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Toluidine blue O and porphyrin-mediated photodynamic therapy on three main pathogenic bacteria of periodontitis using portable LED phototherapy device

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Photodynamic therapy (PDT) has been commonly used in treating many diseases, such as cancer and infectious diseases. We investigated the different effects of PDT on three main pathogenic bacteria of periodontitis — *Prevotella melaninogenica (P.m.)*, *Porphyromonas gingivalis (P.g.)* and *Aggregatibacter actinomycetemcomitans (A.a.)*. The portable red light-emitting diode (LED) phototherapy device was used to assess the exogenous PDT effects with different light doses and photosensitizer concentrations (Toluidine blue O, TBO). The portable blue LED phototherapy device was used to assess the endogenous PDT effects with the use of endogenous photosensitizers (porphyrin) under different light doses. We found out that both exogenous and endogenous PDT were able to restrict the growth of all the three bacteria significantly. Moreover, the optimal PDT conditions for these bacteria were obtained through this *in vitro* screening and could guide the clinical PDT on periodontitis.

Keywords: Photodynamic therapy; periodontitis; toluidine blue O; endogenous photosensitizer; survival rate.

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1. Introduction

Periodontitis is an inflammatory disease of the gingival tissue, which is caused by bacteria residing in the plaque biofilm on the subgingival tooth surface. Periodontitis is considered as the most common dental disease, which affects 30–50% of the adults in industrialized nations.[1] The inflammation leads to pocket formation in the gum tissue, attachment loss, bone destruction and possible tooth loss ultimately. Due to its high prevalence, the disease imposes a serious public-health concern. Periodontitis is a disease caused by numerous risk factors, including bacteria, host conditions and environments.[2] Numerous species of bacteria residing in the plaque biofilm are most responsible for generating and maintenance of the inflammation.[3] *Prevotella melaninogenica* (*P.m.*), *Porphyromonas gingivalis* (*P.g.*) and *Aggregatibacter actinomycetemcomitans* (*A.a*) are three kinds of main pathogenic bacteria.[4] Plaque removal and pathogen killing are the main treatments in the prevention and therapy of periodontitis. The conventional treatments include physical treatment and drug therapy. However, physical treatment might cause gingival damages and root-dentin hypersensitivity. Drug resistance of bacteria limits the drug therapy. Thus, novel treatment strategies have been used in the prevention and therapy of periodontitis, including photodynamic therapy (PDT).

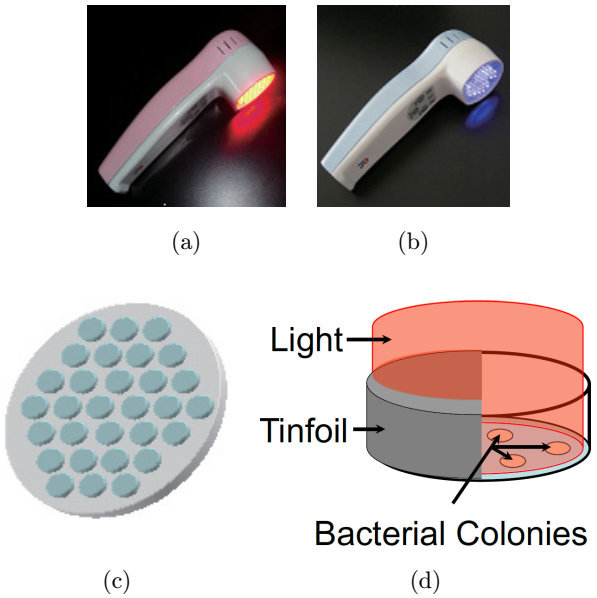
PDT is a non/minimal invasive treatment, which destructs target bacteria/cells by photochemical reaction.[2] PDT involves light-sensitive photosensitizer, light and molecular oxygen.[5] Light-sensitive photosensitizer is the core material of PDT. Exogenous toluidine blue O (TBO) and endogenous porphyrin are the mainly used photosensitizers in bacteria PDT.[1,2,4,6–8] and bring innovative insight in curing bacteria-mediated inflammation diseases, such as periodontitis. While the exogenous or endogenous photosensitizers in target tissues or cells are excited by light of specific wavelength, highly cytotoxic singlet oxygen and other reactive oxygen species (ROS) are generated by either energy or electron transfer, that lead to the inactivation of biomolecules (like proteins, nucleic acids and lipids) and cell death.[4,6]

