

Antimicrobial Effect on *Streptococcus mutans* in Photodynamic Therapy using Different Light Source

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Abstract

In a photodynamic therapy, the difference of antibacterial capacity was compared according to the type of source of light when the same quantity of energy is irradiated.

After *S. mutans* is formed in planktonic state and biofilm state, erythrosine diluted to 40 μM was treated for 3 minutes, and as the type of light source, Halogen, LED, and Plasma arc were used, which were irradiated for 30 seconds, 15 seconds and 9.5 seconds, respectively.

After the completion of the experiment, CFU of each experiment arm was measured to compare the photodynamic therapeutic effects according to each condition.

The CFU of each experiment arm had no statistically significant difference.

Under the same quantity of energy, the photodynamic therapeutic effect can be said to be the same regardless of types of light source, which is a useful result in the clinical field with various light irradiators.

Key words : Photodynamic therapy, Erythrosine, *Streptococcus mutans*, Biofilm, Light source, Energy, Halogen, Light-Emitting Diode, Plasma arc

I. Introduction

Oral diseases mainly occurring in childhood and adolescence include malocclusion, periodontal disease, dental caries, dental erosion, and temporomandibular joint disease. Traditionally, dental caries is one of the biggest contributors to these diseases. Dental caries is a hard tissue disease involving demineralization of the enamel and dentin, which is caused by bacterial metabolism of sugar. Dental caries is one of the two major oral diseases that occur within the oral cavity with

periodontal disease. *Streptococcus mutans*, a gram-positive bacterium, is directly related to dental caries among the many related bacteria[1-3].

Bacteria involved in dental caries grow as surface adherent biofilms. The biofilms are most effectively removed by mechanical methods, such as tooth brushing. The success of tooth brushing depends on compliance by the person. There have also been attempts to use vaccines to inhibit dental caries-inducing bacteria. This strategy has been hampered by technical difficulties in developing vaccines[1]. The use antibi-

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otics has the risks of encouraging antibiotic-resistant bacteria and disruption of the normal microflora[4].

Recently, photodynamic therapy (PDT) has been studied to prevent dental caries[5]. In this approach, active oxygen or free radicals with an affinity for bacterial cell walls and which that can damage bacteria by absorbing light of a specific wavelength are used[6].

PDT is an effective antibacterial therapy that acts only on the dental plaque attached to the photosensitizer and has little effect on the normal bacterial flora in the oral cavity[7]. Previous studies have shown that PDT in the oral cavity is effective for many oral bacteria as well as *S. mutans*. However, PDT requires special light source, such as lasers or light emitting diodes (LED) [8-11]. Recently, however, antimicrobial activity against oral bacteria has been reported to be effective in PDT using erythrosine as a photosensitizer and a halogen or LED light curing unit, which is commonly used in clinical practice[12-14].

During PDT, the light source must match the activation spectrum (the longest wavelength peak) of the photosensitizer to produce the appropriate light potency in this wavelength[15]. Most studies have focused on the absorption spectra of wavelengths and photosensitizer. Little is known of the amount of energy in the light source itself and on the outcome of PDT.

Therefore, the purpose of this study was to investigate the results of PDT using various light sources and to apply the same amount of energy to the planktonic and biofilm populations of *S. mutans*.

II. Materials and methods

1. Bacterial culture

S. mutans ATCC 25175 was incubated under aerobic conditions in brain heart infusion broth (BHI; Becton, Dickinson and Company, Sparks, MD, USA) supplemented with 5% CO₂ at 37°C for 18 hours. A Smart Plus 2700 spectrophotometer (Young - Woo Inst. Seoul, Korea) was used to measure the turbidity of the bacterial suspension. A standard curve related to turbidity and bacterial counts was used for estimating the colony forming units (CFU) of bacterial suspensions. The bacteria were diluted with phosphate buffered saline (PBS) to 10⁵ CFU/mL.

2. Biofilm formation

The CDC Biofilm Reactor was used to prepare *S. mutans*

biofilms. Hydroxyapatite coupons used as the surface for developing biofilms were mounted into eight rods (each rod can hold three coupons) that could be aseptically removed and replaced through the lid. The rod equipped with the specimen was sterilized using ethylene oxide gas to avoid temperature changes that could affect the micro-hardness of the specimen. The CDC Biofilm Reactor was filled with 100 mL *S. mutans* suspension (1 × 10⁵ CFU/mL) and 300 mL BHI broth, and placed on a stir plate at 50 rpm. During the initial 24 hours, only the vortex was formed and the shear stress was maintained without media flow. After 24 hours, the inflow and outflow of BHI medium were induced using a peristaltic pump (Jenie Well, Seoul, South Korea) at a rate of 18.6 mL/min for 72 h. The block was washed twice with 2 ml phosphate buffered saline (PBS) to remove unattached bacteria and the back of the specimen was wiped with sterile gauze.

