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Original Article

Enhancing the Antibacterial Effect of Erythrosine-Mediated Photodynamic Therapy with Ethylenediamine Tetraacetic Acid

MinKi Choi[1], Haeni Kim[1], Siyoung Lee[2], Juhyun Lee[1]

[1]Department of Pediatric Dentistry, Oral Science Research Center, College of Dentistry, Gangneung-Wonju National University, Gangneung, Republic of Korea

[2]Department of Microbiology and Immunology, College of Dentistry, Gangneung-Wonju National University, Gangneung, Republic of Korea

Abstract

This study evaluated the additive impact of ethylenediamine tetraacetic acid (EDTA) on erythrosine-mediated photodynamic therapy (PDT) against *Streptococcus mutans* (*S. mutans*) biofilm by measuring colony-forming units and applying confocal laser scanning microscopy. Fifty-six bovine incisors, free from dental caries or structural defects, were utilized in this study. Dentin specimens were created by cutting with a low-speed diamond disk under a continuous flow of water, resulting in dimensions of 6.0 mm × 3.0 mm × 2.0 mm. The specimens were categorized into 4 groups: Control, EDTA, PDT, and EDTA + PDT. *S. mutans* ATCC 25175 was employed to establish biofilm on the dentin specimens. A 17% EDTA solution was applied for 1 min. For PDT, erythrosine served as the photosensitizer. Finally, a light-emitting diode source (385 - 515 nm) was employed in this study. The PDT group exhibited a significantly lower bacterial count than both the control and EDTA groups ($p < 0.001$). The EDTA + PDT group demonstrated a significantly reduced bacterial count compared to the other 3 groups ($p < 0.001$). This study demonstrated that EDTA enhances the antimicrobial efficacy of PDT on *S. mutans* biofilm. Even at a low concentration of photosensitizer, the combination of EDTA and PDT yields a significant antibacterial effect. [J Korean Acad Pediatr Dent 2024;51(1):32-39]

Keywords

Streptococcus mutans, Erythrosine, Photodynamic therapy (PDT), EDTA

ORCID

MinKi Choi
https://orcid.org/0009-0005-1832-2184
Haeni Kim
https://orcid.org/0000-0002-4390-6270
Siyoung Lee
https://orcid.org/0000-0001-8826-1413
Juhyun Lee
https://orcid.org/0000-0002-7586-9478

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Introduction

One of the most common chronic diseases, dental caries begins in childhood and lasts the entirety of a person's life[1]. According to a meta-analysis, the global

Corresponding author: Juhyun Lee
Department of Pediatric Dentistry, College of Dentistry, Gangneung-Wonju National University, 7 Jukheon-gil, Gangneung, 25457, Republic of Korea
Tel: +82-33-640-2452 / Fax: +82-33-640-3113 / E-mail: ljh55@gwnu.ac.kr

prevalence of this condition is approximately 46% in primary dentition and 53% in permanent dentition among adolescent children[2]. Among the contributing factors to dental caries, bacterial biofilms play a pivotal role, with *Streptococcus mutans* (*S. mutans*) emerging as a significant cariogenic microorganism. This microbe disrupts the oral environment and contributes to the progression of caries[3]. While the standard treatment for dental caries involves the removal of decayed areas followed by the application of restorative materials, this procedure could be particularly challenging for pediatric dentists. Patient cooperation, especially in cases involving young children or individuals with disabilities, could be difficult to obtain.

Photodynamic therapy (PDT) is a treatment method that uses photosensitizers and a specific-wavelength light source directly to the target site. It exerts its antimicrobial effects primarily through the generation of free radicals and reactive oxygen species (Fig. 1)[4]. PDT is currently utilized in the treatment of skin diseases and cancer[5], and it has recently garnered considerable attention in the context of oral diseases such as dental caries, periodontal disease, and oral cancer[6-8]. Various photosensitizers are employed in PDT, including curcumin, erythrosine, toluidine blue ortho, and methylene blue[9]. Among these, erythrosine, which is used as a tooth colorant, efficiently absorbs the blue light commonly used in dental offices[10]. PDT offers the advantage of being less invasive than surgical procedures and enables precise tissue targeting, rendering it an appealing alternative for the treatment of caries in children, adolescents, and individuals with disabilities[11].