In this study, we have used a portable red light-emitting diode (LED) phototherapy device as PDT light source and exogenous TBO as photosensitizer to assess the exogenous PDT effects under different light doses and photosensitizer concentrations. Moreover, we have used blue light source and endogenous photosensitizer to assess the endogenous PDT effects under different light doses.

2. Materials and Methods

2.1. *Portable LED phototherapy device*

The light sources used in this study were blue and red LED portable devices [Figs. 1(a) and 1(b)], which were developed by Rainbow Communications Corp. (CA, USA). The devices were used in acne PDT previously[7,8] and were composed of 30 blue LED lamps in the array of hexagon [Fig. 1(c)] to realize the uniformity of light intensity in illumination area. The blue LED light source emitted 405 ± 10 nm blue light at the power of 30 mW/cm^2 (at the distance of 2 cm away from the face), with the illumination area of about 10 cm^2. The red LED light source was similar to the blue portable device, except for the wavelength and power, which were 630 ± 10 nm and 48 mW/cm^2, respectively. Plates were illuminated in different durations to achieve different light doses, which were reflected by energy



(a) (b) (c) (d)

Fig. 1. Portable LED phototherapy device for *in vitro* periodontitis PDT: (a) Red LED phototherapy device; (b) Blue LED phototherapy device; (c) Schematic of LED array head; (d) Self-checking in *in vitro* periodontitis PDT: After tinfoil covering, only half of the plate was illuminated by LED phototherapy device. The covered half was considered as self-control to reduce the deviation of plating operation.

density (J/cm^2), the product of power density of light source (W/cm^2) and illumination time (s).

2.2. *Bacteria and culture*

The pathogenic bacteria of periodontitis used in this study were *P.m.* (ATCC25845), *P.g.* (ATCC33277) and *A.a.* (ATCC29523), which were obtained from Key Laboratory of Oral Biomedical Engineering, Ministry of Education and Ministry of Health, West China College of Stomatology, Sichuan University, and were analyzed and identified by VITEK® 2 Compact (bioMérieuxsa, Japan) in Department of Stomatology, Huashan Hospital, Fudan University. *P.m.* and *P.g.* were plated on the blood agar culture plate containing 4.6% Anaerobe Basal Agar (CM0972, BioMérieuxsa, Japan) and 5% defibrinated goat blood (Minhang Zhuqu Supply Station of Sterile Animal Blood Reagent, China) and was cultured in the presence of 80% N_2, 10% CO_2, 10% H_2 at 37°C. *A.a.* was plated on the blood agar culture plate containing 3.9% Columbia Blood Agar Base (CM0331, BioMérieuxsa, Japan) and 5% defibrinated goat blood (Minhang Zhuqu Supply Station of Sterile Animal Blood Reagent, China) and was cultured at 37°C. All operations were carried out away from light.

2.3. *PDT treatments*

Self-checking in the same plate was used in our study, which could effectively reduce plating deviation between different plates. As Fig. 1(d) showed, we used tinfoil covering half of the plate, so that this half would not be illuminated during PDT. Bacteria were suspended in normal saline and were quantified to 0.5 MCF (Mcfarland Units, about 10^8/mL) by Densicheck (Rototherm, United Kingdom), then were diluted to 10^3/mL. 0.1 mL bacterium solution were plated onto agar culture plate and illuminated by light source after five-minute culture.