3. Photosensitizer

Erythrosine was used as the photosensitizer for PDT. A stock solution of 1 mM/L erythrosine (Sigma - Aldrich, St Louis, MO, USA) was prepared with PBS. The erythrosine solution was filtered-sterilized and stored at -20°C. Working solutions were obtained by diluting the stock solutions with PBS to 20 mM/L. The application time of erythrosine solution was set to 3 min.

4. Light source

Halogen (XL 3000; 3M ESPE, St. Paul, MN, USA), LED (Blue-phase; Ivoclar Vivadent, Liechtenstein, Austria), and plasma arc (Flipo, Lokki, Les Roches de Condrieu, France) curing unit, which are used in dentistry, were used as light sources. The light irradiation diameter of all the light curing units was set equal to 8 mm. The outputs of halogen, LED, and plasma arc were 600, 1200, and 1800 mW/cm², respectively. To verify the output of each light source, we checked the output by sending a curing unit to the manufacturer before the experiment. The power output of each curing units were checked for every experiment using a radiometer (Light Intensity Meter; Denta-merica, San Jose, CA, USA) and applying the formula: 1 W=1 J/s, 1 J = 1 W x 1 s. Using this formula, the irradiation time of halogen, LED, and plasma arc was set to 30, 15, and 10 s, respectively, to irradiate with the same amount of energy.

5. PDT of planktonic and biofilm populations of *S. mutans*

PDT was carried out using planktonic and adherent (biofilm) populations of *S. mutans*. All processes proceeded under natural light. For planktonic samples, 50 μL *S. mutans* culture was added to each well of a sterile flat-bottomed 24 - well plate. Erythrosine solution (40 μL) was added for groups II, VI, VII, and VIII. PBS was added to a final volume of 1000 μL . Samples were divided into eight test groups. Six experiments per group were repeated. In group I, irradiation and PDT were not performed (P-L-). In group II, photosensitizer treatment was done but irradiation was not (P+L-). In group III (P-LH+) halogen irradiation was done 30 s (P-H+). In group IV, LED irradiation was done for 15 s (P-LL+). In group V, plasma arc irradiation was done for 10 s (P-LP+). In group VI, halogen irradiation was done for 90 s and 30 s photosensitizer treatment was done (P+LH+). In group VII, LED irradiation was done for 15 s and photosensitizer treatment was done (P+LL+). Finally, in group VIII, plasma arc irradiation was done for 10 s and photosensitizer treatment was done (P+LP+). The distance between the light source and the sample was 1 cm. After PDT, diluted sample solution was spread on blood agar (Hanil - KOMED, Seongnam, Gyeonggi - do, Korea) using an Eddy Jet spiral plater (IUL Instruments, Barcelona, Spain). CFU was determined using a Flash & Go colony counter (IUL Instruments) after incubation for 72 h at 37°C in an aerobic condition in an atmosphere of 5% CO₂. The viable count was expressed per mL.

PDT of biofilms was done using the same as for the planktonic samples. Three hydroxyapatite coupons per group were used. In groups I, III, IV, and V, 1000 μL of PBS was added to each well. In groups II, VI, VII, and VIII, 40 μL of 1 mM/L erythrosine and 960 μL of PBS were applied to each well. After the experiment, the blocks were transferred to 2 mL PBS and sonicated with a VC 100 ultrasonic device (Sonics & Materials Inc., Danbury, CT, USA) twice for 10 s to dissipate the biofilm. Each sample was diluted with PBS, spread on duplicate blood agar plates, and incubated for 24 h at 37°C in a CO₂ incubator. Viable cells were determined as CFU/mL.

6. Statistical analysis

Analysis of the PDT effect was performed in duplicate, and all procedures were independently repeated on different days. Statistical analysis was performed using one - way ANOVA (SPSS version 21.0; SPSS Inc., Armonk, New York, USA) with

95% reliability. The differences between the study groups were compared and Scheffe's method was performed for multiple comparison procedures.

III. Results

The antimicrobial effects of 3 light sources with the same energy in the planktonic state and the biofilm state of *S. mutans* were compared with the photosensitizer.

1. Planktonic PDT

The mean and standard deviation of the experimental results for planktonic samples are presented in Table 1 and Fig. 1.

