Photoelimination of *Streptococcus mutans* with two methods of photodynamic and photothermal therapy

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**KEYWORDS**
Photodynamic therapy; Photothermal therapy; *Streptococcus mutans*; Antibacterial

**Summary**

**Background:** Increasing resistance of oral pathogens to conventional antibacterial agents has resulted in finding alternative therapies to overcome resistance development problems; hence this in vitro study was carried out to investigate the efficacy of photoelimination of *Streptococcus mutans* with two methods of photodynamic and photothermal therapy.

**Methods:** Standard Suspensions of *S. mutans* were treated in two groups of photodynamic therapy with Toluidine blue O and Rhadachlorin\textsuperscript{\textregistered} and photothermal therapy by EmunDo\textsuperscript{\textregistered} and their individual light sources, then bacterial suspension from each treatment was subcultured on the surface of Mueller–Hinton agar plates and bacterial growth was assessed. The results were analyzed by analysis of variance and Tukey test ($p < 0.05$).

**Results:** After treatments significant reduction of *S. mutans* viability in planktonic culture was observed in both groups of photodynamic and photothermal therapy with no priority.

**Conclusion:** Photoelimination can be a novel modality in the eradication of *S. mutans* colonies in near future.

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**Introduction**

Antibacterial agents are widely used in the treatment of plaque-related diseases such as dental caries and periodontal diseases but the emergence of bacterial pathogens resistance to commonly used antibiotics and chemotherapeutics has led to search for alternative drugs and/or therapies to overcome resistance development problems \cite{1,2}.

Oral infections primarily result from consortium of bacteria organization in biofilm and one of the main pathogenic bacteria and a key contributor to the formation of cariogenic biofilms is Mutans Streptococci (MS) \cite{3}. The MS is composed of seven species based on gene sequence analysis, including *Streptococcus mutans* and *Streptococcus sobrinus* \cite{4}. These bacteria especially *S. mutans* due to its relatively high numbers in plaque prior to the appearance of carious lesions can produce acid by sugar metabolism and sustain...
acid production at low pH levels, leading to demineralization of tooth [5,6].

Current treatment regimens of plaque-related diseases involve effective mechanical removal of plaque/biofilms and the use of antiseptics. For many patients, mechanical tooth cleaning via brushing alone is not satisfactory to achieve desired plaque control; hence, supplemental chemical methods should be added to compensate mechanical tooth cleaning. There are a number of antiseptic agents that has been clinically effective against MS, such as chlorhexidine but the efficiency of these treatments is hindered by the side effects including bitter taste, teeth staining, non-selective effect, allergic reactions and acquisition of antimicrobial resistance in oral bacteria [7,8].

As minimal intervention is a key issue in today’s dental practice, photodynamic therapy seems to be more appropriate in comparison to antibacterial conventional protocols. PDT is a nonthermal photochemical reaction, with three component of photosensitizing drug (photosensitizer), oxygen and light. In this process, the photosensitizer (PS) is administered to the target then irradiated by light of the proper wavelength leading to production of singlet oxygen and other reactive oxygen species (ROS) such as hydroxyl radicals which can cause oxidative damage on the cell membrane and cell wall and interrupt cell membrane integrity, causing permanent biological damage, respectively [9–11]. All these reaction occur within a limited space, thus this locality making PDT ideal for achieving the selective destruction of the target area [12].

Antimicrobial photodynamic therapy (aPDT) has emerged in dental practice as a powerful technique and potential alternative to antibiotics for reduction of microbiota in the oral cavity and avoid caries progression [13]. It is a non-invasive, repeatable method with high target specificity (localization) which is unlikely to develop bacterial resistance after multiple treatments [14,15].

As aPDT is an oxygen dependent process, it has some limitation in infected sites without oxygen; hence photo thermal therapy (PTT) can be more effective to eliminate microorganism through its local hyperthermic mechanism in such cases.

For a successful bacterial photoelimination some factors must be considered, e.g. selection of appropriate photosensitizer, light source and parameters [13,16]. Common photosensitizers are activated by red light sources (630–700 nm) which can penetrate 0.5–1.5 cm, respectively in biological tissue due to their relatively long wavelengths [10,16,17] in the literature various light sources have been used such as helium–neon, diode and argon lasers [18]. Although, recently some studies have mentioned the application of light-emitting diodes (LED) instead of laser. LED technology has several advantages: being compact and portable, low operation cost and ease of use [19,20].