In exogenous PDT treatments, we used red LED as light source and TBO (Sigma, St. Louis, MO, USA) as photosensitizer. Plates were illuminated for different durations to achieve different light doses. TBO was added into the culture plates in different concentrations during medium preparation. To avoid previous photosensitization, TBO added plates were stored in shading bags before use. In the *P.m.* group, the light doses were $1, 3, 9\,J/cm^2$, respectively, with TBO concentrations being 1, 2.5, 5, 7.5, 10 μg/mL, respectively. In *P.g.* and *A.a.* group, the light doses were 3, 9, $12\,J/cm^2$, respectively, with TBO concentrations being 10, 20, 30, 40, 50 μg/mL, respectively.

In endogenous PDT treatments, we used blue LED as light source and the endogenous porphyrin as the photosensitizer. Plates were illuminated in different durations to achieve different light doses. In the *P.m.* group, the light doses were 10, 20, 30, $40\,J/cm^2$, respectively. In *P.g.* and *A.a.* group, the light doses were 10, 20, 30, 40, $50\,J/cm^2$, respectively.

After PDT treatments, plates were cultured at 37°C in the absence of light. Subsequently bacterial colonies were counted after 48–72 h.

2.4. *Statistical analysis*

Number of colonies on treated-half of the plate was normalized to that on control-half to eliminate the error caused by plating deviation and chemical toxicity of photosensitizer. Thus, the survival rates of bacteria after PDT could be acquired with the equation: Survival rate = (number of colonies on treated-half/number of colonies on control-half) $\times$ 100%. All the data were presented as mean value and standard deviation. Meanwhile, paired t-test was used for statistical analysis to compare the number of colonies on treated-half and control-half of the plates by the SPSS version 16.0 computer program (SPSS, Inc., Chicago, IL). The significance of these differences was defined as a p value < 0.05.

3. Results

3.1. *PDT with red LED and TBO killed three pathogenic bacteria of periodontitis significantly*

To assess the exogenous PDT treatment with red LED as a light source and TBO as a photosensitizer, we used dual-factor (light dose and TBO concentration) gradient screening. As Fig. 2 showed, these exogenous PDT treatments had killing effects on all these three pathogenic bacteria. In *P.m.* group [Figs. 2(a) and 2(b)], the survival rates were about 60–90% after PDT treatments with various light doses and TBO concentrations. The lowest survival rate was $67.31 \pm 7.60\%$ ($p = 0.013$) at $3\,J/cm^2$ light

