The efficacy of photodynamic and photothermal therapy on biofilm formation of Streptococcus mutans: An in vitro study

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Background: The alternative antibacterial treatments of photodynamic therapy (PDT) and photothermal therapy (PTT) significantly affect microbiota inactivation. The aim of the present research was the assessment of the antimicrobial and anti-biofilm effects of PDT with toluidine blue O (TBO) and PTT with indocyanine green (ICG) on Streptococcus mutans as a cariogenic bacterium.

Materials and methods: The S. mutans ATCC 35668 strain was treated with final concentrations of 0.1 mg/mL TBO and 1 mg/mL ICG with energy densities of 17.18 and 15.62 J/cm², respectively. Cell viability was evaluated after culturing and anti-biofilm potential was analyzed using crystal violet assay and scanning electron microscopy.

Results: The number of S. mutans colony forming unit (CFU)/mL was significantly lower in the groups submitted to PDT (12.5–100 μg/mL TBO) and PTT (62.5–1000 μg/mL) compared to the control (untreated group). 0.1 mg/mL TBO-PDT and 1 mg/mL ICG-PTT showed stronger inhibitory effects on biofilm formation in S. mutans than other concentration levels, with a reduction of 63.87% and 67.3%, respectively.

Conclusion: Photo-elimination by high concentrations of TBO-PDT and ICG-PTT exhibited significantly stronger inhibitory effects on biofilm formation and cell viability in S. mutans.

1. Introduction

Dental caries occur as the result of a shift in a complex host-bacterial community interaction that leads to the formation of plaque biofilm on the human tooth surface [1]. Acidogenic communities and acid tolerant species are responsible for the development of caries [2].

Streptococcus mutans as a cariogenic bacterium was found to be the dominant species in many, but not in all, subjects with dental caries [3,4]. S. mutans can degrade the carbohydrates with the formation of abundant acid, and tolerate low pH environments [5]. Anti-caries procedures can be performed using antimicrobial agents and mechanical and chemical disruption of oral microbial biofilm, such as by tooth brushing [6,7]. However, the use of these methods is limited by mechanical damage to the oral mucosa, the disruption of normal bacterial flora, the emergence of drug-resistant strains of microorganisms, and failure to maintain a certain concentration of antimicrobial compounds in the oral cavity [6–8].

Alternative techniques, such as photodynamic therapy (PDT) and photothermal therapy (PTT) can be used to remove biofilm from a tooth surface, considering the limitations of traditional treatments [9,10]. A combination of an appropriate photosensitizer (PS)
and a minimally invasive and nontoxic light source were used in these techniques [9].

Several studies have shown that oral bacteria, including periodontal pathogenic bacteria, cariogenic bacteria, and bacteria involved in endodontic lesions, are susceptible to PDT and PTT [6,8,10]. Since no research on the effects of PDT and PTT with diode laser on biofilm formation of S. mutans has yet been reported, the purpose of our study was to evaluate the PDT and PTT effects with toluidine blue O (TBO) and indocyanine green (ICG), as PSs on viability of S. mutans in the biofilm phase.

2. Materials and methods

2.1. Bacterial strain and culture conditions

The ATCC 35668 strain of S. mutans was obtained from the Iranian Biological Resource Center (Tehran, Iran). Bacteria were incubated in a brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) under aerobic conditions, supplemented with 5% CO2 at 37 °C for 18 h. The initial turbidity of the suspensions was adjusted to A600 nm = 0.5 by spectrophotometry.

2.2. Photosensitizer solutions and light source

For photo-elimination by PDT, TBO powder (Sigma-Aldrich, Steinheim, Germany) was dissolved in sterile 0.9% (wt/vol) NaCl to reach a final concentration of 0.4 mg/mL. Photo-elimination by PTT included ICG (Sigma-Aldrich, Steinheim, Germany) at a final concentration of 4 mg/mL, which was dissolved in distilled water. Both PSs were then filter-sterilized and kept in dark conditions prior to use.

