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The susceptibility of *Streptococcus mutans* to antibacterial photodynamic therapy: a comparison of two different photosensitizers and light sources

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ABSTRACT

Streptococcus mutans is the main etiological agent for dental caries. Recently, photodynamic therapy (PDT) has been introduced as a new modality in bacterial decontamination. Objective: This *in vitro* study was carried out to evaluate the susceptibility of *Streptococcus mutans* to antibacterial PDT using two different photosensitizers and light sources. Material and Methods: Standard suspensions of *S. mutans* were exposed to laser light at 662 nm and Radachlorin® or LED 630 nm in combination with Toluidine blue O (TBO). Radiation-only groups, photosensitizer alone, and groups with no treatment were used as controls. Bacterial suspension from each treatment was subcultured onto 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: PDT with TBO and Radachlorin® significantly reduced *S. mutans* viability, whereas no difference was observed between two groups of PDT. In the groups treated just with the photosensitizer or irradiated alone, no significant reduction of *S. mutans* colonies was observed. Conclusion: *S. mutans* colonies were susceptible to either 662 nm laser or LED light in the presence of Radachlorin® and TBO respectively with no priority.

Keywords: *Streptococcus mutans*. Antibacterial agents. Photodynamic therapy.

INTRODUCTION

Dental caries is the most common disease in the world, and comes about as the result of plaque biofilm formation on the teeth inside the mouth[1,27]. The main etiological factor for caries on smooth surfaces is the group of streptococci, mainly *mutans streptococci* (MS), most notably *Streptococcus mutans*. For the development of cariogenic biofilms, the colony forming of MS on tooth surface has an important role as this bacterium is able to cause oral pH reduction following the production of acids, leading to tooth demineralization[18,23].

Anti caries procedures can be categorized into two phases: prevention and treatment strategies. In the prevention phase, caries control is based on limited consumption of sugar and good oral hygiene, which is achieved through mechanical and chemical removal of the biofilm, including brushing and use of antimicrobial agents[4,21].

However, these methods have some limitations such as mechanical damage to the oral mucosa in patients with mechanoblistering disease caused by brushing or scraping, as well as antibacterial-resistance of biofilm species and the difficulty to maintain therapeutic concentrations of antimicrobials in the oral cavity[18,27].

In the treatment phase and removal of caries, it is difficult to detect the exact caries-removal endpoint. A caries lesion consists of two different layers: the outer layer, known as infected dentin, which is characterized by softened dentin with a large number of bacteria and the inner layer, known as affected dentin, contaminated with

fewer bacteria, that is usually subjected to remineralization. However, the clinical distinction of these two regions is extremely difficult, and usually conventional methods involve the removal of both infected and affected layers which can result in pulp exposure in deep carious lesions. As such, conservative cavity preparations behind affected dentin can cause remineralization and prevent accidental pulp exposure[13].

Since caries are localized infections, as well as considering the limitation of traditional treatments, alternative protocols such as Photodynamic Therapy (PDT) can be proposed[2]. PDT is a therapeutic process, involving the combination of light and photosensitive agents called photosensitizers[14].

The photodynamic process is a two-step protocol, in which target cells are exposed to a photosensitizer and irradiated with a harmless light in the maximum absorption of the sensitizer wavelength, leading to the production of singlet oxygen and free radicals that can damage essential components of the cells, such as plasma membrane and DNA, or of modifying metabolic activities in an irreversible way, thus possibly resulting in cell death[9,13].

PDT has several applications in Dentistry such as the treatment of oral cancer, bacterial, viral and fungal infections and photodynamic diagnosis of the malignant oral lesions[19].

Antimicrobial PDT (a-PDT) is a localized, non-thermal and non-invasive antimicrobial method to decrease bacterial contamination in oral infections[3,14,22].

Several studies have illustrated that PDT has a strong effect on a large number of oral gram-positive and negative bacteria, using different photosensitizers and light sources[7,13,15]. However, the results of these studies are somewhat different and are not always clear. Hence, the purpose of this study was to compare the antibacterial effect of PDT on *S. mutans* with two different photosensitizers and light sources.

MATERIAL AND METHODS

Test microorganism and growth conditions

Lyophilized *Streptococcus mutans* (ATCC 25175, obtained from Rayen Biotechnology Co. Ltd., Tehran, Iran) were rehydrated in brain heart infusion (BHI) broth (Merck KGaA, 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; Conda, Madrid, Spain) plates.