No statistical significance was observed in the group treated without both the photosensitizer and the light source, the group treated with the photosensitizer only, and the group treated with only the three kinds of light sources. No statistical significance was observed in the three groups treated with photosensitizer and light source. However, statistical significance was found between the 3 groups treated with photosensitizer and light source and the rest of the groups.

2. Biofilm PDT

The mean and standard deviation of the experimental results in the biofilm state are specified in Table 2 and Fig. 2.

Similar to the planktonic state, the CFU of the *S. mutans* was significantly decreased when the photosensitizer and 3 kinds of light source were used at the same time as the other groups. In addition, the same amount of energy was irradiated in these 3 groups, but no statistical significance was observed in these 3 groups. When compared to the planktonic state, *S. mutans* in the biofilm state showed a difference of about 100 to 1000 times CFU. It was confirmed that the same degree of antimicrobial effect could be obtained when using the same amount of light source in the planktonic state and the biofilm state of *S. mutans*.

IV. Discussion

Many methods for preventing dental caries have been developed. PDT has been in operation since the 1990s[7,16,17]. PDT is a less invasive and less toxic method of reducing biofilms that cause intraoral diseases. In PDT, the light source,

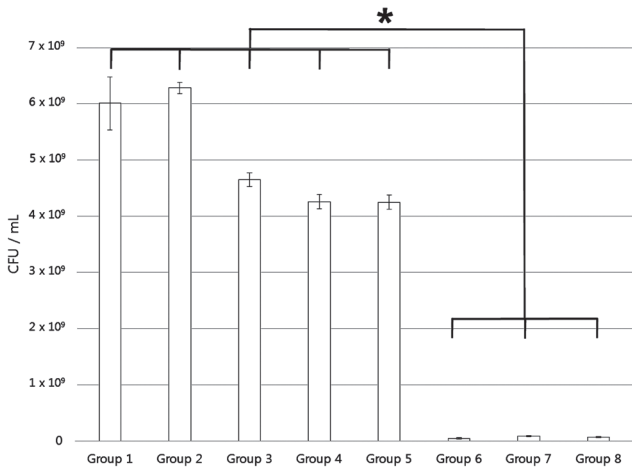


Fig. 1. Mean and standard deviation of *Streptococcus mutans* planktonic cell count. One way ANOVA test (*: $p < 0.05$)

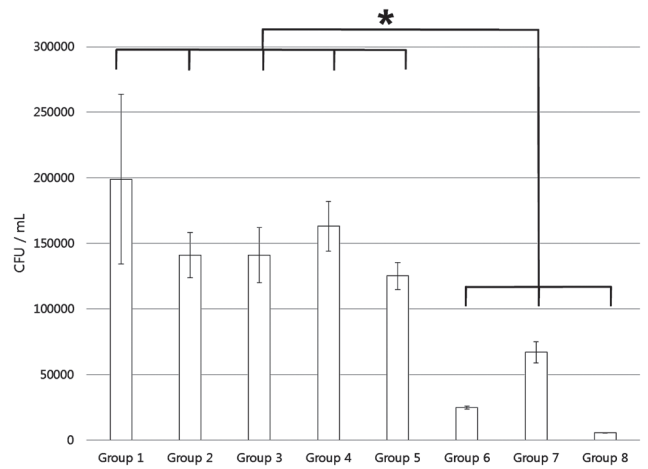


Fig. 2. Mean and standard deviation of *Streptococcus mutans* biofilm cell count. One way ANOVA test (*: $p < 0.05$)

Table 1. *Streptococcus mutans* planktonic viable counts

Group (n = 4)	Bacterial count (mean ± standard deviation CFU/ml)
Group I (P- L-)	$6.01 \times 10^9 \pm 4.68 \times 10^8$ ^a
Group II (P+L-)	$6.28 \times 10^9 \pm 1.05 \times 10^8$ ^a
Group III (P-LH+)	$4.65 \times 10^9 \pm 1.20 \times 10^8$ ^a
Group IV (P-LL+)	$4.26 \times 10^9 \pm 1.27 \times 10^8$ ^a
Group V (P-LP+)	$4.25 \times 10^9 \pm 1.25 \times 10^8$ ^a
Group VI (P+LH+)	$3.36 \times 10^6 \pm 9.85 \times 10^4$ ^b
Group VII (P+LL+)	$4.44 \times 10^6 \pm 4.45 \times 10^4$ ^b
Group VIII (P+LP+)	$1.90 \times 10^6 \pm 7.39 \times 10^4$ ^b

a ,b: statistically significant at $p < 0.05$
 a: No Significance difference between groups I to V
 b: No Significance difference between groups VI to VIII