The light source for activating TBO and ICG was a diode laser (Konftec, Taiwan; and A.R. Clasger GmbH, Nurnberg, Germany, respectively) at a wavelength of 635 nm with output power of 220 mW and 810 nm with output power of 250 mW, respectively. A power meter (Laser Point s.r.l., Milano, Italy) was used to measure the output powers of the laser. S. mutans strains were exposed to 30 s diode laser irradiation time, with an energy density of 17.18 and 15.62 J/cm² at a wavelength 635 and 810 nm for TBO and ICG, respectively, and a room temperature (25 ± 2 °C).

2.3. PDT and PTT against S. mutans strain

PDT and PTT were determined according to a previous study [11]. Briefly, 100 μL of 2X BHI broth was added to each well of sterile 96-well flat-bottom microtiter plates with lids (TPP, Trasadingen, Switzerland). Next, 100 μL of the PSs solution were added to the first well in column one and diluted twofold step-wise from column one to column ten. Then, 100 μL/well of bacterial suspension with concentration of 1.0 × 10⁶ colony forming unit (CFU)/mL was transferred to each well. The microplates were then maintained in the dark for 5 min, in order for PSS to be absorbed by the bacterial cells. Afterward, bacterial suspensions were subject to illumination (17.18 and 15.62 J/cm² for TBO and ICG, respectively). After treatment, in order to assess bacterial viability, the 10 μL aliquots of each dilution were plated in triplicate on BHI agar (Merck, Darmstadt, Germany) and incubated for 48 h at 37 °C in a partial atmosphere of 5% CO2. After incubation, the number of CFU/mL was determined using the Miles and Misra Method [12].

2.4. Evaluation of biofilm formation ability of treated S. mutans by crystal violet

S. mutans biofilms were formed in a flat-bottomed sterile polystyrene microplate (TPP, Trasadingen, Switzerland) according to previous studies [11,13,14]. Briefly, 200 μL aliquots of S. mutans suspension was treated with PDT and PTT in a final concentration of 5.0 × 10⁶ CFU/mL, incubated at 37 °C in a 5% CO2 aerobic atmosphere for 24 h. After incubation, the contents of the microplate were decanted, and the plates gently washed twice with 200 μL of phosphate-buffered saline (PBS) (2 mM NaH2PO4, 10 mM Na2HPO4, 137 mM NaCl, 2.7 mM KCl; pH 7.4), to remove planktonic and loosely-bound cells. Subsequently, the adherent bacteria were stained with 200 μL of 0.1% (wt/vol) crystal violet solution for 15 min at room temperature. After rinsing twice with 200 μL of PBS, the bound dye was extracted from the stained cells using 100 μL of 96% ethanol. Thereafter, the wells were rinsed once with PBS and air-dried. 150 μL of 33% (wt/vol) acetic acid was then added to each well. Biofilm formation was then quantified by measuring the absorbance of the solution at 570 nm using a microplate reader (Thermo Fisher Scientific, US).

2.5. Visualization of treated S. mutans biofilms by scanning electron microscope (SEM)

SEM was used to demonstrate the morphology and the biofilm formation ability of S. mutans, as previously described [13]. Briefly, after treatments, bacterial suspension were grown on pegs of MBEC™ high-throughput (HTP) plates (Innovotech, Alberta, Canada) for 24 h, following the manufacturer’s protocol, and SEM was done using previously reported method [13]. In brief, following incubation, separated pegs were rinsed in separated pegs were sterile PBS for one min to remove planktonic bacteria. Pegs were then fixed with glutaraldehyde (2.5% in 0.1 M cacodylic acid; pH 7.2) at 4 °C for 16 h. Each Peg was then rinsed once with the same buffer for approximately 10 min, immersed in 1% aqueous osmium tetroxide, and incubated for one hour at 25 °C. After being washing with de-ionized water for 15 min, dehydration with ethanol, and 1.5 h drying (Bal-Tec CPD 030, Netherland) followed, and the pegs were then coated with gold-palladium by a coater (Bal-Tec SCD 005, Netherland) and tested with a SEM (S800LV, JEOL, Japan).

2.6. Statistical analysis

The results were expressed as mean values ± standard deviations (mean ± SD) and analyzed by two-way analysis of variance (ANOVA) and Tukey’s test in SPSS statistical software version 21. All experiments were performed in at least triplicate. The level of significance for all analyses was set at p < 0.05.

