluted in sterile PBS to obtain the desired concentration. The light source was a diode laser (Sanyo Electric Co., Tokyo, Japan) with a maximum output of 260 mW and a wavelength of 635 nm. The light was distributed by a fiberoptic applicator with a 0.8-mm cylindrical diffusing tip. Before irradiation, the output power was adjusted to 50 mW, and the generated light was optically collimated to a spot size of 0.8 cm in diameter. The laser output energy was carefully calibrated with a power meter to avoid overexposure (PS10, Coherent Inc., Santa Clara, CA, USA).

Bacteria adsorption of hematoporphyrin monomethyl ether

The suspension specimens and HMME solutions were mixed well to obtain the final concentrations of 10, 20, 30, and 40 μg/ml. The mixtures were kept in the dark for 2 h (Zou et al. 2008) at room temperature in the presence of 85 % N_2, 10 % H_2, and 5 % CO_2, subsequently, centrifuged (at 5,000×*g* for 1 min), and removed the medium. The microorganisms were rinsed and resuspended in sterile PBS, centrifuged again to eliminate unabsorbed HMME. The above washing steps were repeated three times. The

processed samples were illuminated with blue LED source (Sunlight Shenzhen Opto-Electronic Technology Co. Ltd), which has the wavelength of 405 nm and a bandwidth of 20 nm, to measure fluorescence of HMME adsorbed with the bacterial cells. We detected the resulting fluorescence by a multimode optical fiber (Ocean Optics Inc OFLV-200–1100), which was coupled to an 8-nm resolution spectrometer composed of a monochromator and a 2,048-element CCD-array detector (Ocean Optics Inc USB2000).

Photodynamic antimicrobial chemotherapy assay

The bacterial suspensions were incubated with different concentrations of HMME, ranging from 10 to 40 μg/ml, in the dark for 2 h at room temperature under the mixed anaerobic gas atmosphere. Afterwards, aliquots of 200 μl were inoculated in 96-well microtitration plate (well diameter, 8.0 mm). The power output of light sources was 50 mW and the laser beam illuminated an area of 0.5 cm^2, resulting in an energy intensity of 100 mW/cm^2. The irradiation times were: 5, 10, 15, 20, and 25 min, while the corresponding light dosage were 30, 60, 90, 120, and 150 J/cm^2, respectively. The PACT experimental groups were designated as P^+L^+. Untreated control groups were the suspensions in the absence of the agent and light (P^-L^-). Two negative controls were photosensitizer alone groups (P^+L^-) and light alone groups (P^-L^+). Each group contained samples from 15 patients, and three replicates were performed.

Statistical analysis

After irradiation, tenfold serial dilutions were carried out, and 25-μl aliquots were plated onto Brain Heart Infusion agar containing supplements, which were then cultured in the presence of 85 % N2, 10 % H2, and 5 % CO2 at 37 °C for 5 days. After incubation, the viable colonies were photographed, and the numbers of colony-forming units were counted automatically by Image Pro Plus 5.0 software. Data were subsequently collected. The differences between each two groups were statistically analyzed by Tukey test. *P* values of less than 0.05 were considered statistically significant.

Results

Three 16S rRNA gene libraries were constructed from pyrosequencing of sample 1, sample 2, and sample 3 communities with 4,747, 3,543, and 3,199 high-quality sequences, respectively. Rarefaction curve was used to estimate species richness. The more flattened the slope, the greater the proportion of the discovered species diversity had, thus causing the fewer species richness. The sample 1 curve showed the most stable slope, followed by sample 2 and sample 3 (Fig. S1), indicating that their species richness also followed this trend.

We obtained 782 (sample 1), 754 (sample 2), and 778 (sample 3) operational taxonomic units (OTUs) at a 3 % distance. The sum of total observed OTUs in all three samples was 1,520, and about 160 OTUs or 10.5 % of the total OTUs were shared by them (Fig. S2). *Firmicutes* was the most dominant population (55.6 %) and *Synergistetes* was the fewest (0.6 %) in the shared OTUs. Sample 1 and sample 2 had more common OTUs (327, 21.5 % of total) than any of them with sample 3 (sample 1/sample 3, 230, 15.1 %; sample 2/sample 3, 274, 18.0 %). OTUs that were unique to each community numbered 385 (sample 1), 381 (sample 2), and 357 (sample 3), and together, they accounted for 73.9 % of the total number of observed OTUs.