Ethylenediamine tetraacetic acid (EDTA) is a colorless polyaminocarboxylic acid that has found effective application in endodontics as a chelating agent[12]. EDTA reacts with metal ions, such as calcium ions, to form water-soluble calcium chelate compounds[13]. It also removes magnesium ions and lipopolysaccharides found in the cell membranes of gram-negative bacteria[14]. In this way, it eliminates the inorganic components of the smear layer and exhibits sterilization and disinfection properties[12].

Numerous studies have explored the effects of PDT on *S. mutans* biofilms under varying conditions related to photosensitizers and light sources[15-17]. Nevertheless, when PDT was applied to biofilm conditions, its therapeutic effectiveness was suboptimal, giving rise to concerns regarding improving its efficacy. Among these concerns, there are reports suggesting increased effectiveness when EDTA is added to PDT[18,19]. To the best of our knowledge, no studies have looked at the efficacy of erythrosine-mediated PDT to *S. mutans* in conjunction with EDTA. Therefore, this study aimed to ascertain whether EDTA enhances the effect of PDT using erythrosine as the photosensitizer.

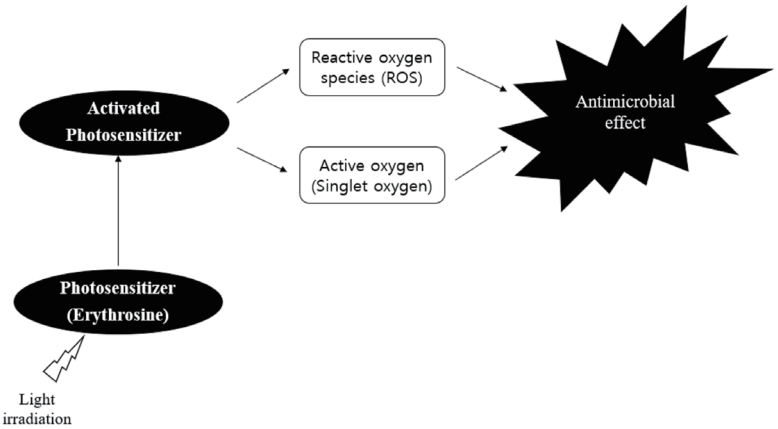

Fig. 1. Schematic diagram of erythrosine-mediated photodynamic therapy.

Materials and Methods

1. Specimen preparation

56 bovine incisors devoid of caries and structural defects were used for this study. The bovine incisors' enamel was removed, and the dentin was sectioned into specimens measuring 6.0 mm × 3.0 mm × 2.0 mm using low-speed diamond disks under a continuous flow of water. The specimen surfaces were then polished with 1000 grit sandpaper. A silicone impression was applied to cover all surfaces, leaving only 1 side exposed, followed by sterilization with ethylene oxide gas.

2. Preparation of bacterial suspension

S. mutans ATCC 25175 was cultured for 18 h in brain heart infusion (BHI) broth (Becton, Dickinson and Company, Sparks, MD, USA) at 37°C under 5% CO_2. The bacterial turbidity was subsequently assessed with a spectrophotometer (Smart Plus 2700, Young-woo instrument, Seoul, Korea), and the *S. mutans* suspension was prepared to a concentration of 1.0×10^8 colony-forming units (CFU)/mL.

3. Biofilm formation

The prepared specimens were arranged in a 12-well plate and inoculated with a mixture consisting of 1980 μL of BHI broth containing 1% sucrose and 20 μL of *S. mutans* suspension. The final bacterial culture concentration reached 1.0×10^6 CFU/mL. The specimens were then incubated for 24 h at 37°C in an aerobic environment supplemented with 5% CO_2.

4. Photosensitizer preparation and light source

Erythrosine powder (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS) to achieve a concentration of 20 μM. This solution was freshly prepared immediately before the experiment, and the polyethylene tubes were shielded with silver foil to minimize light exposure. The 385 - 515 nm light emitting diode (LED, VALO™, Ultradent Products Inc., South Jordan, UT, USA) served as the light source, positioned at a distance of 1 mm employing the extra power mode. The light output (2000 mw/cm^2) was verified with a photometer (DigiRate radiometer, Monitex, Taiwan).

5. Group distribution and biofilm treatment

The specimens were divided into 4 distinct groups. Control group; undergoing no treatment other than washing with PBS. EDTA group; with 40 μL of EDTA applied using a sterilized fine-sized microbrush for 1 min, followed by washing with sterilized saline solution. PDT group; with 40 μL of the 20 μM photosensitizer applied for 3 min, followed by 17 s of light irradiation. EDTA + PDT group; in which specimens were treated with 40 μL of EDTA for 1 min using a sterilized fine-sized microbrush, followed by a saline solution wash, drying with sterile gauze, and application of 40 μL of the 20 μM photosensitizer for 3 min, culminating in 17 s of light irradiation. The groups were summarized in Table 1.

