THE EFFECT OF PHOTODYNAMIC THERAPY ON THE VIABILITY OF STREPTOCOCCUS MUTANS ISOLATED FROM ORAL CAVITY

Ji-Sook Jung¹, Ho-Won Park¹, Ju-Hyun Lee¹, Hyun-Woo Seo¹, Si-Young Lee²

¹Department of Pediatric Dentistry, ²Department of Microbiology and Immunology, Oral Science Research Center, College of Dentistry, Gangneung-Wonju National University

Abstract

Photodynamic therapy (PDT) is a technique that involves the activation of photosensitizer by light in the presence of tissue oxygen, resulting in the production of reactive radicals capable of inducing cell death. The aim of this study was to evaluate the effect of PDT on Streptococcus mutans in planktonic conditions, previously treated with different photosensitive concentrations of erythrosine, using halogen and LED curing unit as a light source. And we compared the effects of PDT on six strains of S. mutans isolated from oral cavity and reference strain.

As a result, S. mutans was susceptible to the combination of hand held photopolymerizer (HHP) and erythrosine. The higher concentration of erythrosine in the presence of light irradiation induced greater effects in reduction of viability of S. mutans. Isolated S. mutans showed a significant reduction in bacterial counts of the groups submitted to PDT compared to the control groups. And they appeared to be similar or slightly lower antimicrobial effect compared with reference strain. However, the difference was not significant (p < 0.05).

In conclusion, PDT using erythrosine as a photosensitizing agent and HHP as a light source could be an efficient option for diseases caused by S. mutans.

Key words: Photodynamic therapy, Erythrosine, Halogen, LED, Streptococcus mutans

I. Introduction

Many of oral diseases are caused by microorganisms. Dental caries is among the most significant human chronic contagious diseases¹⁻³. The Gram positive bacteria Streptococcus mutans is a substantial part of the dental plaque microbiota and its importance in the dental caries etiology is unquestionable⁴. Mechanical removal of the biofilm, fluoride therapy, and adjunctive use of antibiotics have been conventional methods to control bacterial proliferation in the mouth environments⁵⁻⁶. But in practice, antibiotics are rarely used due to problems such as production of drug - resistant organisms and disruption of the normal microflora. As a result, there is a pronounced interest in the development of alternative antimicrobial concepts⁷⁻⁹.

Photodynamic therapy (PDT) could be an alternative to conventional therapeutic methods. PDT, also known as photoradiation therapy, phototherapy, or photoschemotherapy, involves the use of a photoactive dye (photosensitizer) that is activated by exposure to light of a specific wavelength in the presence of oxygen¹⁰. The transfer of energy from the activated photosensitizer to available oxygen results in the formation of toxic oxygen species, such as singlet oxygen and free radicals. These very reactive chemical species can damage proteins,
lipids, nucleic acids, and other cellular components. Among the various photosensitive agents used in PDT, there are merocyanine derivatives, phthalocyanines, hematoporphyrin and xanthenes dyes. Erythrosine belongs to a class of cyclic compounds called xanthenes, which absorb light in the visible region, and the ability of erythrosine to initiate photochemical reactions is well documented. Erythrosine has an advantage over other photosensitizers, as it already targets dental plaque and has full approval for use in the mouth.

Historically, large complex lasers which need certain technical support were required for PDT. In clinical settings, these lasers have been replaced by reliable, easy-to-use light sources which no longer require complex technologies and expensive maintenance. The hand held photopolymerizer (HHP) used in dentistry for the light-curing of restorative materials has been suggested as an alternative to the use of lasers because of their low cost and simplicity.

Previous studies have shown that PDT using erythrosine is capable of killing oral bacteria. Recently, Park et al. proved that PDT effect of erythrosine as a photosensitizer and dental halogen curing unit as a light source to the planktonic condition of S. mutans. And they compared the susceptibility of S. mutans in different light irradiation time and distance.

In the present study, we evaluated the PDT effect on S. mutans, previously treated with different photosensitive concentrations of erythrosine, using two types of HHP as a light source. The goal of our study was to compare the viability of S. mutans according to concentration of photosensitizer. Furthermore, we compared the effects of PDT on six strains of S. mutans isolated from oral cavity and reference strain.

Ⅱ. Materials and methods

1. Bacterial strains and culture conditions

Seven S. mutans strains, including one reference strain (ATCC 25175) and six clinical strains isolated from oral cavity were used in this study. The isolated strains were obtained from the Laboratory of Microbiology and Immunology, School of Dentistry of Gangneung - Wonju National University.