Table 2. *Streptococcus mutans* biofilm count

Group (n = 4)	Bacterial count (mean ± standard deviation CFU/ml)
Group I (P- L-)	$1.99 \times 10^5 \pm 6.47 \times 10^4$ ^a
Group II (P+L-)	$1.41 \times 10^5 \pm 1.73 \times 10^4$ ^a
Group III (P-LH+)	$1.41 \times 10^5 \pm 2.10 \times 10^4$ ^a
Group IV (P-LL+)	$1.63 \times 10^5 \pm 1.91 \times 10^4$ ^a
Group V (P-LP+)	$1.25 \times 10^5 \pm 1.04 \times 10^4$ ^a
Group VI (P+LH+)	$2.47 \times 10^4 \pm 1.29 \times 10^3$ ^b
Group VII (P+LL+)	$6.67 \times 10^4 \pm 8.12 \times 10^3$ ^b
Group VIII (P+LP+)	$5.36 \times 10^3 \pm 2.30 \times 10^2$ ^b

a ,b: statistically significant at $p < 0.05$
 a: No Significance difference between groups I to V
 b: No Significance difference between groups VI to VIII

photosensitizer, and singlet oxygen interact to reducing the number of biofilms in the oral cavity[18].

The photosensitizer used in PDT has an affinity for bacterial cell walls and is activated by light irradiation, which damages the cell wall. Activated photosensitizer molecules can transfer energy to neighboring cell wall molecules, producing active oxygen or free radicals that can damage or kill bacteria[19]. In this study, the photosensitizer was erythrosine. The compound is commonly used as a disclosing agent in dental practice. Erythrosine is effective in the treatment of intraoral bio-

films[12]. PDT using a halogen curing unit and erythrosine for *S. mutans* biofilms optimized the concentration of erythrosine (> 20 - 40 μ M) and application time (>2.5 min) for the prevention of dental caries[20].

Previous studies on PDT have used lasers as a light source for activating photosensitizers[21-23]. Use of a laser has advantages that include monochromaticity and high potency. However, the procedure is expensive, involves only a single wavelength, and requires a separate unit for each photosensitizer. To circumvent these disadvantages, PDT uses an easy-to-

use and low-cost light source. The three light sources (halogen, LED, and plasma arc) used in this study are also commonly used in dental practice. The photodynamic response of erythrosine induced by a halogen light source produces a sufficient amount of reactive oxygen that is lethal to *S. mutans*[24]. However, disadvantages of a halogen curing unit is the decreased light output over time, heat generation, and reduction of curing efficiency over time.

To overcome these drawbacks, plasma arc curing units and blue light LED devices have begun to be used. Plasma arc lighting devices have been introduced that provide high light output for fast curing. The development of LEDs operating at 470 nm wavelength has provided another alternative to standard halogen curing units. These light-curing units have recently become commercially available. In addition, LED curing devices have been developed as an alternative to halogen curing devices, and these two types of curing units have recently been widely used[25]. A study on PDT using halogen as a light source and erythrosine as a photosensitizer for planktonic *S. mutans* reported an effective reduction in viability[26].

Presently, compared with no treatment (Group I), photosensitizer alone (Group II), and light source alone (Groups III - V), statistically significant decrease in the number of microorganisms was observed in the group using photosensitizer and light source at the same time (Group VI - VIII) in both planktonic and biofilm state. This confirms the usefulness of the antibacterial effect of PDT in both conditions[17]. There was no statistical significance in the three groups using both light source and photosensitizer. The PDT light source should match the activation spectrum of the photosensitizer and produce the appropriate light effects at this wavelength. At the end of the 19th century, Ewald developed the opponency theory[27]. The theory is consistent with PDT, in which the photosensitizer should have the maximum absorbance achieved by a complementary and appropriate light source; for example, a blue photosensitizer must be irradiated by a red light, which is more absorbed, and both of which are complementary colors[27]. In this study, red erythrosine agent and a blue light source were used. The effect of the light source on PDT is influenced by affinity with the photosensitizer, wavelength, and power density[17]. Although the wavelengths of the three kinds of light sources differed from each other, the amount of energy was set to be the same. The same amount of PDT treatment results when the amount of energy applied is the same. Additional studies with the same wavelength and

energy will be needed.

