Inactivation of Streptococcus mutans Using Photo-activated Disinfection Therapy with Methylene Blue and Indocyanine Green Photosensitizers

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Abstract:
Background: Streptococcus mutans is one of the species in the biofilm adjacent to cariogenic lesions. Photo-activated disinfection (PAD) is a new method which reduces cariogenic bacterial without common side effects. The success of PAD depends on the sensitivity of the organism, type of dyes, a dose of dyes, and the depth of emitted laser. In this study, effect of PAD on S. mutans reduction using indocyanine green (ICG) compared with methylene blue was evaluated.

Materials and Methods: The standard turbidity (0.5 McFarland) of S. mutans suspension was prepared. Bacterial suspensions were transferred to the wells. Then, samples were subsequently divided into 7 groups. 1: Negative control, 2: Only methylene blue without laser, 3: Only 640 nm laser, 4: PAD by methylene blue, 5: Indocyanine without laser, 6: Only 810 nm laser, and 7: PAD by ICG. Subsequently, the bacterial suspension from each well was cultured, colony counts were determined, and data were analyzed using one-way ANOVA and Tukey’s tests (P < 0.05).

Results: There was a significant decrease of S. mutans colony counts after PAD using both methylene blue and ICG photosensitizers. The highest decreased amount belonged to the ICG (PAD group). Methylene blue group (without laser application) also had a significant decrease of S. mutans colony counts (less effective than both PAD groups). Other groups (laser applications alone and ICG dye without laser) showed no significant bacterial reduction.

Conclusion: With the parameters of this study PAD by ICG seems to be more effective than PAD by methylene blue, in reducing S. mutans bacteria as etiological factors of dental caries.

Key Words: Photodynamic therapy, photosensitizers, Streptococcus mutans

Introduction
Dental caries as a multifactorial chronic disease is produced by the acid formation of cariogenic biofilm on the tooth surface. Streptococcus mutans is one of the most important cariogenic bacterial species in such biofilm.¹ High S. mutans counts are responsible for catalyzing carbohydrates into acids, which results in demineralization of tooth structure. Progression of bacterial invasions on dental surfaces will cause more destruction and cavity formation.³,⁴ Competition of cariogenic and non-cariogenic biofilm is a crucial point in caries prevention which is an innovative approach rather than conventional symptomatic treatments using tooth drillings and fillings.⁴

Various methods have been used for the elimination and reduction of cariogenic bacteria, including consumption of antibacterial agents (like chlorhexidine rinses), using systematic antibiotic and ozone therapy.⁵,⁶ Systemic toxic effects and interaction with other drugs and producing bacterial resistance are some common side effects of such techniques.⁵,⁷

In recent studies photo-activated disinfection (PAD) as a new antibacterial method is introduced.⁶ This technique relies on using special dyes which could release free radicals such as oxygen singles when irradiated with specific laser wavelengths.⁴ As a result of local application, this method has no systemic side effect and by providing the chance of localized application and laser targeting, adverse effects of local antibacterial agents and rinses like oral flora destruction will not happen.⁴

This method could be employed in carious prevention and treatment procedures even after removing infected tissue for disinfection of remaining dentin to reduce the chance of secondary caries reproduction. The bacterial reduction could reduce dentin hypersensitivity and could increase the success rate of treatments in which dentinal carious layer is not completely removal like indirect pulp capping techniques.⁴,⁷,⁸

Efficiency of photodynamic therapy depends on several factors, including the sensitivity of the organism, the type of the photosensitizer and parameters of applied laser.⁴ Several Photosensitizers have been used in PAD researches (like photofrin, tolonium chloride, and methylene blue).⁴,⁶
These studies have shown the effects of different photosensitizers activated with a laser on protozoa, \textit{S. mutans}, \textit{Candida albicans} as oral pathogens.\textsuperscript{9,12}