The bacteria were incubated in brain heart infusion broth (Becton, Dickinson and Company, Sparks, Maryland, USA) at 37°C for 18 hours under aerobic condition supplemented with 5% CO₂. The turbidity of bacterial suspensions was measured by spectrophotometer (Smart Plus 2700, Young - Woo Inst. Seoul, Korea). A standard curve relating the culture turbidity and bacterial cell numbers was established and utilized. The bacteria was diluted to 10⁷ colony-forming units (CFU) / mL with phosphate buffered saline (PBS).

2. Photosensitizer

Erythrosine (Sigma-Aldrich, St. Louis, MO, USA) was used as photosensitizer. A stock solution of 2 mM erythrosine was prepared in PBS. This solution was filter-sterilized and stored at -20°C in the dark.

In order to evaluate the characteristic absorption spectra for erythrosine solution, it was examined using a spectrophotometer (Optizen 3220 UV, Mecasys, Daejeon, Korea). Fig. 1 shows the characteristic absorption spectra for an erythrosine solution. It is worth emphasizing that this xanthene derivate presents an absorption band in the range of 460 - 550 nm, which is similar to the emission spectra of conventional HHP (400 - 500 nm), suggesting that it can be used to photoactivate the dye. The erythrosine absorption spectra, obtained in the presence of 5 × 10⁴ CFU / mL of S. mutans, does not show any alteration (data not shown).

3. Light source

The light sources used in this study were conventional halogen curing unit (XL 3000, 3M ESPE, St. Paul, MN USA) and HHP. A standard curve relating the light intensity and bacterial cell numbers was established and utilized. The light intensity was measured using a light meter (Lutron, LP-300D, Korea). The light intensity was measured at 400 - 500 nm, which is similar with the emission spectra of conventional HHP. The light intensity was measured at 400 - 500 nm, which is similar with the emission spectra of conventional HHP (400 - 500 nm). Inset : Erythrosine structure.

Fig. 1. Absorption spectra of erythrosine in PBS. Cuvette with bacterial suspension was used as reference. The erythrosine presents an absorption band in the range of 460 - 550 nm, which is similar with the emission spectra of conventional HHP (400 - 500 nm).
USA) and light-emitting diodes (LED) curing unit (Bluephase, Ivoclar Vivadent, Liechtenstein, Austria). The light beam of halogen curing unit irradiated diameter of 8 mm and that of LED curing unit was 10 mm. The power output of the halogen light was 600 mW/cm² and that of LED light was 900 mW/cm², checked by radiometer (Light intensity meter, Dentamerica, San Joes, California, USA). The halogen unit produces light spectrum of 370–530 nm with maximum at 470 nm, and that of LED unit is 380–515 nm with maximum at 480 nm according to the manufacturer.

4. Photodynamic therapy

1) PDT according to the concentration of erythrosine
An aliquot (17.5 μL) of S. mutans suspension (reference strain, final concentration of 5 × 10⁵ CFU/mL) was added to each well of sterile flat-bottomed 96-well plate. Next, the erythrosine (3.5 μL) was added for group 3 and group 4. PBS was added for final volume of 350 μL. Samples were divided into four test groups.

• Group 1 (P-L-) – Neither irradiation nor photosensitizer treatment
• Group 2 (P-L+) – Irradiation only
• Group 3 (P+L-) – Treatment with photosensitizer only, no irradiation (Subgroups were divided by concentration of erythrosine)
• Group 4 (P+L+) – Irradiation using photosensitizer (Subgroups were divided by concentration of erythrosine)

Each group was duplicated. The distance between the light tip and sample was 1 cm. The light irradiation time was 30 seconds and it was performed immediately after addition of erythrosine. The final concentration of erythrosine of subgroups is as follows: 20, 10, 5, 2.5, 1.25, 0.625 μM. Each experiment was performed with the halogen curing unit and the LED curing unit in the same way. After irradiation, each sample was diluted and incubated in the same way as reference strain. And the bacterial viability was determined by colony forming unit (CFU).

3) Statistical analysis
Statistical analysis was done by using the Software Package for Social Sciences (SPSS, version 12.0, SPSS Inc., USA). The arithmetic average and standard deviation were calculated in each group. Mann Whitney non-parametric tests were utilized for assessing the data. The level of significance was p < 0.05. One-way analysis of variance was used to analyze differences among subgroups and the Bonferroni method was performed for multiple comparison procedures.