Several studies have investigated the efficacy of PDT for microbial disinfection on oral pathogen [20–23]. However, only little published data is available on the effect of PTT on oral microorganism inactivation and comparison of these two methods; hence further studies are required as there is no precise protocol and clear method yet structured.

The aim of this in vitro study was to compare the efficacy of Photoelimination of S. mutans as a main etiological agent in tooth caries by two methods of PDT (with Toluidine blue O and Radachlorin®) and PTT (with EmunDo®).

Materials and methods

Test microorganism and growth conditions

Lyophilized S. mutans (ATCC 25175, obtained from Rayen Biotechnology Co. Ltd., Tehran, Iran) was rehydrated in brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) and incubated in an aerobic atmosphere at 37°C for 48 h. For experiments requiring cultures on plates, cultures grown in BHI broth were transferred onto brain heart infusion (Mueller–Hinton) agar plates.

Photosensitizers and light sources

For photoelimination by PDT, two different photosensitizers and light sources were used as explained below:

- Toluidine blue O (TBO) powder (Certistain®, Merck, Frankfurt, Germany) that was dissolved in distilled water to reach the concentration of 0.1 mg/ml and then filter sterilized to obtain clear and homogenous solution. The light source for activating was Light Emitting Diode (LED) 620–640 nm, peak 630 nm (FotoSan®630 LAD, CMS dental, Denmark) with output intensity of 2.000–4.000 mW/cm², within 30 s using disposable blunt tip (8 mm diameter).
- Radachlorin® solution 0.35% (Rada-Pharma Ltd., Russia) that was activated by a red Diode Laser of 662 nm (LAKHTA-MILON, Russia) with fiber optic of 800 μm with power of 300 mW and irradiation time of 30 s and energy density of 24 J/cm².

Photoelimination by PTT included one individual photosensitizer and related light source as follow:

- EmunDo® (indocyanine green) solution (A.R.C laser GmbH, Nurnberg, Germany) in combination with an infra-red laser diode of 810 nm (A.R.C laser GmbH, Nurnberg, Germany) with EmunDo® handpiece with recommended parameters of manufactures: 300 mW, continuous, 30 s and energy density of 24 J/cm².

Photoelimination process

Fresh colonies of S. mutans from Mueller–Hinton (MH) agar plates were suspended in BHI broth, and bacterial density was visually adjusted to a turbidity of 0.5 McFarland standard reagents. The exact density (CFU/mL) of each suspension was verified on MH agar plates.

S. mutans solution was prepared for ten 96-well (7 mm diameter) flat-bottom plates with lids (Orange Scientific, Belgium) separately as below:

PDT1: 1. LED + TBO (LEDT)
2. LED (LED)
3. TBO (T)
PDT2: 4. Red Laser + Radachlorin® (RLR)
5. Red Laser (RL)
6. Radachlorin® (R)
PTT: 7. Infra-Red Laser + EmunDo® (IRLE)
8. Infra-Red Laser (IRL)
9. EmunDo® (E)
10. Control (no light, no photosensitizer) (CO)

In all study groups, 200 μL of S. mutans suspension was added to 15 wells of each 96-well plate. In the groups of 1, 3, 4, 6, 7, 9: 200 μL of appropriate photosensitizer was added to suspension, while in the groups of 2, 5, 8 and 10, 200 μL of the sterile phosphate-buffered saline (PBS) was added instead in order to equalize the level of all wells. All the plates were shaken so that the samples were well mixed. The plates were then kept at dark for 5 min before irradiation letting time to photosensitizers absorption in the bacterial cells.

Irradiation was performed in a laminar flow hood (Besat, Tehran, Iran) in the dark under aseptic condition. Light devices were placed in a fixed vertical position, tangent to the surfaces of the wells which was the same in all the study groups. To prevent light transmission into neighboring wells, 15 wells of each plate with 2-well distance between them were selected and plates were covered using a black shield with an orifice corresponding to the diameter of the wells.

After the treatment, the plates were overnight incubated and the samples were then serially diluted in PBS. In order to evaluate the bacterial viability, 50 μL of each dilution was cultured on Mueller Hinton agar and incubated for 24 h at 37°C in a partial atmosphere of 5% CO2. After incubation, the number of colony forming units per milliliter (CFU/ml) was determined by a blind examiner.

