

The Effect of Antimicrobial Photodynamic Therapy with Radachlorin and Toluidine Blue on Streptococcus Mutans: An in Vitro Study

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Abstract:

Objectives: Dental caries and periodontal diseases are caused by infection of teeth and supporting tissues due to complex aggregate of bacteria known as biofilm, firstly colonized by streptococci. The main purpose of this in vitro study was to evaluate the antimicrobial effects of toluidine blue O (TBO) and Radachlorin® in combination with a diode laser on the viability of Streptococcus mutans.

Materials and Methods: Bacterial suspensions of Streptococcus mutans were exposed to either 0.1% TBO associated with (20 mW, 633 nm diode laser, continuous mode, 150 s) or 0.1% Radachlorin® and laser irradiation (100 mW, 662 nm diode laser, continuous mode, 120 s). Those in control groups were subjected to laser irradiation alone or TBO/Radachlorin® alone or received neither TBO/Radachlorin® nor laser exposure. The suspensions were then spread over specific agar plates and incubated aerobically at 37°C. Finally, the bactericidal effects were evaluated based on colony formation.

Results: Potential bacterial cell killing was only observed following photosensitization with TBO and 3 j/cm² laser exposure (p<0.05), whereas Radachlorin® showed significant reduction in dark condition compared to laser exposure (p<0.05).

Conclusion: TBO-mediated photodynamic therapy seems to be more efficient than Radachlorin® in significantly reducing the viability of Streptococcus mutans in vitro.

Key Words: Toluidine Blue; Radachlorin®; Antimicrobial Photodynamic Therapy (APDT); Diode Laser; Streptococcus Mutans

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INTRODUCTION

The human oral cavity is colonized by a highly diverse community of bacteria. Most of the bacteria existing in the oral cavity are present as complex aggregates known as biofilms on the surfaces of the teeth and these biofilms are

termed “dental plaques” [1-2]. Streptococci are said to be the first bacteria which colonize in oral surfaces and may consist 70% of the cultivable bacteria existing in the human dental plaque and Streptococcus mutans as the primary odontopathogen presents in the supragingival

plaque and results in one kind of oral disease known as dental caries [3-4].

Since both dental caries and periodontal disease start initially by plaque accumulation on the oral soft and hard tissues, conventional mechanical debridement and good oral hygiene may accomplish a temporary decrease of microorganisms in dental plaques [5]. To overcome these problems, it is essential to develop new antimicrobial therapeutic approaches. Recently, vaccines for dental caries and periodontal diseases have been produced and applied on patients [6-7]. One alternative method is photodynamic therapy (PDT), showing great potential for the treatment of neoplastic and non-neoplastic diseases which was firstly demonstrated by Jodlbauer and von Tappeiner in 1904 [8-9]. In this method, a photoactive dye, termed a photosensitizer [PS] is taken up into the cells and is then irradiated with light of an appropriate wavelength. This may end in cell death through the production of active oxygen molecules [10]. Generally, in photosensitization processes, the laser or PS alone are not toxic [11] and only cells that contain the photosensitizer and also receive laser are affected by the treatment finally. Thus, the use of this method provides an opportunity to achieve selectivity and to target specific sites of the mouth or the plaque [12]. Antibacterial photosensitizers currently under investigation for use in the mouth include TBO and chlorin e6 [13-14]. These agents show great promise, but will necessarily be subjected to lengthy experimental and clinical assessments. However, more benefits could be derived from photosensitizers recently certified for oral use. One such photosensitizer is Radachlorin® which is a chlorophyll *a* derivative, including mainly sodium chlorin e6, having been successfully applied in diagnosing tumors and treating surface tumors [15].

There have been only a few studies on the antimicrobial photodynamic therapeutic (APDT) effects of Radachlorin®, although there have

been several studies on chlorin e6, which is a major component of Radachlorin® [14,16]. On the other hand, TBO is a widely known photosensitizer that has been in use for many years and is efficient in producing singlet oxygen under the maximum absorption wavelength of 630 nm. TBO has also been reported as an effective dye for inactivation of yeasts, gram positive and gram negative bacteria in assistance with laser irradiation [17-19]. Therefore, this prompted us to carry out an in vitro study on the subject of the antimicrobial photodynamic effect of Radachlorin® in comparison with TBO on the viability of *Streptococcus mutans* to enhance PDT application in plaque-related disease treatment.