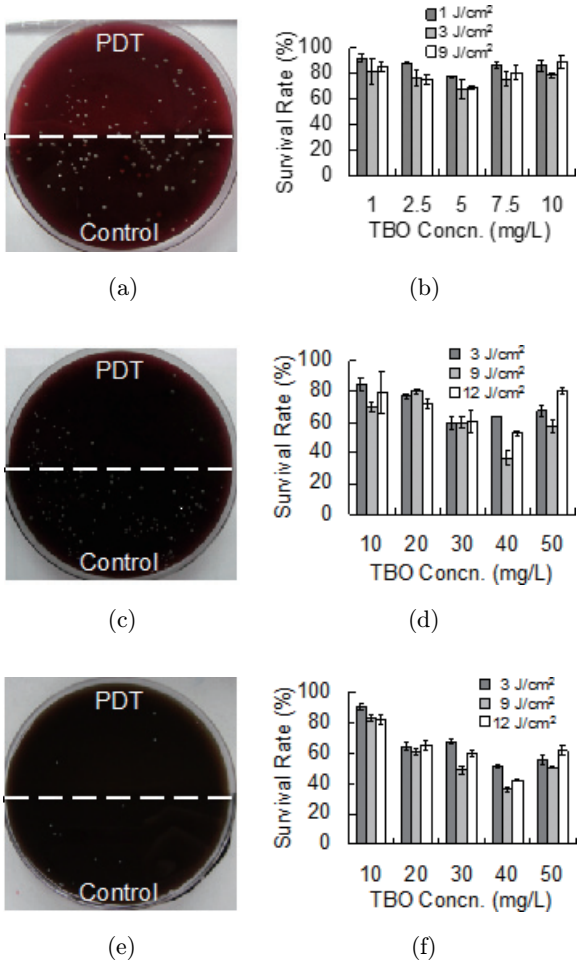


Fig. 2. Exogenous PDT (with red light and TBO as photosensitizer) effects on three pathogenic bacteria of periodontitis: (a) Typical *P.m.* plate after exogenous PDT; (b) *P.m.* survival rates after exogenous PDT with various light doses and TBO concentrations; (c) Typical *P.g.* plate after exogenous PDT; (d) *P.g.* survival rates after exogenous PDT with various light doses and TBO concentrations; (e) Typical *A.a.* plate after exogenous PDT; (f) *A.a.* survival rates after exogenous PDT with different light doses and TBO concentrations. Mean values and standard deviations (SD) were shown ($n \geq 3$) in (b), (d) and (f).

dose and 5 μg/mL TBO concentration, which was defined as best killing effect point (BKEP). Interestingly, BKEP was in the middle of the dual-factor gradient. With the same light dose, the survival rate decreased with increasing TBO concentration until 5 μg/mL, and then increased. On the other hand, with the same TBO concentration, the survival rate decreased with increasing light dose until 3 J/cm^2, and then increased. These results indicated that, after achieving the best PDT effect, increasing light dose or photosensitizer concentration did not increase PDT effects.

In comparison to *P.m.*, *P.g.* and *A.a.* were less sensitive to PDT in our preliminary experiments (data not shown). Thus, higher light doses and TBO concentrations were used in *P.g.* gradient screening. In the *P.g.* group [Figs. 2(c) and 2(d)], the survival rates were about 40–90% after PDT treatments. The survival rate of BKEP was 36.81 ± 4.24% ($p = 0.0005$) with 9 J/cm^2 light dose and 40 μg/mL TBO concentration. Similar to *P.m.* results, the light dose and TBO concentration of BKEP were not the largest ones. However, lower correlation of survival rate and dual-factor gradient were shown in *P.g.* group — volatility of survival rates was exhibited when dual-factor changed.

In the *A.a.* group [Figs. 2(e) and 2(f)], the survival rates were about 40–80% after PDT treatments. The survival rate of BKEP was 36.26 ± 1.63% ($p = 0.0014$) with 9 J/cm^2 light dose and 40 μg/mL TBO concentration, which were similar to those of *P.g.* BKEP. Similar to the previous two bacteria, the light dose and TBO concentration of BKEP were not the largest ones. Meanwhile, similar to *P.m.*, not to *P.g.*, good correlation of survival rates and PDT conditions was presented: with the same light dose, the survival rate decreased with increasing TBO concentration until 40 μg/mL, and then increased. With the same TBO concentration, the survival rate decreased with increasing light dose until 9 J/cm^2, and then increased.

In conclusion, exogenous PDT treatments had killing effect on all the three main pathogenic bacteria of periodontitis. However, different sensitivities to PDT treatment were shown (Table 1): *P.m.* was most sensitive to the PDT treatments, which

Table 1. Best exogenous and endogenous PDT effects of three pathogenic bacteria of periodontitis.

	Survival rate (%)	p value of paired t-test	Light dose (J/cm^2)	Photosensitizer concentration (mg/L)
Exogenous PDT				
P.m.	67.31 ± 7.60	0.013	3	5
P.g.	36.81 ± 4.24	0.0005	9	40
A.a.	36.26 ± 1.63	0.0014	9	40
Endogenous PDT				
P.m.	62.86 ± 2.80	0.0014	20	—
P.g.	70.83 ± 1.03	0.0017	20	—
A.a.	77.68 ± 0.56	0.0007	40	—