In one study, Williams reported that the laser system in association with tolonium chloride photosensitizer produced significant reductions of bacteria. In other study, Fekrazad \textit{et al.} mentioned a decrease in \textit{S. mutans} counts after using radachlorin photosensitizer activated with 600 nm laser irradiation.\textsuperscript{9,11} Fekrazad \textit{et al.} studied the effects of methylene blue and indocyanine green (ICG) on \textit{C. albicans} counts and reported a significant reduction of colony counts.\textsuperscript{12} Rolim \textit{et al.} studies showed the effects of methylene blue, erythrosine and photofrin dyes on decreasing \textit{S. mutans} counts in bacterial biofilms.\textsuperscript{13} Furthermore, a study by Pereira \textit{et al.} showed the effect of methylene blue on decreasing bacterial biofilms containing \textit{S. mutans}, \textit{C. albicans}, and \textit{S. aureus}.\textsuperscript{14}

Recently, there is an emphasis on using ICG dye as new material for PAD. It is biocompatible nontoxic dye which has been used in several cardiovascular and internal diseases diagnosis. This material could be activated with 810 nm laser which penetrates more deeply in tissues in comparison with other visible wavelengths used in routine PAD procedures (for example 640 nm which is needed to activate methylene blue and tolonium chloride dyes).\textsuperscript{15,16} It is one of wavelengths which is generally used in other dental procedures as diode laser units, so a provides benefit of using PAD with the same device.\textsuperscript{15,16} This study examined the effect of PAD using ICG and 810 nm laser in comparison with methylene blue dye and 640 nm laser application.

\textbf{Materials and Methods}

\textbf{Photosensitizers and light sources}

Methylene blue photosensitizer was prepared by adding 0.096 g powder (Sigma Chemical Co., St. Louis, MO, USA) to 100 mL of distilled water repeatedly to achieve a concentration of 10 µmol/L. GaliumArc LED (RJ Laser, Waldrich, Germany) with 640 nm was used to activate this photosensitizer with a 20 j/cm\textsuperscript{2} radiation energy density and 0.5 W power radiation for 2 min and from 2 mm distance (Figures 1 and 2).\textsuperscript{17}

Emendo (prefabricated ICG) solution (A.R.C Lasers GmbH Nurnberg, Germany) with 1 mg/ml concentration was used with 810 nm GaAl As diode laser (GIGAA, Wuhan 430206, China) with a 150 j/cm\textsuperscript{2} radiation energy density and 10-15 W power for 2 min and from 2 mm distance (Figures 1 and 2).\textsuperscript{18}

\textbf{S. mutans suspension samples}

\textit{S. mutans} suspension samples were prepared using standard \textit{S. mutans} clones (Pastour Institute, Iran) which are cultured in Triptic Soy Agar medium (Merck, Germany) as a liquid medium and Mitis Salivaris Agar (MSA) (Que Lab, Canada) as a solid medium.\textsuperscript{19} Both media were autoclaved at 121°C for 15 min before use.\textsuperscript{11} The cultures were maintained in anaerobic jars under anaerobic or microaerophilic conditions at 36°C after the bacteria were cultured. A standard turbidity of the bacteria (0.5 McFarland) containing 1/5 \times 10\textsuperscript{8} bacteria/mL was prepared.\textsuperscript{11} Then, 15 µL of the solution was added to previously autoclaved Falcon tubes (graduated plastic tubes) containing 4 mL of Tryptic Soy Broth medium as a transitional media. The initial colony counts were determined in each 50 µL of this solution after incubation and anaerobic growth of bacteria on MSA medium at 37°C for 36 h.\textsuperscript{11}
About 50 µL of the achieved solution were transferred to each of the 105 polystyrene sample wells in three series of 96-well microplates. The samples were divided into 7 groups:

Group 1: No photosensitizer and laser irradiation were used as negative control.