MATERIALS AND METHODS

Bacterial culture: The standard strain of *Streptococcus mutans* (ATCC 35668, PTCC 1683) was purchased from the Iranian Science Organization of Science and Technology (IROST) in Tehran, Iran.

The bacterium was subcultured on mitis salivarius agar (Quelab, Canada) and then incubated at 37°C in the presence of 10% CO₂ for 24 hours. Overnight cultures were prepared in Trypticase Soy Broth (Merk, Germany) by transferring a few colonies grown on mitis salivarius agar. The bacterial suspensions were then diluted in broth to an optical density of McFarland No: 0.5 (approximate numbers 1.5×10^8 bacteria mL⁻¹).

Photosensitizers and laser sources: Radachlorin® gel (0.1%, 25 g) was obtained from RADA-FARMA Ltd, Russia and stored at 0-8 °C in the dark (Fig 1).

Toluidine blue powder was taken from Micromedia chemicals-Hungry, dissolved in sterile saline firstly to reach the final concentration of 0.1% and then subsequently kept in the dark (Fig 2).

The laser sources used for each photosensitizer were a diode laser (Milon-LAHTA, Russia)



Fig 1. Radachlorin®



Fig 2. Toluidine Blue



Fig 3. Diode laser (Milon-LAHTA, Russia)



Fig 4. Diode laser (Mustang 2000, Russia)

(Fig 3) with a fiber optic diameter of 800 micrometer, a maximum output of 2.5 W and a predominant wavelength of 662 nm for Radachlorin® and the irradiation of a diode laser (Mustang 2000, Moscow, Russia) (Fig 4) with a hand held probe of KLO4 and a maximum power output of 30 mW at a wavelength of 633 nm for TBO. The distance between the laser tips and the illuminated area was adjusted to create a spot light of 1 cm in diameter with a fixed power density for each photosensitizer.

Photodynamic therapy: The laser parameters used in this study for bacterial suspension were 100 mW/cm² (power density) and 12 J/cm² (energy density) and continuous mode

for Radachlorin® and 30 mW/cm², 3 J/cm² and continuous mode for toluidine blue O.

The concentration of 0.2 ml of each photosensitizer was applied on 0.2 ml of the bacterial suspensions. The following groups were used: (I) L⁻ PS⁻ (no laser, no photosensitizer), (II) L⁻ PS⁺ (treated only with PS), (III) L⁺ PS⁻ (treated only with laser) and (IV) L⁺ PS⁺ (treated with laser and PS: photodynamic therapy group). Group I and II were kept in the incubator at 37°C in the presence of 10% CO₂.

Bacterial suspensions in group III and especially group IV which were incubated with PS for 10 minutes in the dark at room temperature, were exposed to 662 and 633 nm laser from above for 120 and 150 seconds in the

dark at room temperature and subsequently transferred to the incubator. After overnight incubation of all groups, they were cultured on mitis salivarius agar and viable microorganisms grown on the plates were counted in the next day.

Statistical analysis: In order to access the differences between the groups, the variable bacterium reduction promoted by each treatment was analyzed by Kruskal-Wallis and Mann-Whitney U test. Statistical significance was accepted at $p < 0.05$. The Statistical Package for Social Sciences (SPSS)16 for Windows, (SPSS Inc, Chicago, IL, USA) was used for data analysis.