Data analysis

The results were log10 transformed and analyzed by oneway analysis of variance (ANOVA) and Tukey test in SPSS statistical software version 20. Statistical significance was defined as p < 0.05.

Results

The means and standard deviations of the number of log10 CFU/ml obtained for the studied groups after treatments are demonstrated in Table 1.

Base on the results of this study, photoelimination of S. mutans with EmunDo® (PTT group), TBO and Radachlorin® (PDT groups) were statistically significant, whereas no significant differences was observed between these three groups although significant difference with other groups was obvious (Fig. 1).

In the groups treated just with the photosensitizer or irradiated only, no significant reduction of colonies was seen.

Discussion

Current study demonstrated that Photoelimination of S. mutans with Radachlorin® , TBO and EmunDo® promoted significant microbial reduction of S. mutans colonies.

TBO is a thiazine dye of the quinine-imine family and one of the major photosensitizers applied clinically in the medical field capable of inactivating both gram positive and negative bacteria in the maximum absorption wavelength of 630 nm [24,25]. Williams et al. stated that TBO mediated PDT by laser of 633 nm, energy density of 1.8 J or more produced log reduction of 8–10 cfu/ml with antibacterial action directly proportional to energy dose in planktonic suspension of S. mutans [25]. The bacterial reduction in our study was about 2 logs which was significant but lower than Williams’ results. One possible explanation is the light sources differences as we used LED instead of laser; however the amount of this bacterial reduction is acceptable in clinical practice. Zanin et al. reported that S. mutans in biofilms can be killed when treated with TBO with the concentration of 0.1 mg/ml, similar to the current study, irradiated by either a He–Ne laser or an LED interestingly with similar results [6,26]. The result of current study confirmed the positive bactericidal effect of PDT with TBO and LED similar to Zanin’s papers.

Chlorins are second generation of photosensitizers, absorbing light in the range of 640–700 nm. In this article we used Radachlorin® which is a chlorophyll a derivative, mainly including sodium chlorine e6 with low toxicity in dark, high contrast of tumor accumulation and much more rapid body evacuation [27,28]. We used this photosensitizer with 662 nm diode laser and observed significant reduction of bacterial load.

Researches on Radachlorin® are limited. Fekrazad et al. stated that combination of He–Ne laser with Radachlorin® gel 0.1% was effective on the reduction of S. mutans colonies, although cytotoxic effect of Radachlorin® was seen in dark [28]. This result is similar to the current finding but in our study Radachlorin® did not show any cytotoxic effect, maybe because of different form and concentration of Photosensitizer used in our research.

Yahabi et al. declared that PDT with TBO 0.1% and 633 nm laser at 3 J/cm2 was effective on eliminating S. mutans, whereas PDT with Radachlorin® plus 662 nm laser had no effect on reducing the viability of S. mutans [29]. Results of PDT with TBO confirmed the current finding but the result of the present study with Radachlorin® was different maybe due to the different laser parameters in these two researches.

Another P5 which was inserted to the study was EmunDo® as the new presented photosensitizer in the field of dentistry. The manufacturer has stated that EmunDo® Include mainly indocyanine green dye. Indocyanine green (ICG) is a water-soluble tricarboxycin dye that strongly absorbs in near infra-red (NIR) region. ICG is approved by the US Food and Drug Administration for medical diagnostic studies, such as visualizing retinal and choroidal vasculatures [30,31]. ICG exhibits a high absorption cross-section in the infra-red region at 805 nm leading to more penetration depth in comparison to many other sensitizers [32,33]. The antibacterial mechanism of ICG is controversial. W Bäumler et al. evaluated the mechanism of ICG cell killing on human colonic cancer cells and stated that the absorbed energy of the laser light by ICG was converted either to heat or transferred to molecular oxygen via triplet-state and concluded that second procedure was more effective in cell death [33]. In contrast with previous article Gomes et al. reported that Photodamage of cells after incubation with ICG solution is due mainly, to free radical formation from excited states in comparison to the formation of singlet oxygen [34]. While in acne treatment studies, photothermolysis is the main mechanism of ICG for destroying sebaceous glands [35,36]. Nagahara
et al. investigated the bactericidal effect of PDT on Porphyromonas gingivalis using ICG-loaded nanospheres photosensitizer with an 805 nm diode laser and observed significant reduction of P. gingivalis (approximately 2-log 10 bacterial killing). They also examined changes in temperature during irradiation and reported at least increase of 4.23 °C at 1 min [31]. In agreement to our study EmunDo® solution with laser 810 nm led to similar reduction in S. mutans colonies.