6. Bacterial colony count

After processing all specimens, the bacterial broth was washed twice with PBS and subjected to 20 s of sonication to obtain the bacterial broth. It was then diluted to a 1/1000 concentration using a PBS solution and 50 μL of the broth was evenly spread on a blood agar plate. After incubating the blood agar plate for 3 days at 37°C with 5%

Table 1. Group distribution and biofilm treatment in this study

Group (n = 56)	Treatment
Control (n = 14)	PBS
EDTA (n = 14)	EDTA 40 μL for 1 min + saline
PDT (n = 14)	Erythrosine 20 μM, 40 μL for 3 min + light irradiation
EDTA + PDT (n = 14)	EDTA 40 μL for 1 min + saline + erythrosine 20 μM, 40 μL for 3 min + light irradiation

PBS: phosphate-buffered saline; EDTA: Ethylenediamine tetraacetic acid; PDT: Photodynamic therapy.

CO_2, the colony count was taken with the naked eye, and the number of bacteria was converted to a log scale.

7. Confocal laser scanning microscopy (CLSM) analysis

CLSM analysis was done to confirm the outcomes. After washing each specimen with PBS, a 200 μL solution of the LIVE/DEAD KIT was applied and incubated in the dark to observe the stained biofilm.

8. Statistical analysis

Statistical analysis was performed with SPSS software program version 28.0 (SPSS Corp., Armonk, NY, USA). The Shapiro-Wilk test was employed for data normalization. Subsequently, the Kruskal-Wallis test was used for statistical significance between the groups. The Mann-Whitney test was utilized for post-hoc analysis. The results were corrected by the Bonferroni correction method.

Results

1. CFU count

The CFU counts for all groups are summarized in Table 2. No significant differences were observed between the control and EDTA groups. The PDT group exhibited significantly lower microbial counts than the control and EDTA groups, whereas the EDTA + PDT group resulted in significantly fewer microorganisms than the other 3 groups (Fig. 2).

Table 2. Bacterial count for each group in this study

Group (n = 56)	Bacterial count (Mean ± Standard deviation)	
	Log_{10} CFU/mL	*p* value
Control (n = 14)	5.43 ± 0.73[a]	
EDTA (n = 14)	5.27 ± 0.57[a]	0.603
PDT (n = 14)	4.10 ± 0.74[b]	< 0.0001
EDTA + PDT (n = 14)	1.41 ± 1.49[c]	< 0.0001

p values from Bonferroni's post hoc analysis.
a, b, c: Different letters indicate statistical significance ($p < 0.0083$).
EDTA: Ethylenediamine tetraacetic acid; PDT: Photodynamic therapy.

2. CLSM analysis

In the CLSM analysis, live bacteria are represented by green fluorescence, whereas dead bacteria are indicated by red fluorescence. CLSM images of the groups are presented in Fig. 3. The control group exhibited the highest

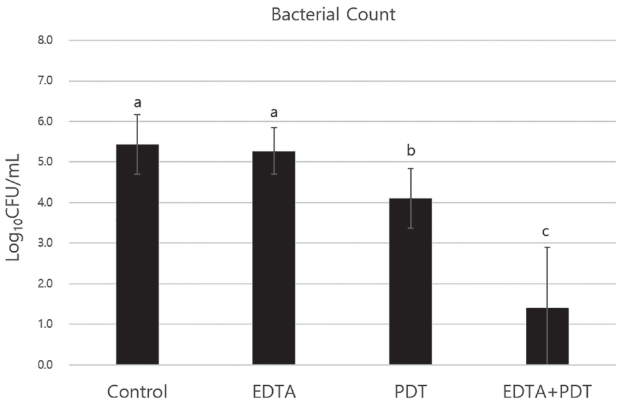

Fig. 2. Bacterial count for each group by using Bonferroni's post hoc analysis.
a, b, c: Different letters indicate statistical significance ($p < 0.0083$).
EDTA: Ethylenediamine tetraacetic acid; PDT: Photodynamic therapy.