Photosensitizers and light sources

In this study two different photosensitizers and light sources were used:

Toluidine blue O (TBO) powder (Certistain®, Merck KGaA, Darmstadt, Germany) was used and dissolved in distilled water to reach the concentration of 0.1 mg/ml, where the filter was sterilized to obtain clear and homogenous solution. The light source for activation was Light Emitting Diode (LED) (FotoSan® 630 LAD, CMS dental, Copenhagen, Denmark) with output intensity of 2.000-4.000 mW/cm^2, within 30 seconds.

Radachlorin® solution 0.35% (Rada-Pharma Co, Ltd., Moscow, Russia) was used which was activated by Diode Laser 662 nm (LAKHTA-MILON, Saint-Petersburg, Russia) with fiber optic of 800 µm with power of 300 mW and irradiation time of 30 seconds and energy density of 24 J/cm^2.

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 reagent. The exact density (CFU/mL) of each suspension was verified on MH Agar plates.

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

1) Laser+Radachlorin® (L^+ Rad^+)
2) LED+TBO (LED^+ TB^+)
3) Laser (L^+ Rad^-)
4) LED (LED^+ TB^-)
5) Radachlorin® (L^- Rad^+)
6) TBO (LED^- TB^+)
7) Control (no light, no photosensitizer)

In each study well of the plates, 200 µL of *S. mutans* suspension plus 200 µL of related photosensitizer were added. In the groups of 3 (Laser), 4 (LED) and 7 (Control), 200 µL of the sterile phosphate-buffered saline (PBS) was added to equalize the level of all the wells. Samples were then kept in the dark for 5 minutes before irradiation.

Irradiation was performed in a laminar flow hood (Besat, Tehran, Iran) in the dark under aseptic conditions. Light devices were fixed in vertical positions at the level of the wells. To prevent light transmission into neighboring wells, 15 wells of each plate, with 2-well distance between them, were selected and plates were covered with a black shield with an orifice corresponding to the diameter of the wells.

After the treatment, the plates were incubated overnight. The samples were then serially diluted in PBS. In order to evaluate bacterial viability, 50 µL of each dilution were cultured on Mueller Hinton Agar and incubated for 24 hours at 37°C in a partial atmosphere of 5% CO_2. After incubation, the number of colony forming units per milliliter (CFU/ml) was determined. The results were log-transformed and analyzed by analysis of variance

Table 1- Means±Standard deviation and P-value of the number of log10 obtained for the different groups

Group	Mean ± Standard Deviation	P Value
L+ Rad+	6.43 ± .47	.000*
LED+ TB+	6.34 ± .40	.000*
L+ Rad-	7.71 ± .05	0.889
LED+ TB-	7.70 ± .07	0.772
L- Rad+	7.65 ± .11	0.414
LED- TB+	7.69 ± .09	0.711
Control	7.79 ± .09	-

*significant difference between study groups and control ($p<0.05$, Tukey test)

(ANOVA) and Tukey test using SPSS statistical software version 20. Statistical significance was defined as $p<0.05$.

RESULTS

Table 1 shows the means and standard deviations of the number of log10 obtained for the studied groups.

The reduction of *S. mutans* viability in groups of PDT with TBO and Radachlorin® was statistically significant. There was no significant difference between the two groups of PDT, however significant difference was observed between the PDT and other groups.

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

DISCUSSION

The current study illustrated that Photodynamic Therapy (PDT) with both photosensitizers (PS) and their individual light sources led to a significant reduction in the viability of *S. mutans* with no significant difference between two groups of PDT (LED+TBO and Radachlorin®+Laser).

One of the photosensitizers that were used in this study was TBO. TBO is a cationic phenothiazine derivatives photosensitizer of affordable cost and with maximum absorption wavelength in the red light spectrum of 630 nm, capable of inactivating both gram positive and gram negative bacteria. This is mainly due to its physical and chemical properties and hydrophilic features that allow its free passage across the bacterial membrane and consequently the attraction to the negatively charged potential of mitochondria, which allows direct action on this organelle[16,21]. Another article proposed that TBO may bind to the polyphosphates of the outer membrane and produce molecular damage to lipids and proteins, including membrane-bound enzymes[24].