In order to identify the phylogenetic composition of bacterial communities in the three samples, we assigned qualified sequences to known phyla, classes, and genera (Fig. S3). Approximate bacterial phyla were detected in the three samples, 12 (sample 1), 11 (sample 2), and 12 (sample 3), respectively. The three samples showed a similarity to a certain degree in proportion of phyla, reflected in the fact that *Firmicutes* and *Bacteroidetes* were preponderant communities in almost all of samples. The sum of two phyla accounted for 54.2 % (sample 1), 74.0 % (sample 2), and 60.4 % (sample 3) of the total reads. The clearest difference between samples was the different distribution of phylum *Fusobacteria* in the total community composition. It was the lowest in relative abundance in sample 2 (6.6 %), higher in sample 3 (13.3 %), and highest in sample 1 (21.3 %; Fig. S3a). The class level identification of the bacterial communities in the three samples is illustrated in Fig. S3b. The majority of reads, detected by pyrosequencing, belonged to 13 classes in all three communities. *Bacteroidia* accounted for a roughly similar proportion, 14.2 % (sample 1), 16.1 % (sample 2), 12.5 % (sample 3). Sample 2 and sample 3 had a similar bacterial community composition and highly enriched with *Bacilli*. The preponderant classes in sample 1 were *Clostridia* (23.6 %), *Fusobacteria* (21.3 %), and *Bacilli* (14.4 %). We could infer further about the compositions of samples on the basis of the genus level (Fig. S3c). The three samples were dissimilar in differ degree on the scales of each kind of pathogens, but these bacteria all were in the majority of samples, 63.9 % in sample 1, 81.9 % in sample 2, and 88.0 % in sample 3.

As shown in Fig. 2, fluorescence emission profiles of bacterial cells after 2 h incubated with different concentrations of HMME (10–40 μg/ml). The area beneath the curve could be used for comparison for the dosage of HMME within the microorganisms. The characteristic fluorescence emission profile is broad and centered at both 630 and

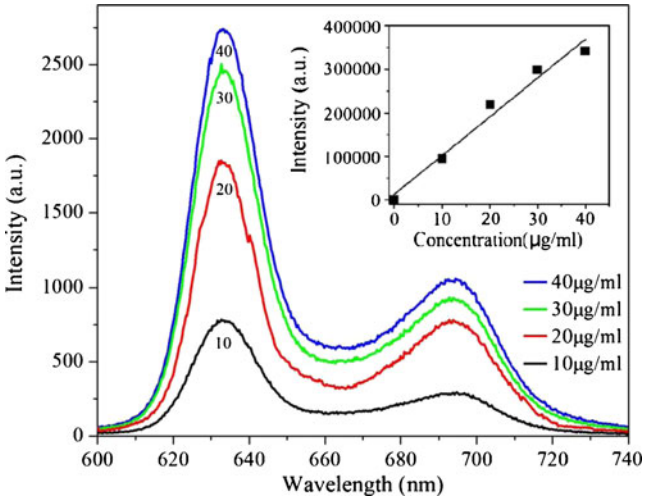

Fig. 2 Fluorescence emission profiles of bacterial cells after 2 h incubation with different concentrations of HMME (10–40 μg/ml). After stimulated by laser at λ=405 nm, HMME emit fluorescence centered at both λ=630 nm and λ=690 nm. Intracellular HMME content increases with HMME concentration and reached the maximum at 40 μg/ml in our study. The data points are marked as *filled squares* in the figure, and the relationship between concentration and fluorescence intensity is linear in the observation range

690 nm. The amount of intracellular accumulation of HMME steadily increased as the augment of photosensitizer concentration, and the relationship is approximately linear (slope= $8.90(0.85)\times10^3$). The peak value of fluorescence intensity was obtained at 40 μg/ml and the lowest at 10 μg/ml.

Figure 3 illustrates the effect of HMME-mediated lethal photosensitization on survival fraction of pathogenic microbes. Neither the irradiation of light alone (P^-L^+) nor the incubation with photosensitizer alone (P^+L^-) showed a significant bactericidal effect on the bacterial viability (P>0.05). However, when 30 μg/ml HMME was in association with a light exposure of 25 min (P^+L^+), about 97.2 % reduction in bacterial colonies was obtained, which had remarkable statistical differences from other groups (P<0.001). It suggested that the PACT method has great bactericidal ability.