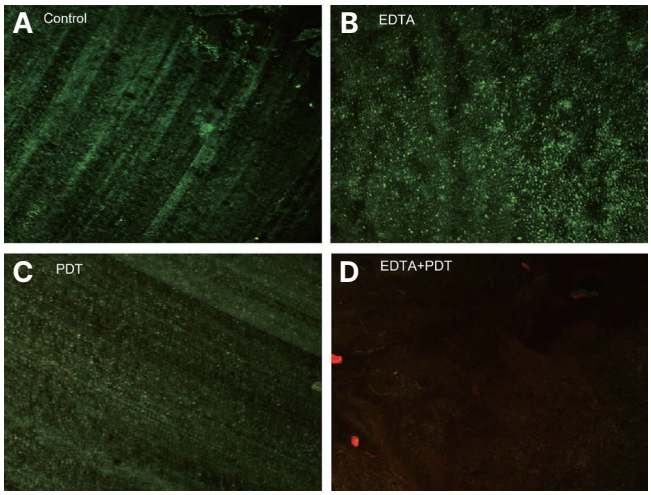

Fig. 3. Confocal laser scanning microscopy images of each group. (A) Control group, (B) EDTA group, (C) PDT group, (D) EDTA + PDT group.
Live bacteria: green fluorescence, Dead bacteria: red fluorescence.
EDTA: Ethylenediamine tetraacetic acid; PDT: Photodynamic therapy.

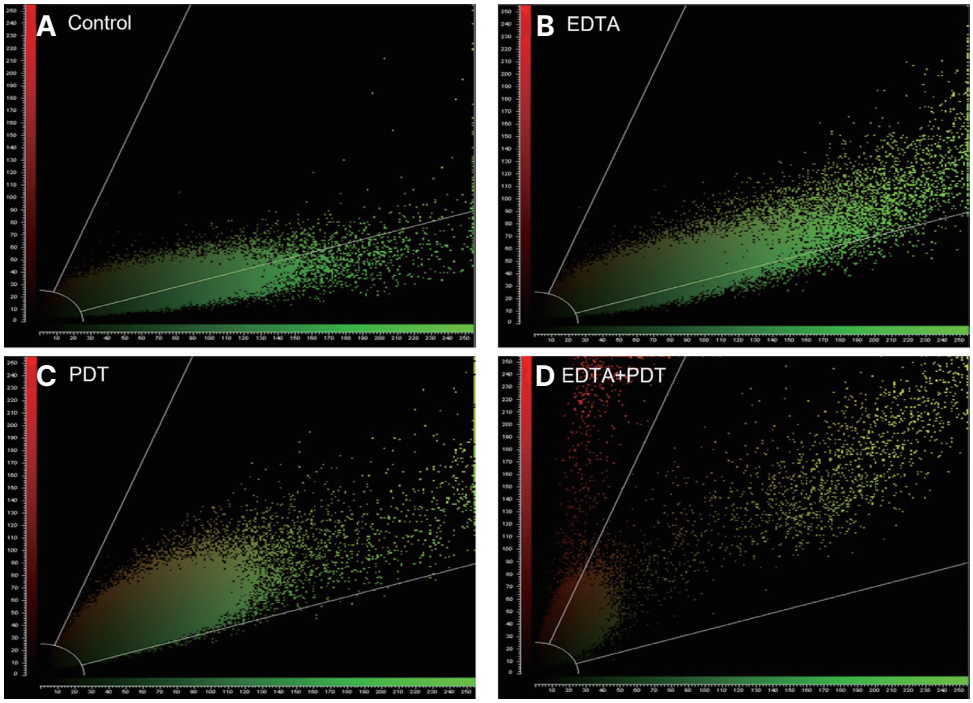

Fig. 4. Scatter plots (pixel distribution diagrams) for each group. (A) Control group, (B) EDTA group, (C) PDT group, (D) EDTA + PDT group. Live bacteria: green fluorescence, Dead bacteria: red fluorescence. EDTA: Ethylenediamine tetraacetic acid; PDT: Photodynamic therapy.

degree of green fluorescence, whereas the EDTA + PDT group exhibited an increased presence of red fluorescence. Scatter plot analysis of green and red fluorescence demonstrated that the percentage of red fluorescence progressively increased in the direction of the EDTA + PDT group, signifying a significantly heightened antibacterial effect (Fig. 4).