III. Results
The reduction of CFU in each of the test groups is tabulated for each concentration of erythrosine. Mean and standard deviation values of the CFU obtained are shown in Table 1 and Table 2. Comparison between group 1, 2 and 3 reveals that treatment with light irradiation in the absence of photosensitizer (group 2) or photosensitizer alone without light (group 3) does not have antibacterial effect. A decrease in the number of colony counts was only verified when they were exposed to both the light and the photosensitizer at the same time (group 4).

When using halogen curing unit, in concentrations of erythrosine above 2.5 μM, significant decreases in colony counts were observed (p < 0.05). In concentrations above 5 μM, the bactericidal rate went up above 90% (Table 1). There was a significant decrease in colony counts at 2.5 μM concentration (p < 0.05), but even then, about 63.6% of tested organisms survived. No statistically significant difference between 1.25 and 0.625 μM of erythrosine.

When using a LED curing unit, in all tested concentrations of erythrosine, significant decreases in colony counts were observed (p < 0.05). In concentrations above 5 μM, the bactericidal rates went up to above 90%.
There was a significant decrease in colony counts at 0.625 μM (p < 0.05), but even then, about 67.2% of tested organisms survived.

Fig. 2 shows a direct comparison of the efficacy of erythrosine concentration in the PDT of S. mutans. It shows percentage of bacteria killing based on colony counts of control group. In cut off level of p < 0.05 of significance, using halogen curing unit at above 2.5 μM and using LED curing unit at all tested concentrations showed significant decreases of colony counts.

Table 3 and Fig. 3 show the PDT effect on six strains of S. mutans isolated from oral cavity. A significant reduction in bacterial counts was observed for the groups submitted to PDT (P+L+) when compared to the control groups (P-L-). Table 3 shows the CFU / well reduction and killing rate observed from the P+L+ groups compared to the control groups. Whereas the killing rate of reference S. mutans went up to above 90% at 5 μM, the isolated S. mutans appeared to be similar or slightly lower killing rate than the reference strain (ranging from 69.7% to 92.45%). However, the difference was not significant (p < 0.05).

(Table 2). There was a significant decrease in colony counts at 0.625 μM (p < 0.05), but even then, about 67.2% of tested organisms survived.

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IV. Discussion

Photodynamic action has been used to kill oral microorganisms since the beginning of the 1990s, when studies demonstrated that some photosensitizers show affinity for bacterial walls and can be photoactivated to cause the desired damage\(^{19-21}\). Excited photosensitizer molecules can transfer energy to nearby molecules, resulting in the formation of reactive molecules as singlet oxygen, superoxide, and other free radicals, capable of causing damage and even death of cells and bacteria\(^{22-25}\).

The successful application of PDT in inactivating microorganisms mainly depends on photosensitizer and light source. Dental practitioners currently use erythrosine to stain and visualize dental plaque in the form of disclosing solution or tablets. And noncoherent blue light sources such as halogen lamp and LED are commonly used in dentistry for photopolymerization of tooth-colored restorative materials. By applying the same light sources and photosensitizer, we can now demonstrate a phototoxic effect on the Gram-positive bacteria S. mutans associated with dental caries.

Historically, lasers are the most common light sources used to activate the photosensitizers. However, recently, reliable and easy-to-use light sources which no longer require complex technologies and expensive maintenance have replaced conventional lasers. In this study, light sources of low cost, simple technology without UV-A or UV-B radiation, and photosensitive dye (erythrosine) which shows strong absorbance in the green region (maximum about 520 nm, wide spectra) were used. Since it was observed that the dye has not altered its absorbance spectra in the presence of the S. mutans, we might suggest that its molecular structure was not altered and there is no aggregation process which could reduce its efficiency in the reactive oxygen species (superoxide, hydroxyl radicals, hydrogen peroxide) liberation after irradiation with the HHP. Upon irradiation with light corresponding to an absorption maximum of the photosensitizer, cytotoxic reactive oxygen species are produced that can cause rapid oxidation of cellular constituents and cell death\(^{26}\).