DISCUSSION

The growing bacterial resistance in spite of antibiotic drugs, conventional mechanical debridement and chemical agents has questioned the efficiency of these therapies. To overcome these problems, photodynamic therapy has become a possible alternative antibacterial therapy for plaque-related diseases such as dental caries. The advantages of PDT over conventional antimicrobial agents are non-invasive nature, ease of reaching deeply situated areas, repeatability, high selectivity, no resistance to drugs, rapid killing of target microorganisms in a few minutes depending mainly on the energy densities delivered (on the contrary, in conventional antimicrobial agents hours or even days are necessary) and finally that antimicrobial effects may be limited to the site of the lesion by careful topical application of photosensitizers and the site of irradiation may even be restricted further by using an optical fiber [21-22]. In fact, in the human oral cavity, there are very large numbers of bacterial species, which comprise a complex ecosystem. Thus, the response of the bacterial community to photodynamic treatment may differ greatly from that of their in vitro cultured isolates in many aspects, such as growth rate, metabolic

activity and gene expression [23-24]. Wainwright also demonstrated that photodynamic inactivation (PDI) of microorganisms depends on the chemical structure of PS and the incubation time of the drug with the bacterial cells. Damage to the bacterial cell wall, increased permeability of cytoplasmic membrane and nucleic acid strand breakage may be resulted following with PDI [25]. Based on these advantages, several studies were carried out using PDT approving that oral bacteria are susceptible to PDT [26-27]. Photosensitizers are vital elements in PDT; several studies have demonstrated the efficacy of a range of photosensitizers in the elimination or reduction of oral bacteria [11,28-29]. TBO is an attractive option because of its affordable cost and intense absorption wavelength in the red light spectrum (> 600 nm) [30], while Radachlorin® is a chlorophyll *a* derivative, including mainly sodium chlorin e6, which has already passed complete pre-clinical assessments. These clinical trials 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 [15,31]. Soukos, Rovaldi and Pfitzner determined the antimicrobial activity of chlorin e6 derivatives like Poly-L-Lysine chlorin e6 conjugates and new photosensitizer BLC1010, BLC 1014 on anaerobic bacteria compared with pure chlorin e6 [14,32,33]. Risovannaia also reported that Radachlorin®-mediated photodynamic therapy could eliminate *Streptococcus pyogenes* in the animals infected tissues [34]. The results obtained in this study demonstrated that TBO-mediated photodynamic therapy was more successful compared to Radachlorin® in effective bacterial reduction. The most effective combination is 0.1% TBO with 3 J/cm^2 laser at 30 mW. Our findings regarding TBO are in accordance with

those previous studies which have shown that it is possible to kill periodontal bacteria by using low concentration of toluidine blue and low energy densities [35-37]. Although the bacterial count reduction in 3 J/cm² laser irradiation alone was more than our expectation, it may be explained by attenuation in bacterial growth as fastidious microorganism. One explanation for the decreased photodynamic effect of Radachlorin® may be that extra Radachlorin® molecules which could not bond to the bacterium excessively spend the limited oxygen molecules dissolved in the suspension and would reduce available oxygen for the photosensitizer molecules inside or close to the bacterium.

Another explanation may be defined according to Wainwright's study which was carried out on fotolon as a major component of Radachlorin® indicating that high power density over a short time period may give different antimicrobial effects in comparison with lower power density over a longer time even with the same energy density in both cases.

He also declared that the reduced photobleaching rates for higher chlorin e6 concentrations may be explained by the self-shielding effect. In a higher concentration of the dye, the distance traveled by the excitation light may be reduced due to its loss in intensity. In such a case, superficial layers of the dye absorb the laser very efficiently, but they prevent its penetration into deeper layers.

Finally, the photobleached superficial layers become transparent while deeper layers still strongly absorb the laser.

This is why radiant exposures for a highly concentrated photosensitizer may be underrated, leading to reduced PDI efficiency. During the antibacterial experiments, the energy density of 15 J/cm² under 60 sec illumination could not completely photobleach chlorin e6 solution and in fact, a higher dose (such as a dose higher than 30 J/cm²) should be used for further enhancement of PDI [25].

CONCLUSION

In conclusion, our results demonstrate that the association of TBO with a diode laser and the energy density of 3 J/cm² may be more effective in reducing the viability of streptococcus mutans pure cultures compared with Radachlorin®-mediated 12 J/cm² laser irradiation.

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