Discussion

EDTA is widely utilized in endodontic treatments, such as regenerative endodontic procedures, owing to its ability to effectively remove the smear layer. The degree of smear layer removal varies depending on factors such as pH, concentration, and application time of EDTA. In a study by Serper and Calt[20], phosphorus liberation from dentin was found to be more pronounced under neutral pH and higher concentration conditions. They also reported that a 10-min application of 17% EDTA resulted in more extensive erosion of the dentin compared to a 1-min application[21]. In the study conducted by Lee et al.[22], EDTA applied for 30 s, 1 min, or 2 min almost completely eliminated the smear layer in the middle third of the root. The degree of erosion gradually increased as the application time increased. Therefore, in our study, we chose to apply a 17% EDTA solution, a common practice in endodontic treatment, to the specimens for 1 min.

The effectiveness of PDT relies heavily on 2 key factors: the photosensitizer and the light source. Erythrosine is FDA-approved and widely used in food, drugs, and cosmetics[23]. Choi et al.[24] revealed that PDT with erythrosine exhibited a significant effect when the photosensitizer concentration was 20 - 80 μM, with efficacy improving as the concentration increased. Furthermore, the photosensitizer demonstrated a notable PDT effect when applied for a minimum of 2.5 min[24]. Chequer et al.[25] reported significant DNA damage at erythrosine concentrations exceeding 50 μg/mL. In our study, we employed a 20 μM concentration of erythrosine, which represents the minimum concentration for a significant effect. This concentration, approximately 0.2 μg/mL, is likely safe for use as a photosensitizer. The application duration of the photosensitizer in this study was set at 3 min.

To the best of our knowledge, a consistent protocol

for the power and duration of light sources has not been established. Choi et al.[24] observed that a minimum of 30 s of irradiation was necessary to achieve a significant effect when employing 600 mW/cm^2 for PDT, equivalent to at least 18 J/cm^2 of irradiation. Nima et al.[26] reported that PDT treatment with curcumin could yield effective results without causing DNA damage when applying 33.5 J/cm^2 at a distance of 1 mm. Curcumin absorbs light in the wavelength range of 400 - 500 nm[26,27], whereas erythrosine absorbs light in the approximately 450 - 550 nm wavelength region[28]. Despite the difference in photosensitizers, PDT effects might still be expected when employing blue light. In this study, we utilized LED blue light as the light source, with a light irradiation output of at most 2000 mW/cm^2 and an application time of 17 s. Based on the literature, the irradiation energy was calculated as a maximum 34 J/cm^2.

EDTA exerts its antibacterial effects by dissociating divalent ions from the cell membrane of gram-negative bacteria[29]. We found no discernible differences between the control group and the EDTA group in our study. We speculate that *S. mutans*, being a gram-positive bacterium, might be more resistant to EDTA, which aligns with previous studies demonstrating that EDTA alone has limited antibacterial activity[30,31].

The PDT group demonstrated a significantly reduced number of *S. mutans* microorganisms compared to the control and EDTA groups. This result supports the effectiveness of PDT against cariogenic bacteria, consistent with prior research[15-17,32,33]. However, PDT is less effective when dealing with mature biofilm state. Garcez et al.[34] reported that PDT was most effective in the outermost layer of biofilms, suggesting that photosensitizers need to penetrate deeper to be effective within bacterial biofilms.

The EDTA + PDT group displayed significantly fewer microorganisms than the other 3 groups, confirming the enhancing effect of EDTA on PDT. The chelating properties of EDTA removed the smear layer, enhanced dentin permeability, and allowed the photosensitizer to penetrate deeper, thus increasing the effectiveness of PDT[35]. This finding aligned with the study employing different photosensitizer[36].

To the best of our knowledge, this study is the first to confirm the enhancement of erythrosine-mediated PDT to S.mutans using EDTA, highlighting the efficacy of achieving effective PDT with a lower photosensitizer concentration. Nevertheless, because dental caries could be attributed to various microorganisms, the first limitation of this study was that it focused on the initial biofilm of a single cariogenic bacterium. The second limitation of this study was that it was an in vitro study using bovine teeth. Future investigations should explore the potentiation antibacterial effect of PDT in multispecies cariogenic bacteria through the inclusion of EDTA, as well as assess its impact on bond strength in restorations following combined PDT with EDTA treatment for clinical use.

Conclusion

Despite the limitations of this study, it conclusively demonstrated that EDTA significantly enhances the efficacy of low-concentration erythrosine-mediated PDT against *S. mutans*. To pave the way for future clinical applications, further research is imperative to explore the impact of this combined treatment on multispecies cariogenic bacteria and to assess its effects on the bond strength of restorations.

Conflict of Interest

The authors have no potential conflicts of interest to disclose.

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