Previous study reported that PDT with erythrosine and dental halogen curing unit is available for killing S. mutans in planktonic state\(^{15}\). According to this study, PDT effect was more effective with increasing irradiation time and closer to the light source. Under irradiation for

<table>
<thead>
<tr>
<th>Strains of S. mutans</th>
<th>5 × 10^4 CFU / well P-L-</th>
<th>P-L+</th>
<th>P+L-</th>
<th>P+L+ (r)</th>
<th>Killing rate (n=4)</th>
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</thead>
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<tr>
<td>KN 537</td>
<td>10.01</td>
<td>8.19</td>
<td>8.24</td>
<td>1.16</td>
<td>88.48</td>
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<tr>
<td>KN 542</td>
<td>4.66</td>
<td>6.02</td>
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<td>KN 558</td>
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<td>3.33</td>
<td>0.33</td>
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<td>1.35</td>
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<tr>
<td>KN 577</td>
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<td>2.12</td>
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<tr>
<td>KN 580</td>
<td>7.61</td>
<td>8.70</td>
<td>7.95</td>
<td>0.69</td>
<td>90.99</td>
</tr>
<tr>
<td>ATCC 25175</td>
<td>22.99</td>
<td>23.45</td>
<td>20.30</td>
<td>2.01</td>
<td>91.40</td>
</tr>
</tbody>
</table>

* Comparing to control group (P-L-), statistically significant with \(p < 0.05\).
30 seconds at 1 cm distance, approximately 90% of the bacteria showed decrease, which is an appropriate level to evaluate the effect of erythrosine concentration on PDT. Therefore, we used the light irradiation for 30 seconds at the distance of 1 cm in this present study. We have performed these experiments with variable concentrations of erythrosine (ranging from 0 to 20 μM) and two types of light sources (halogen and LED). Significant bactericidal effects were observed at concentrations of erythrosine above 2.5 μM with halogen and all concentrations with LED. The reductions in viability of more than 90% with both light sources were observed using erythrosine concentration of 5 μM.

We noted that the general effect of toxicity with exposure to light increases with increasing concentration of photosensitizer. Previous studies reported the similar results. Goulart et al. verified that rose Bengal at 0.1 μM, associated with 0.65 J / cm² light irradiation, reduced the Aggregatibacter actinomycetemcomitans biofilm by approximately 45%. This reduction was significantly dependent on concentration of rose bengal and dose irradiation. According to Jucaira et al., Photogem / toluidine blue O mediated PDT effect on the viability of S. mutans and Lactobacillus acidophilus in planktonic state was dependent on both photosensitizer concentration and light dose.

In PDT, the dye should not cause cell damage, that is, without light exposure: an ideal dye has no toxic effect on the cells, and the conditions used for the photoinactivation of any pathogen with PDT (light dose and dye concentration) should not affect the neighboring human tissues either. Erythrosine in concentrations ranging from 9 to 15 mM is used in dentistry procedures to visualize dental plaque. All concentrations of erythrosine used in the present study are much lower than the currently acceptable clinically used concentration. Also, there are protection and repair systems in the eukaryotes like superoxide dismutases enzymes present in the cytoplasm and mitochondria, as well as some catalases. In addition to that, the presence of metal – substituted poliene such as carotene and lycopene, and vitamins that are often absent in prokaryotes such as vitamin E and C, would work as anti – free radical agents in the eukaryotes, providing a higher protection against reactive species generated by PDT.

The efficacy of PDT with erythrosine in S. mutans has been previously studied. Wood et al. compared the use of three different photosensitizing agents: erythrosine, Photofrin, and methylene blue at 22 μM for each dye concentration to photosensitize the S. mutans biofilm, using 400 W tungsten lamp. Erythrosine was more effective than Photofrin or methylene blue, reducing S. mutans biofilm up to 48%, compared with methylene blue (41%) and Photofrin (just 0.04%). Metcalf et al. has also verified the PDT effect on biofilm formed by S. mutans using 22 μM of erythrosine and a light dose of 6.75 J / cm²; it induced 57% of biofilm cell reduction.

In this study, we demonstrated the antimicrobial efficacy of PDT using erythrosine and HHP against the seven S. mutans strains studied, including one reference strain (ATCC 25175) and six S. mutans strains previously isolated from the oral cavities of different individuals. We included these clinical strains of S. mutans to confirm that the effects of PDT would be more biologically relevant. To evaluate the effects of PDT on the isolated strains, we used erythrosine concentration of 5 μM, which showed bacterial reduction about 90% in the reference strain. A significant reduction in bacterial counts of the groups submitted to PDT in the isolated S. mutans, when compared with the control groups, was observed. And the reduction rate in the isolated S. mutans appeared to be similar or slightly lower than the reference strain. Carolina et al. has verified the effect of photodynamic therapy with erythrosine using a LED on planktonic cultures of ten S. mutans strains, including nine clinical strains and one reference strain (ATCC 35688). The results showed that PDT with erythrosine exerted an antimicrobial effect on all S. mutans strains studied. They reported that no significant difference was observed between the isolated strains and the reference strain. The results agreed with our study. However, while they irradiated the light for 3 minutes, we acquired a similar antibacterial effect with reduced irradiation time (30 seconds).