The comparison result of the isolated photosensitizer group (P^+L^-) and PACT group (P^+L^+) by changing HMME concentration is presented in Fig. 4. The HMME concentrations were ranged from 10 to 40 μg/ml in both groups. Although bacterial survival fractions were decreased slightly as concentration of HMME increased in P^+L^- group, they did not achieve the level of statistical significance (P>0.05). However, after irradiated by the light source of 100 mW/cm^2 for 20 min, the PACT group displayed very prominent effect in killing bacteria compared with the isolated group (P<0.001), and the effect became more obvious with higher concentration of HMME (P<0.001). Therefore, we could conclude that the effect is not apparently available unless proper concentration of HMME is in conjunction with appropriate lighting illumination.

Figure 5a reveals the relationships among irradiation time, HMME concentration, and bacterial survival fraction. As the irradiation time and drug concentration increased, the survival rate of bacteria in every group declined. It reached the lowest value of 1.0 % when using 40 μg/ml HMME and light exposed for 25 min. It's worth noting that there was no statistical significances among 30 μg/ml HMME with 25 min irradiation and 40 μg/ml HMME with 15, 20, 25 min irradiation (P>0.05). Therefore, we inferred that

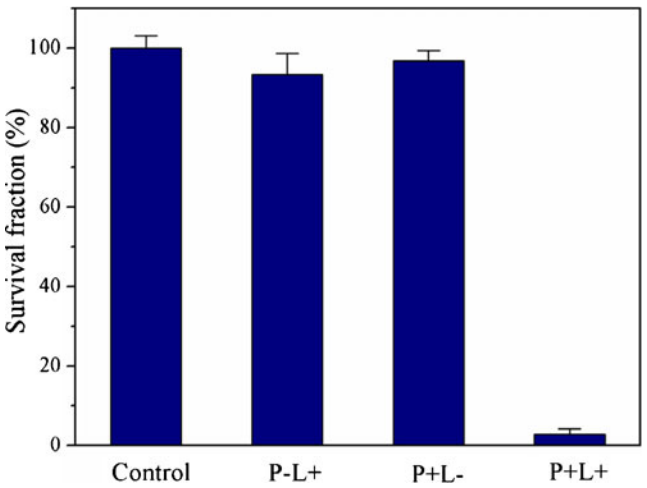

Fig. 3 Bactericidal effect of HMME-mediated photodynamic therapy (P^+L^+) against pathogenic communities from supragingival plaque. Negative control groups consisted of samples incubated with HMME in the absence of light (P^+L^-), irradiated with light in the absence of HMME (P^-L^+), and without light and HMME treatment (P^-L^-), respectively. Data represent mean values (n=15), and *error bars* represent standard deviations

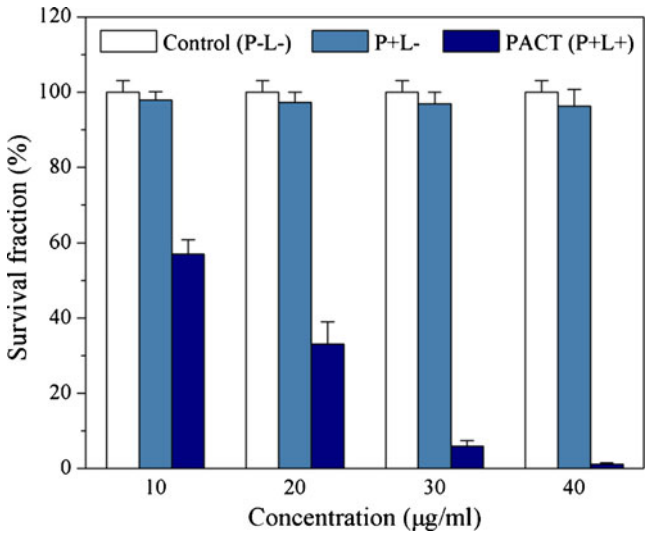

Fig. 4 A comparative study on the livability of pathogenic communities among the control group (P^-L^-), photosensitizer group (P^+L^-), and HMME-mediated photodynamic group (PACT). The concentrations of HMME were ranging from 10 mg/L to 40 μg/ml, and with light intensity of 100 mW/cm^2 exposed for 20 min in the PACT group. Data represent mean values (n=15), and *error bars* represent standard deviations