3. Results

The count of bacteria decreased with increasing concentration of PSSs in both groups (PDT and PTT), so that the significant decrease was observed at concentrations of 12.5–100 μg/mL of TBO and 62.5–1000 μg/mL ICG (p < 0.05). In the groups treated only with the PSSs or only irradiated, no significant reduction of CFU/mL was observed (p > 0.05) (Fig. 1).

Base on the results of Fig. 1, photo-elimination of the S. mutans strain with TBO (PDT group) and ICG (PTT group) were statistically significant, whereas no significant differences were observed between these two groups.

As shown in Fig. 2, the biofilm formation ability of S. mutans decreased gradually with increasing doses of PDT and PTT. Significant differences in reducing biofilm formation was seen in the concentration of 100 μg/mL TBO (63.87%) and 1000 μg/mL ICG (67.3%) compared with other groups (p < 0.05).

The S. mutans morphology of the PDT and PTT untreated and untreated cells were studied using SEM (Fig. 3). PDT and PTT treated cells showed reduction in the numbers of bacterial cells as well as
significant changes in morphology, in contrast to untreated bacterial cells.

4. Discussion

PDT and PTT might be optional therapeutic methods to reduce biofilms that cause oral disease via pathogenic bacteria [9]. They involve electron transfer reactions and energy transfer from the PSs with the participation of a light, to release oxygen and produce cytotoxic reactive oxygen species (ROS), such as superoxide and hydroxyl radicals, and produce excited singlet oxygen that can destroy the biological material of bacteria, including proteins, nucleic acids, and lipids [9,10,15].

We used TBO and ICG as PSs and a diode laser as a light source, since these are already safely in the oral environment [9]. To date, several studies have been conducted on the PDT effects against streptococcal species involved in dental caries and showed the possibility for targeting this cariogenic microbiota [16–20]. However, to the best of our knowledge, the present study is the first report of the effects of PDT and PTT on S. mutans biofilm formation using TBO.
and ICG combination with diode laser, respectively. The results of this study indicate that after treatments with high concentrations of PSs significant reduction of S. mutans viability was observed in both groups of PDT and PTT with no priority (p < 0.05).

Similar results were also observed. Fekrazad et al. [16] showed that 0.1 mg/mL TBO and EmunDo® (ICG) solution without significant differences reduced the CFU/mL of S. mutans. Hakimih et al. [17] reported that PDT with 0.1 mg/mL TBO significantly reduced S. mutans viability. Vahabi et al. [18] also stated that PDT with TBO 0.1% and 633 nm laser at 3 J/cm² effectively eliminated S. mutans. Williams et al. [21] reported that PDT in combination with TBO by energy density of 1.8 J/cm² produced log reduction of 8–10 CFU/mL of planktonic suspension of S. mutans.

There are some reports that bacteria in biofilms are more resistant to aniseptic agents than to planktonic forms owing to the structure of the polymeric matrix of biofilm, which prevents the penetration of antimicrobial agents [9,11,22].

In this study, S. mutans biofilms exposed to PDT and PTT with 100 and 1000 μg/mL concentrations of TBO and ICG, respectively are more sensitive than other biofilms that have received lower concentrations of PSs (0.19–50 μg/mL TBO and 1.9–500 μg/mL ICG) (p < 0.05). These results appear to be due to detachment of the biofilms by the disintegration of the interaction between bacteria, owing to ROS exposure falling PDT and PTT. High cell density in large cellular aggregates was observed in the untreated group, whereas the cell density after PDT and PTT was reduced. Owing to the loss of cell–cell interaction and physical contact between cells, irregular biofilm shapes and sizes and cell loss were evident [23].

5. Conclusion

Our in vitro results indicate that both methods of photoelimination by high concentrations of TBO mediated PDT and ICG-mediated PTT exhibited significantly stronger inhibitory effects on biofilm formation and consequently the CFU/mL in S. mutans than lower concentrations. Thus, PDT and PTT represent novel preventive strategies to control caries in those at high risk.

Disclosure statement

No competing financial interests exist.

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References