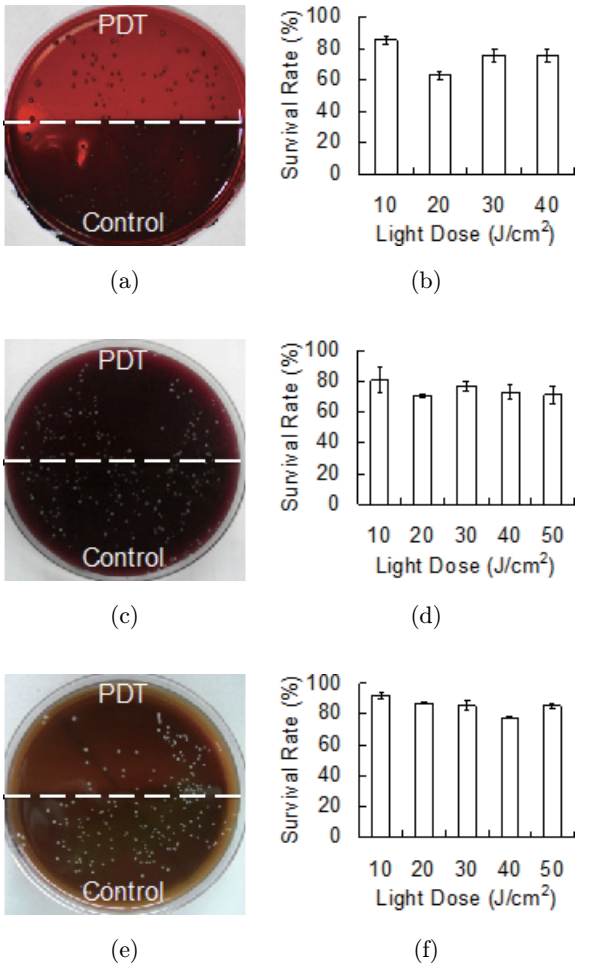


Fig. 3. Endogenous PDT (with blue light and endogenous photosensitizer) effects on three pathogenic bacteria of periodontitis: (a) Typical *P.m.* plate after endogenous PDT; (b) *P.m.* survival rates after endogenous PDT with various light doses; (c) Typical *P.g.* plate after endogenous PDT; (d) *P.g.* survival rates after endogenous PDT with various light doses; (e) Typical *A.a.* plate after endogenous PDT; (f) *A.a.* survival rates after endogenous PDT with various light doses. Mean values and standard deviations (SD) were shown ($n \geq 3$) in (b), (d) and (f).

showed lowest light dose and TBO concentration to achieve BKEP. According to our experimental data, there was no or rare colony present when we used similar PDT conditions to *P.g.* and *A.a.* groups. *P.g.* and *A.a.* presented similar PDT effects.

3.2. *PDT with blue LED significantly killed three pathogenic bacteria of periodontitis endogenously*

Many bacteria, such as *Propionibacterium acnes* in acnes[7,8] and a number of pathogenic bacteria in periodontitis,[9,10] have higher endogenous porphyrin photosensitizer level than normal tissues, which could be used for endogenous PDT. Thus, endogenous PDT might have higher therapy specificity and safety than exogenous PDT. To assess the endogenous PDT treatment with blue LED as a light source and endogenous photosensitizer, we used single-factor (light dose) gradient screening. As Fig. 3 showed, the endogenous PDT treatments had killing effects on all the three pathogenic bacteria. In the *P.m.* group [Figs. 3(a) and 3(b)], the survival rates were about 60–90% after PDT treatments with various light doses. The survival rate of BKEP was $62.86 \pm 2.80\%$ ($p = 0.0014$) with $20\,\mathrm{J/cm^2}$ light dose. Interestingly, as observed in exogenous PDT, the BKEP in this experiment occurred in the middle of the light dose gradient. With light dose increased, the survival rate decreased until $20\,\mathrm{J/cm^2}$, and then increased. These results indicated that, after achieving the best PDT effect, increasing light dose might not be able to further increase endogenous PDT effects.