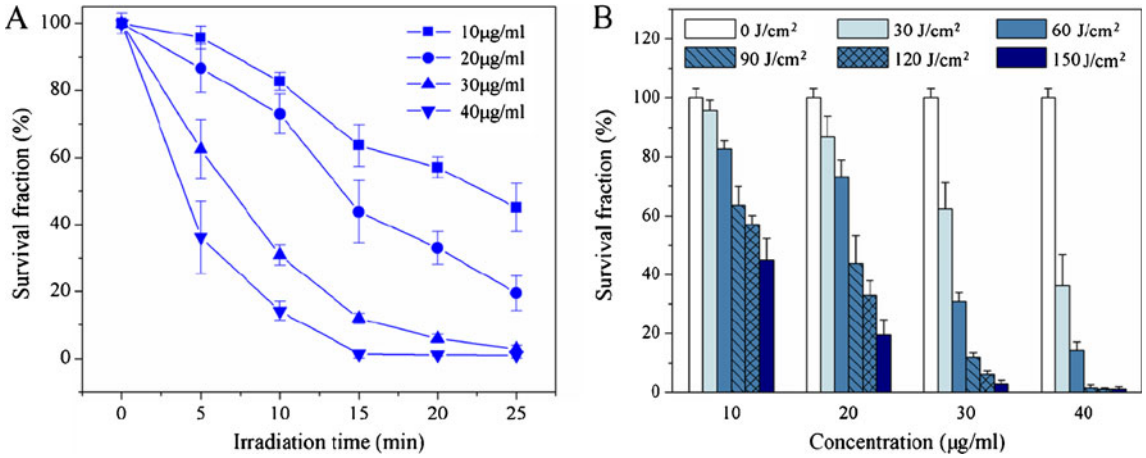

Fig. 5 The effect of variation of irradiation time/light dosage and HMME concentration on survival fraction of pathogenic communities. **a** Irradiation time ranged from 5 to 25 min was used in combination with HMME at a light intensity of 100 mW/cm^2, and *filled squares* represented 10 μg/ml. HMME, *filled circles* 20 μg/ml, *upper filled triangles* 30 μg/ml, *down filled triangles* 40 μg/ml; **b** HMME concentrations were ranged from 10 to 40 μg/ml, and the laser output remained 100 mW/cm^2. In every concentration group, the samples were exposed to a laser energy from 30 to 150 J/cm^2. Data represent mean values (n=15), and *error bars* represent standard deviations

both 10 and 20 μg/ml HMME might achieve the lowest survival fraction, differing only in the length of irradiation time. In other groups, the amounts of survived bacteria reduced obviously following the increase of HMME concentrations at the same irradiation time (P<0.05). The survival fractions of microbes all showed downward trends with increasing exposure time at the same HMME concentration. The relationship of irradiation time–survival fraction could also be converted into light dosage–survival fraction (Fig. 5b). Based on the results of Fig. 5b, there was a remarkable inverse relationship between laser dosage and bacterial survival in 10 to 30 μg/ml groups (P<0.05). The inverse relationship seemed weaker in 40 μg/ml group, even no statistical significance among 90 to 150 J/cm^2 groups (P>0.05).

Because both HMME concentration and irradiation time were inversely proportional to bacterial survival rate, we defined the product of HMME concentration and irradiation time as Ct. It was found that the relation between survival fraction of microorganism and the Ct value could be exactly fitted to an exponential function. The fitting precision of the functions was relatively high, and the R^2 was 0.93 and t1 was 3.25(0.59)×10^2 μg/ml·min. A typical recording is shown in Fig. 6. There is a negative correlation between the survival fraction and the Ct value in the exponential curve. It tended to a straight line from 600 to 800 μg/ml·min.

Discussion

Majority of PACT studies in the literature focused on the effect of single bacteria strain (Nagahara et al. 2013; Orlandi et al. 2012; Park et al. 2012); the pathogenic microorganisms of periodontitis and other oral diseases in vivo were presented as an ecosystem. Oral micro-ecological niche consists of hundreds of bacterial species, which inhabit, grow, multiply, compete, and inhibit with each other (Wright et al. 2013). Hence, there might be some differences between bacteria in an ecosystem and simple single-species microbes when killed by photosensitization. In our study, we took pathogenic communities from supragingival plaque as the research object and carried PACT mediated by HMME on them, which provided more natural oral pathogen samples and obtained more practical experimental results. Pyrosequencing revealed that though different patients' supragingival samples showed diversities to varying degrees, they all shared many kinds of algogens, some