Many works have demonstrated that bacteria of the oral cavity grown in planktonic media are sensitized by PDT. However, microorganisms that cause oral disease are organized in biofilm, which presents some different characteristics from those observed in planktonic growth, such as the presence of extracellular polymeric substances (EPS), as well as different cell wall composition, growth, metabolic activity, and gene expression. Because the bacteria grown in the biofilm may be more resistant to PDT, further study is required to evaluate the effect of PDT with erythrosine and HHP to S. mutans in vitro or vivo biofilm condition. Based in this
study, it is possible to suggest that the condition of this experiment should be a good model in more extensive experiments using erythrosine and HHP.

V. Conclusion

We studied PDT effect of erythrosine as a photosensitizer and HHP (halogen curing unit and LED curing unit) as a light source on the planktonic condition of S. mutans. We concluded the followings.

1. *S. mutans* was susceptible to the combination of HHP (halogen curing unit and LED curing unit) and erythrosine in planktonic conditions.
2. The higher concentration of erythrosine in the presence of light irradiation induced greater effects in reduction of viability of *S. mutans*. That is, the antibacterial effect of PDT was photosensitizer concentration dependent.
3. The activity of erythrosine or HHP (halogen curing unit and LED curing unit) alone was not able to reduce the number of *S. mutans*.
4. Isolated *S. mutans* showed a significant reduction in bacterial counts of the groups submitted to PDT when compared to the control groups. And they appeared to be similar or slightly lower antimicrobial effect compared with reference strain. However, the difference was not significant ($p < 0.05$).

The results of this study proved the PDT using erythrosine as a photosensitizing agent and HHP (halogen curing unit and LED curing unit) as a light source routinely used in dental clinic could be an efficient option for *S. mutans*. Based on these results, further study is required to evaluate the effect of PDT with erythrosine and HHP to *S. mutans* in biofilm conditions, in vitro and in vivo.

References


광역 동 치료가 구강 내에서 분리한 수종의 Streptococcus mutans의 생존력에 미치는 영향

정지숙1∙박호원1∙이주현1∙서현우1∙이시영2

강릉원주대학교 치과대학1소아치과학교실, 2미생물학 및 면역학 교실 및 구강과학연구소

광역 동 치료는 광감각제가 빛에 의해 활성화되면서 발생하는 화학 반응을 이용한 것으로, 치료 원리는 광화학 반응으로 자유 라디칼 및 반응성 산소가 생성되고 이 산물들에 의한 세포 독성으로 항균 효과를 가지게 되는 것이다.

이 연구의 목적은 치과 임상에서 널리 사용되는 광원(할로겐, LED)과 광감각제(erythrosine)를 이용하여 치아 우식증과 연관된 세균인 Streptococcus mutans에 대한 광역 동 치료의 항균 효과를 알아보고, 광감각제의 농도에 따른 광역 동 치료의 효과를 평가하기 위함이다. 또한 임상 분리 균주와 표준 균주에 대한 광역 동 치료의 효과를 비교해 보았다.

연구 결과, 표준 및 임상 분리 균주 모두 광감각제 처리 후 광조사를 시행한 군에서만 대조군에 비해 S. mutans의 유의한 감소가 나타났다. 또한 광조사를 시행한 군에서 침의 감소가 나타나고, 표준 균주와 비교시 임상 분리 균주에서는 표준 균주와 비슷하거나 약간 낮은 S. mutans의 감소가 나타났고, 이는 통계적으로 유의한 차이를 보이지 않았다(p < 0.05).

이상의 결과들로 보아 광감각제로 에리스로신의 사용과 광원으로 치과용 광중합기를 사용한 광역 동 치료는 S. mutans 연관 질병에 대한 효과적인 치료 방법이 될 수 있을 것으로 사료된다.

Key words: 광역 동 치료, 에리스로신, 할로겐, LED, Streptococcus mutans