Although there have been a few studies on PDT with different types of light sources, most did not quantify the amount of energy applied. One study used a 650 mW/cm² halogen curing unit as an experimental group and 67 mW/cm² LED as a control group. The irradiated energy was 36 J/cm² and 4 J/cm², respectively. Due to the differences in the amount of energy irradiated, the LEDs failed to show the results of effective photodynamic therapy[24]. Others conducted a study on PDT using 600 mW/cm² of halogen and 900 mW/cm² of LED, and irradiated 18 J/cm² and 27 J/cm² respectively[26]. Both light sources were effective.

In this study, 18 J/cm² of energy was irradiated in all light sources. The optimal dental caries preventive effect in PDT using erythrosine and halogen has been determined to be an irradiation time exceeding 30 s, with an energy output of the halogen of 600 mW/cm²[20]. Presently, the result of PDT was the same for the three groups with the same amount of energy was used along with photosensitizer. Further studies on the results of photodynamic therapy with different energy irradiation will be needed.

The energy of the light source is affected not only by the intensity of the light source but also by the irradiation time. Choi *et al.*[20] reported that the PDT was performed while changing the light irradiation time to the *S. mutans* biofilm using a 600 mW/cm² halogen light source and the antibacterial effect was significantly increased when the irradiation time was 30 seconds or more. On the basis of this result, 30 seconds of light irradiation was performed using the same Halogen curing unit as used in Choi *et al.* The output of the Halogen curing unit used was 600 mW/cm² and the total energy applied was 18000 mW/cm². In order to irradiate the same amount of energy, an LED and a plasma arc curing unit with an energy amount of 1200 and 1800 mW/cm² were used, respectively, and the irradiation time was set to 15 seconds and 10 seconds, respectively.

PDT outcome differed between the planktonic and biofilm states. In the planktonic state, compared with the group that had no treatment, the use of both light source and photosensitizer reduced bacterial viability by about 1000 times more. However, in the biofilm state, the reduction was only 10 to 100 times. Other studies have shown similar experimental results in *Aggregatibacter actinomycetemcomitans*,[28] *Porphyromonas gingivalis* and *Fusarium nucleatum*[29].

Planktonic oral bacteria are sensitized by PDT. However,

the microorganisms responsible for oral diseases are formed in the biofilm state, which is different from that of planktonic bacteria, such as the presence of extracellular polymeric material, differences in cell wall composition, metabolism activity, growth, and gene expression[30]. The bacteria grown in the biofilm are more resistant to antimicrobial agents like antibiotics and biocides. The use of PDT could be prudent in reducing viability of biofilms of bacteria involved in dental caries.

V. Conclusion

In the planktonic and biofilm states of *S. mutans*, erythrosine was used as a photosensitizer and the antimicrobial effects of photodynamic therapy were compared using Halogen, LED, and plasma arc with the same energy as light source.

In the planktonic and biofilm states of *S. mutans*, the CFU of *S. mutans* was significantly reduced only when the light source and photosensitizer were simultaneously applied. The same energy was applied considering the output of the light source and the irradiation time, and the total amount of energy applied was 18000 mW/cm². There was no statistically significant difference in the CFU of *S. mutans* among the three experimental groups using the photosensitizer and halogen, LED, and plasma arc sources, respectively.

Therefore, the effect of PDT on the same amount of energy in both planktonic and biofilm states of *S. mutans* was the same regardless of the type of light source.

This is a useful result in a clinical practice with various dental curing units.

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국문초록

광원의 종류에 따른 광역동 치료시의 *Streptococcus mutans*에 대한 항균 효과

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본 연구의 목적은 치면세균막 착색제인 erythrosine을 광감각제로 사용하여 *S. mutans*에 광역동 치료를 시행하였을 때, 동일한 에너지량을 조사시 광원의 종류에 따른 항균능의 차이를 비교해보고자 함이다.

*S. mutans*를 각각 planktonic 상태와 biofilm 상태로 형성한 후 40 µM로 희석한 erythrosine을 3분간 처리한 다음 광원의 종류로 Halogen, LED, Plasma arc를 사용하였고 각각 30초, 15초, 9.5초의 광조사를 시행하였다.

실험 종료 후 각 실험군의 CFU를 측정하여 각 조건에 따른 광역동 치료 효과를 비교하였다. 각 실험군의 CFU는 통계적으로 유의한 차이가 없었다.

동일 에너지량을 조사시 광역동 치료의 효과는 광원의 종류와 관계없이 동일하다고 할 수 있으며, 이는 다양한 광 조사 기기를 가진 임상현장에서도 유용한 결과라 할 수 있다.