Compared to *P.m.*, *P.g.* was less sensitive to endogenous PDT [Figs. 3(c) and 3(d)] — the survival rates were about 70–80% after PDT treatments with various light doses. The survival rate of BKEP was $70.83 \pm 1.03\%$ ($p = 0.0017$) with $20\,\mathrm{J/cm^2}$ light dose, which was similar to that for *P.m.* BKEP. Similar to the results of treating *P.m.*, the light dose of BKEP for treating *P.g.* was not the largest one. However, less correlation of survival rate and dual-factor gradient was shown in *P.g.* group — volatility of survival rate was exhibited when light dose changed, which was also shown in exogenous PDT treatment of *P.g.*

Interestingly, *A.a.* was less sensitive to endogenous PDT than the other two bacteria [Figs. 3(e) and 3(f)]. The survival rates were about 80–90% after PDT treatments with various light doses. The survival rate of BKEP was $77.68 \pm 0.56\%$ ($p = 0.0007$) with $40\,\mathrm{J/cm^2}$ light dose. Similar to the previous two bacteria, BKEP occurred in the middle of light dose gradient. Meanwhile, similar to *P.m.*, not to *P.g.*, good correlation of survival rate and light dose for *A.a.* treatment was obtained: with the increase in light dose, the survival rate decreased until $40\,\mathrm{J/cm^2}$, and then increased. These results indicated that, after achieving the best PDT effect, increasing light dose might not be able to further increase endogenous PDT effects.

In conclusion, endogenous PDT treatment has significant killing effects to all the three main

pathogenic bacteria of periodontitis, although less effective than exogenous PDT. However, different sensitivities to PDT treatment were shown (Table 1): *P.m.* was most sensitive to the endogenous PDT treatments, which showed lowest survival rates; *P.g.* was a little less sensitive than *P.m.* with higher survival rates but similar BKEP; *A.a.* was least sensitive, which required twice the light dose to achieve BKEP and showed a higher survival rate than the other two bacteria.

4. Discussion

Many studies have shown that PDT, with non/minimal invasiveness and side-effect treatment, has great potential in periodontitis therapy.[9–12] In our study, using portable LED phototherapy devices, which are much smaller and less costly with easier access than the light sources in previous studies, we have demonstrated that both exogenous and endogenous PDT have killing effects *in vitro* on *P.m.*, *P.g.* and *A.a.* — the three main pathogenic bacteria in periodontitis. Meanwhile, BKEPs were screened out, which would be helpful to further preclinical or clinical studies.

In our study, we found out that, in both exogenous and endogenous PDT, BKEP occurred in the middle of the PDT condition gradient, which indicated that increasing light dose or photosensitizer concentration might not further increase PDT effects after achieving the best effect. This phenomenon was also observed in previous studies.[11,13] The causes of this phenomenon might be complex: firstly, in exogenous PDT, the chemical toxic effect from the photosensitizer might overwhelm the PDT effect when the concentration is higher than BKEP concentration, which leads to the decrease in colony number in both PDT and control half-plate; secondly, higher concentration might cause dimer or multimer formation of the photosensitizer, which might inhibit singlet oxygen production in the bacteria, consequently decrease the PDT effects.

Comparing our exogenous and endogenous PDT results, we found out that exogenous PDT was more effective than endogenous PDT. In exogenous PDT, we used TBO as photosensitizer, which was commonly used in periodontitis PDT studies.[6,9] We found out that different kinds of bacteria responded differently to TBO-dependent PDT — *P.m.* was most PDT sensitive in our study. This was consistent with the previous finding that the effect of periodontitis PDT depended on bacterium species.[14] In endogenous PDT, the PDT effect depended on the endogenous photosensitizer porphyrin.[15–17] Endogenous PDT was safer than the exogenous one, because exogenous photosensitizer might have chemical toxicity and cause more side effects.[18,19] Endogenous PDT was much more commonly and effectively used in acne therapy.[7,8,20,21] However, endogenous PDT effects in periodontitis therapy remained controversial.[22–24] Here we have demonstrated that, although less effective than exogenous PDT, endogenous PDT has killing effects to the three main kinds of pathogenic bacteria in periodontitis.

In conclusion, a portable, affordable, and easy-to-use LED light source developed in this study would be useful in periodontitis PDT in a comfortable way. An *in vitro* study showed that both exogenous and endogenous PDT treatments were effective in killing pathogenic bacteria of periodontitis. Further investigation is underway to study the PDT effects of the LED light source developed here in periodontitis animal model, and further in patients.

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