There is a large number of studies on the efficacy of TBO in PDT and most of them have confirmed the effectiveness of this photosensitizer[6,11,25,26,28,29].

In the current study the light source for activating TBO was LED, while in other studies Laser was used instead. LED is a non-monochromatic light that has become a practical technology for PDT in the last few years, especially for irradiation of easily accessible tissue surfaces. The main advantages of LED over Laser are their low cost and ease configuration of LED arrays into different irradiation geometries[16].

Rolim, et al.[21] (2012) examined the antimicrobial activity of various photosensitizers against *S. mutans* with the same concentration by analyzing the generation of O_2 and reported that amongst the photosensitizers of methylene blue (MB), toluidine blue ortho (TBO), malachite green (MG), eosin (EOS), erythrosine (ERI) and rose Bengal (RB), TBO is the only photosensitizer that effectively reduced 99.9% of these microorganisms.

Zanin, et al.[29] (2006) used TBO with the concentration of 0.1 mg/ml, similar to the current study, combined with LED in order to achieve the photodestruction of oral biofilm and reported that TBO-mediated PDT can significantly decrease the load of *S. mutans* in the biofilm. Giusti, et al.[8] (2008) also stated that the greatest effect on *S. mutans* was obtained with TBO at 0.1 mg/ml in combination with LED which was in agreement with our results.

Furthermore, in another research, Lima, et al.[13] (2009) confirmed that PDT with TBO was effective in killing oral microorganisms present in dentin caries produced *in situ* and may be a useful technique for eliminating bacteria from dentin carious lesions before restoration.

Radachlorin® was the other photosensitizer which was used in combination with laser 662 nm. It is a chlorophyll *a* derivative, including mainly sodium chlorin e6[5]. Research on PDT with Radachlorin® is limited. However, some clinical trials on this sensitizer have clarified significant advantages such as very low toxicity in the dark, high contrast of tumor accumulation, much more

rapid body evacuation (only two days), intensive absorption band at relatively large wavelengths where tissues are more transparent and finally the high phototoxicity[10,20].

Fekrazad, et al.[6] (2011) studied the antibacterial effect of PDT on *S. mutans* with He-Ne Laser (633 nm) and reached the conclusion that a combination of this laser with Radachlorin® 0.1% gel photosensitizer was more effective than single use of laser or Radachlorin® alone on reduction of *S. mutans* colony count (although cytotoxic effect of Radachlorin® was seen in the dark). This result is similar to our findings, but in our study Radachlorin® did not show any cytotoxic effet; this may be due to the different form and concentration of PS used in these two studies.

Vahabi, et al.[26] (2011) declared that only PDT with TBO 0.1% and 633 nm diode laser at 3 J/cm^2 was effective in eliminating *S. mutans*, whereas PDT with Radachlorin® plus 662 nm laser had no effect on reducing the viability of *S. mutans*. Results of PDT with TBO confirmed the current finding, but the result of the current study with Radachlorin® was different. One possible explanation is that the laser parameters were not the same in these researches.

This study showed that neither of the light sources nor photosensitizers alone had any effect on *S. mutans* viability, which is in accordance to other similar articles[12,17].

Based on the results of the current research and other *in vitro* and *ex vivo* studies, it appears that PDT can be helpful for elimination of cariogenic bacteria prior to restorative proceduth while minimizing the excavation of the affected dentine. Consequently, this can allow maximal preservation of tooth tissue and long term prognosis of the restored tooth can be predicted with minimal risk of secondary caries formation. In addition, in patients with high risk of caries, PDT can be a novel approach as a preventive protocol to control caries, although the high cost of PDT must be considered.

In addition to what is stated above, selection of an appropriate photosensitizer with related light source considering bacteria species should be taken into account in any photodynamic therapy and further studies on the effect of a-PDT, not only on single species of bacterial but on oral biofilms, are still required to obtain more definitive and certain results.

CONCLUSION

The results of the present study in its conditions demonstrated that *S. mutans* colonies were susceptible to either 62 nm laser or LED light in the presence of Radachlorin® and TBO respectively with no significant difference. Therefore, we can conclude that PDT with these photosensitizers may be helpful in caries preventive and treatment protocols.

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