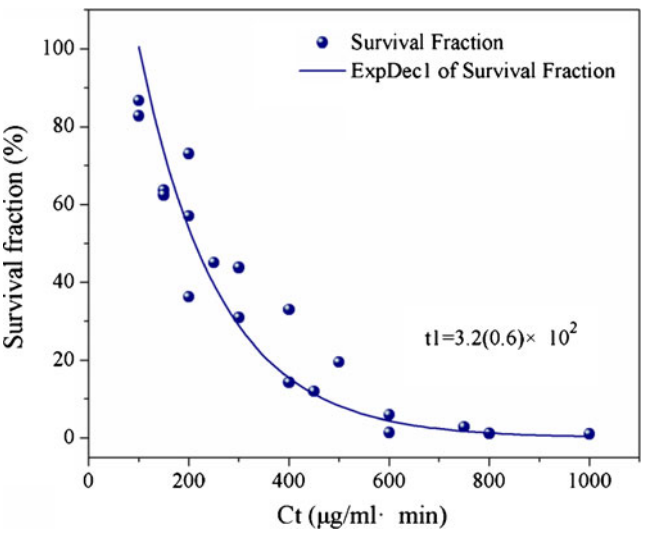

Fig. 6 Survival fraction of microbes showing an exponential fit. The recorded data points are marked as *filled circles* in the figure. The *solid curve* shows the exponential fit for the product of HMME concentration and irradiation time (Ct). The t1 was 3.25(0.59)×10^2 μg/ml·min for this case

of which could result in dental caries (*Actinomyces*, *Rothia*, *Veillonella*, *Leptotrichia*, *Granulicatella*, *Streptococcus*, etc.) and some of which should be responsible for periodontopathy (*Porphyromonas*, *Prevotella*, *Fusobacterium*, *Capnocytophaga*, *Treponema*, *Filifactor*, etc.; Figs. S1, S2, S3). We chose oral collective media BHI, supplemented with sterile defibrinated sheep blood, hemin, and menadione which could cultivate the major kinds of microbes in supragingival plaque. For experimental purposes, we amplified oral bacterial communities in vitro according to cultural rules, which would result in some changes comparing to original communities. However, the culture conditions could meet the growth of dominant pathogens, and that PACT mediated by HMME performed well on lethal photosensitization despite increase of microbiological quantity by enrichment in BHI broth (the lowest survival fraction of pathogens only 1.0 %).

The PACT effect is determined by the combination of photosensitizer and the energy input of the illumination. The interaction of light and photosensitizer increases its energy state, leading to the production of highly cytotoxic singlet oxygen ($^1O_2$) and other reactive oxygen species (ROS), which promotes phototoxic response to treat microbes in general via oxidative damage (Kharkwal et al. 2011; Kurek et al. 2011). The sensitizer has come to play an important role in the process. As a first-generation photosensitizer, hematoporphyrin derivative (HpD) is a multicomponent mixture, which has been applied in clinic for years and widely acknowledged for its good treatment effect (Patrice et al. 1990; Ward et al. 1997). HMME, a novel second-generation photosensitizer, has $^1O_2$ and ROS output higher than that of HpD by about eight times in vitro (Fu et al. 2000). Furthermore, HMME possesses a stable structure, higher singlet oxygen yield, higher photoactivity, lower dark toxicity, and faster clearance rate, especially for its cheaper price, has been widely used in photodynamic therapy treatment of tumors in China and has got good performances (Song et al. 2007; Song et al. 2011; Tian et al. 2010; Wang and Liu 2005; Zhang et al. 2011). In our study, we have demonstrated that low-cost HMME has an equally excellent action in killing bacteria in supragingival plaque. The experimental results showed that this drug also works well in PACT group in conjunction with 635 nm red light from a diode laser, but no obvious effects were found in photosensitizer alone group or light alone group (Figs. 3 and 4), which means that neither an isolated photosensitizer nor an isolated laser application can kill bacteria effectively. And the HMME mediating photodynamic inactivation is found to be in a concentration-dependent and light dose-dependent manner, with the highest concentration of HMME tested (40 μg/ml) and irradiation time of 25 min achieved a 98.97 % reduction in activity compared to control samples (Fig. 5a, b).