Group 2: 10 µ mol/L of methylene blue solution (Sigma Chemical Co., St. Louis, MO, USA) was added to sample wells (with no light activation) and was shaken to insure that suspension was well mixed. Mixtures were kept in dark environment at 28°C for 2 min before culturing (Figure 3).

Group 3: A 640 nm GaliumArc laser (RJ laser, Waldrich, Germany) with a 20 j/cm² energy density and at 0/5 W power irradiated for 2 min and from 2 mm distance (positive control group with no photosensitizer) (Figure 3).

Group 4: PAD was done using methylene blue (Sigma Chemical Co., St. Louis, MO, USA) and laser application. Methylene blue was used by the concentration of 10 µ mol/ml and shaken and then 640 nm laser was applied on the sample wells for 2 min with the same protocol of group 3 (Figure 3).

Group 5: 1 µg/mL of EmenDo (ICG) solution (A.R.C Lasers, GmbhH Nurnberg, Germany) added to samples without any light application. The wells were shaken for 2 min to insure that suspension was well mixed. The mixture of the suspension and ICG was kept in a dark environment at stored in the dark at 28°C for 2 min (Figure 4).

Group 6: Only GaAl As Diode laser (GIGAA, Wuhan 430206, China) at a wavelength of 810 nm at a power of 10-15 W and 150 j/cm² energy density, irradiated for 2 min was used (positive control group) (Figure 4).

In Group 7: PAD was done using EmenDo (ICG) solution (A.R.C Lasers GmbH, Nurnberg, Germany) and laser application. ICG was used by the concentration of 1 µg/mL and shaken and then 810 nm laser was applied on the sample wells for 2 min with the same protocol of Group 6 (Figure 4).

Using a digital sampler (Socorex, Isba S.A, Switzerland) device, 50 µL of the cellular suspension was transferred and directly spread on MSA medium using a cotton swap. Plates were incubated for 36 h at 37°C in a dark field candle jar to protect them from light and the air. A 1/8th part of each plate was randomly selected to colony count, and result was multiplied by 8 as the count of whole plate. Bacterial colonies that had been formed were counted and calculated as Log CFU/mL. Data were analyzed with one-way ANOVA Tukey’s test. A statistical significance was set at \( P < 0.05 \).

**Results**

The means and standard deviations of the number of Log CFU/mL values obtained from this study are demonstrated in Table 1.
Based on the results of this study, photodynamic therapy with two photosensitizers was significantly decrease *S. mutans*; furthermore, the most reduction of *S. mutans* was shown in the photodynamic therapy by ICG photosensitizer (Graph 1).

Photosensitizers only and lasers only had no significant effect on decreasing *S. mutans* counts, except methylene blue. Application of methylene blue alone (without laser irradiation) could result decrease of *S. mutans* colony counts.

**Discussion**

PAD is a method of bacterial reduction on dental surfaces. Local application and laser targeting of this technique provide a unique method in which normal flora is not affected and systemic side effects are not seen. The effects of photo-activated compounds on bacteria, depend on several factors, including the sensitivity of the organism, the type of photosensitizer and the doses of these agents. Beside these factors laser parameters like wavelength, dose and time of application should be selected properly to maximize photoreceptor and laser interaction.

The results of the present study showed that photodynamic therapy with both methylene blue and ICG resulted in a significant decrease in colony counts of *S. mutans*, with a greater decrease in colony counts with the use of ICG as a photodynamic therapy agent.

Methylene blue is a quinoneimine dye which is extensively used clinically as a major photosensitizer against Gram-positive and Gram-negative bacteria, with the maximum absorbing at a wavelength of 636 nm.

In our study, methylene blue was activated with a 640 nm laser (0.5 W power) producing Log of reduction of 1/5 cfu/ml in
planktonic suspension that was parallel the results of Soria’s study which reported a 6 cfu/ml log reduction of *S. mutans* bacteria, but the count reduction in our study is 4 log less than Soria’s study which was significant; this might be explained by different parameters in two studies such as using laser instead of application of LED light source and lower power (0/5 W) in our study, in addition, a lower concentration of methylene blue (10 µmol/l) was used in our study compared to Soria’s study (15 µmol/l).  