Based on the EmunDo® manufacture, the bactericidal effect of this PS is due to the photothermal action; however photodynamic mechanism has not been mentioned. To our knowledge, this present research is the first study which has investigated the antibacterial feature of EmunDo®; hence

Table 1 Means ± standard deviation and $P$ value of log10 CFU/mL obtained for all study groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean</th>
<th>Std. deviation</th>
<th>Mean difference with control</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDT1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEDTA</td>
<td>6.3440</td>
<td>.40518</td>
<td>−1.4527</td>
<td>.000</td>
</tr>
<tr>
<td>LEDB</td>
<td>7.6927</td>
<td>.02915</td>
<td>−.1040</td>
<td>.983</td>
</tr>
<tr>
<td>TC</td>
<td>7.6900</td>
<td>.03000</td>
<td>−.1067</td>
<td>.980</td>
</tr>
<tr>
<td>PDT2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RLD</td>
<td>6.4347</td>
<td>.47245</td>
<td>−1.3620</td>
<td>.000</td>
</tr>
<tr>
<td>RLE</td>
<td>7.7013</td>
<td>.05927</td>
<td>−.0953</td>
<td>.991</td>
</tr>
<tr>
<td>RLF</td>
<td>7.6533</td>
<td>.05715</td>
<td>−.1433</td>
<td>.877</td>
</tr>
<tr>
<td>PTT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRLE</td>
<td>6.4073</td>
<td>.46063</td>
<td>−1.3893</td>
<td>.000</td>
</tr>
<tr>
<td>IRL</td>
<td>7.7280</td>
<td>.20557</td>
<td>−.0687</td>
<td>.999</td>
</tr>
<tr>
<td>E</td>
<td>7.6200</td>
<td>.04629</td>
<td>−.1767</td>
<td>.677</td>
</tr>
<tr>
<td>CO</td>
<td>7.7967</td>
<td>.06309</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Significant difference between study groups and control ($p < 0.05$, Tukey test).
A LED + TBO.
B LED.
C TBO.
D Red Laser + Radachlorin®.
E Red Laser.
F Radachlorin®.
G Infra-red Laser + EmunDo®.
H Infra-Red Laser.
I EmunDo®.
J Control.

Figure 1 Effects of treatments in each group of study (PDT1, PDT2 and PTT) on the viability of S. mutans (log-transformed mean CFU/ml).
more studies should be carried out to achieve more reliable results but PTT as a non-oxygen dependent method may has some advantages over PDT especially the combination of ICG with 810 nm laser can enhance elimination of bacteria in deeper and low oxygen areas such as deep periodontal pockets, Although thermal damage of surrounding tissue should be taken in account.

The results demonstrated that neither of light sources nor photosensitizers alone had any effect on S. mutans viability, which is in accordance to other similar articles [37,15].

Current study investigated the antibacterial effects on S. mutans in planktonic cultures not in biofilms. As biofilms offer some advantages for microorganism such as resistance to antimicrobial agents and increased protection against the host immune system, bacteria biofilms have been shown to be less susceptible by a photodynamic procedure than bacteria in planktonic phase [38] thus we suggest further studies on biofilms to reach more definite conclusion.

Application of microbial photoloinization methods in dentistry is growing rapidly and based on the results of this study and other similar experiments it can be proposed as a novel modality adjuvant to conventional methods in prevention and treatment of dental plaque-related disease like rampant caries cases and decreasing the load of bacteria in cavity preparation in order to avoid secondary caries developments.

Although the results of this study are encouraging, more evidence-based data about the light parameters and photosensitizer property is still required for clinical dental practice.

Conclusion

Within the limitations of this in vitro study, we can conclude that both methods of photoloinization by PDT and PTT could significantly reduce the load of S. mutans with no priority; although neither of light sources nor photosensitizers alone had any effect on S. mutans viability.

Conflicts of interest

The authors reported no conflicts of interest related to this study.

Acknowledgements

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