When the HMME concentration increased from 10 to 40 μg/ml, the number of surviving bacteria decreased obviously under the same light condition (Fig. 5a). There was no noticeable decrease in bacteria survival fraction though concentration increased in the absence of light (Fig. 4). The relationship between photosensitizer concentration and bactericidal effect is in agreement with the result of previous reports about other homologous agents (Giusti et al. 2008; Jori 2006). In Fig. 2, we can see that the fluorescence intensity of 40 μg/ml group was stronger than 10 μg/ml group by 3.6 times, which represents that the bacteria in 40 μg/ml group absorbed much more HMME. It might mean that more ROS were produced in higher concentration group. Therefore, higher concentration group led to a stronger photodynamic bactericidal effect. However, the photosensitizer must be excited by corresponding laser to generate chemical reactions because there was no effect if only using HMME. The results also proved that HMME is rather safe for use since the bactericidal effect is localized only at sites under light irradiation.

The irradiation time also had a determinant effect on lethal photosensitization of the organisms (Fig. 5a). The survivor bacteria decreased markedly as the illumination time increased in the presence of 10 and 20 μg/ml HMME. The consequences may attribute to the fact that greater numbers of photons were taken by HMME molecules with longer light irradiation time. In the 30 and 40 μg/ml HMME groups, the bacterial survival rates were also declined obviously from 5 to 15 min illumination, but did not show a significant change from 15 to 25 min. The reason for this outcome may be due to the fact that all photosensitizer molecules in the samples were activated adequately up to a certain level of light illumination, i.e., the absorption of photons by photosensitizer reached its saturation. And notably, though in 10 and 20 μg/ml HMME groups the lowest survival rates were not the optimal, they still showed a descending trend; the survival rate of 30 and 40 μg/ml HMME groups have entered into a plateau and even no statistical significances among 25 min group using 30 μg/ml HMME, and 15 to 25 min group using 40 μg/ml HMME (2.8±1.4, 1.4±0.6, 1.2±0.4, and 1.0±1.0 %, respectively). Based on the above analysis, we inferred that the lower HMME concentrations may attain equally satisfied effect like the higher concentrations if there was long enough irradiation time. In clinical applications, light intensity would be expected to prominently affect light penetration into the periodontal tissue. Hence in our study, we opted to use 100 mW/cm^2 light intensity to provide improved penetration. It is generally known that the results of irradiation time are consistent with light dosage when light intensity is invariant according to the physics formula. Therefore, it is easy to understand why the survival fraction decreased obviously from 30 to 90 J/cm^2, but not from 90 to

150 J/cm^2 (Fig. 5b). These tested results were also similar to that of Wilson and Pratten (1994), who used higher light intensity (66, 132, and 264 J/cm^2).

Since both HMME concentration and irradiation time have inverse relationships with bacterial survival fraction, we defined their product Ct to describe how the two factors influence the experimental results. We found that the relationship between the survival fraction of microbes and Ct value is exponential (Fig. 6). It showed a decay curve from 50 to 600 μg/ml min. It means that no matter how long the irradiation time is or what HMME concentration is in this range, we can obtain similar bactericidal effect as long as their products are equal. The figure presented an approximate linear relation from 600 to 1,000 μg/ml min. The results suggest that we can get the same effect by either higher drug concentration but shorter irradiation time or lower concentration but longer irradiation time. Accordingly, the Ct value can be chosen from 600 to 1,000 μg/ml·min for obtaining satisfactory treatment effect. For example, it can be 40 μg/ml HMME irradiated for 15, 20, 25 min or 30 μg/ml HMME irradiated for 25 min. In order to shorten the treatment time or reduce drug dosage for practical clinical applications, we can employ either irradiating 15 min with 40 μg/ml HMME or 25 min with 30 μg/ml.

In conclusion, the results of our study demonstrated that PACT using HMME with appropriate light irradiation is effective against the experimental pathogenic communities from supragingival plaque. Both illumination 15 min with 40 μg/ml HMME and 25 min with 30 μg/ml can obtain satisfactory effects. Consequently, we conjecture that the second-generation photosensitizer may be a worthy candidate agent of antibacterial therapy for plaque-related diseases. Most importantly, PACT using HMME can reduce the emergence of antibiotic resistance and promote stronger effect of photodynamic therapy. For clinical application, one could either prolong the irradiation time with lower HMME concentration, or shorten the irradiation time with higher HMME concentration according to the patient's condition. To verify and improve our experimental conclusion further, we plan to perform HMME-mediated PACT on supragingival plaque in situ of animal model and estimate which bacterial species are most effected or most resistant to treatment in a forthcoming experiment.

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