In the present study, lasers alone did not decrease *S. mutans*, counts. Thermal and photo destructive effect of laser could damage cariogenic species including *S. mutans*, and *Lactobacillus* cell wall integrity destruction and denaturation of proteins of bacterial cells are results of this irradiation. The laser application with parameters used for PAD in this study has not enough thermal effects to induce such cellular damages and laser application alone did not show any effect on bacterial growth.  

In Group 2: Adding methylene blue photosensitizer alone in dark filed showed a decrease of colony count but significantly less than PAD groups. This limited bacterial reduction would be related to its bacteriotoxic effect, methylene blue low molecular weight and ability to target Gram-positive bacteria was responsible for this action in some of studies.  

A more significant bacterial reduction was reported in Group 4 which methylene blue was irradiated with lasers (PAD by methylene blue), methylene blue concentration was 10 µmol/l in present study which was close to concentration reported in a parallel study as an optimum concentration needed for PAD.  

The results of our study was showed that methylene blue photosensitizer with application (PAD) resulted in a significant decrease in *S. mutans* counts compared to the positive control group (only methylene blue), which was not parallel to results of Rolim’s study, which reported no significant differences between photodynamic therapy (methylene blue in combination laser irradiation) and methylene blue alone. The different results of two studies might be explained by the different parameters of them such as using higher concentration of methylene blue in Rolim’s study which was selected to produce higher radicals but resulted in decreased laser effect by higher interacation of dye and laser in superficial layers of high concentration dye in PAD group high concentration methylene blue dye was more cytotoxic and resulted in same bacterial reduction of PAD group in that study.  

Another photosensitizer evaluated in the present study was indocyanine blue, which has recently been introduced to dentistry. ICG is a water-soluble tricarbocyanine dye with maximum absorption at 800-805 nm. In Group 5 (Indocyanine in dark field without laser application) of this study, there was no significant bactericidal effect, several studies of indocyanine in medical field reported biocompatibility and nontoxicity of this material on biologic cells, when it was used routinely in diagnostic procedures even intraocular and retinal surgeries. Inertness of the material was seen in our study when it was used in dark field without any light application.  

Indocyanine was activated with an 810 nm laser in this study. This wavelength has higher tissue penetration in comparison with the wavelength used for methylene blue dye (640-670 nm) so may have better effect on bacterial cells in dentinal tubels or affected dentin. Wide crystalin head used in this study for laser application provided less temperature increase and higher irradiated area and significant colony count decrease in this group; this result was parallel to the results of PAD effects of this combination which was used in a similar study on porphyromonas bacteria.  

Colony count reduction of PAD with indocyanine and 810 nm laser showed more antibacterial effect than methylene blue PAD group this difference could be explained by the higher depth of laser penetration in indocyanine group which affects more photosensitizer molecules, while superficial molecules of photosensitizers in methylene blue group interacted and absorbed more light in superficial area and deeper layers molecules were not completely activated.  

The results of the present study showed that lasers alone and photosensitizers alone had no effect on the vitality of *S. mutans* bacterial species, except for methylene blue dye which affected *S. mutans* when used alone. In the present study, the highest decrease in *S. mutans* counts occurred with photodynamic therapy using ICG.  

More studies with different dyes and wavelengths and bacterial fields (like biofilm) could be designed to have more comprehensive results.  

**Conclusion**  
By limitated data provided from the results of this study, it can be concluded that photodynamic therapy with both methylene blue and ICG photosensitizers, showed a significant decrease in *S. mutans* counts. Application of ICG and laser irradiation with indocyanine had the highest decreasing amount. Photosensitizers alone and lasers alone did not have a significant effect on *S. mutans*, except methylene blue dye. Methylene blue dye alone (without laser application) could decreased *S. mutans* counts.  

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References