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Antimicrobial Effects of 5-Aminolevulinic Acid Mediated Photodynamic Therapy against Pathogenic Bacteria

Pil Seung Kwon*

Department of Clinical Laboratory Science, Wonkwang Health Science University, Iksan, Republic of Korea

***Corresponding author:** Pil Seung Kwon, pskwon@wu.ac.kr

Abstract

This study evaluates the improved effect of photodynamic therapy (PDT) by subjecting pathogenic bacteria to a combination of 630 nm light-emitting diode (LED) and 5-aminolevulinic acid (ALA). Bacterial suspensions of 1.5×10^4 cells/mL were diluted and exposed to ALA concentrations of 10, 5, 2.5, 1.25, and 0.625 mg/mL, incubated for 30 minutes, followed by irradiation with 630 nm LED (18 J/cm^2). The non-irradiated *P. aeruginosa* group and the group administered only LED light averaged 415 and 245 colonies, respectively. Conversely, the PDT group showed an average of 109, 225, 297, and 285 colonies at concentrations of 10, 5, 2.5, and 1.25 mg/mL of ALA. Evaluating the effect on *E. faecalis* revealed an average of 8,750 and 8,000 colonies in the group that did not receive the control photosensitizer and the group exposed to light alone, respectively. However, an average of 0, 2,350, 4,825, and 7,475 colonies at concentrations of 5, 2.5, 1.25, and 0.625 mg/mL ALA were determined for the PDT groups. In conclusion, better inhibitory effects were observed for *E. faecalis* than for *P. aeruginosa*. Moreover, our results validate the possibility of improved PDT efficacy using a combination of ALA and 630 nm LED.

Keywords

5-aminolevulinic acid
Enterococcus faecalis
Photodynamic therapy
Pseudomonas aeruginosa

1. Introduction

The cellular mechanism of photodynamic therapy (PDT) is understood through photophysical phenomena. The administered photosensitizer, in its ground state, is activated by light of a specific wavelength, transforming it into an excited state, either a singlet state or a triplet state. When it returns to the ground state, it emits energy that reacts with oxygen to generate active oxygen species (O_2^-), a Type II reaction. In cases where the photosensitizer is involved in the electron transport system, it forms free radicals, resulting in a Type I reaction, causing cell damage [1,2].

Superficial skin conditions are treated by directly illuminating the tissue with light, while deep-seated conditions within the body are reached using a fiber catheter for treatment and then illuminated with light. The laser light source primarily used in photodynamic therapy emits collimated light and offers excellent therapeutic effects, making it an ideal choice, but its high cost makes it less accessible. As a result, many researchers have shown great interest in low-cost light-emitting diodes (LEDs). They can cover a wider area of lesions, receive less light per unit area, and are used extensively in research for extracorporeal photodynamic therapeutic research [3,4]. PDT is known as one of the cancer treatment methods and is used in clinical treatments. However, there is limited active research on its effectiveness against clinical microorganisms such as bacteria and fungi [5]. Bacterial PDT traces back to the year 1900 when Oscar Raab, a medical student in Munich, Germany, experimented with acridine dye. During his experiments, he discovered that acridine red-stained protozoa exposed to light would die. This discovery led to initial attempts at extracorporeal methods, and since then, many studies have been conducted using various photosensitizers [6,7].

5-aminolevulinic acid (ALA) is a natural photosensitizer that is converted into protoporphyrin IX (PPIX) within target cells. ALA possesses several advantageous characteristics, and α-linolenic acid in the heme biosynthesis pathway is a natural intermediate product that can be rapidly removed from target cells [8]. ALA, as the actual precursor of the photosensitizer PPIX, is small enough to penetrate the cell's surroundings and accumulate in the target cells [9]. Finally, the photodynamic effects of ALA are limited to surface lesions (1–2 mm) due to the restricted penetration of the light source [10]. These characteristics can shorten the light exposure time, reduce tissue damage, and enhance the effectiveness of PDT, and all of this ensures the safety and efficacy of PDT in clinical applications.

In recent years, lasers and LEDs used in lighting devices have shown promising results against various types of bacteria and yeast [11-15]. This study applied PDT as an alternative to antibiotics for treating bacteria by activating reactive oxygen species (ROS) through appropriate wavelengths of light (red) and ALA. This approach takes advantage of the lethal action of ROS within target cells. This study aimed to demonstrate its therapeutic effects on *Pseudomonas aeruginosa* (green pus bacillus) and *Enterococcus faecalis* [16].

P. aeruginosa is a bacterium responsible for approximately 10% of hospital infections and regional infections. Infections caused by *P. aeruginosa* can often be severe, endanger lives, or present treatment difficulties due to the emergence of antibiotic resistance during therapy, posing a high risk. Consequently, it is a bacterium that can lead to serious consequences [17,18]. Additionally, *P. aeruginosa* is a common bacterium responsible for wound infections. Current treatments for skin wound infections involve systemic antibiotics, topical dressings, surgery, and other methods, but they have disadvantages such as time, cost, natural resistance, and resistance due to biofilm formation. Furthermore, vancomycin-resistant enterococci (VRE) have become a prominent pathogen in healthcare-associated infections since their first report in Europe in 1986, with their global isolation frequency increasing over the past three decades [19].

The purpose of this study was to explore the potential of ALA-based PDT for antimicrobial effects against the challenging bacteria *P. aeruginosa* and *E. faecalis*, intending to address the difficulties in their treatment.

2. Materials and methods

2.1. Target strains and culturing

The standard strain of *P. aeruginosa* (ATCC 27853) was inoculated into 50% glycerol-brain heart infusion broth and stored at -70°C. It was then subcultured onto

MacConkey agar and incubated for one day at 37°C in an aerobic incubator (Vision Scientific, Daejeon, Korea) before use. The standard strain of *E. faecalis* (ATCC 29212) was subcultured on trypticase soy agar and used.

2.2. Light source and photosensitizer

The photosensitizer used in this study, 5-aminolevulinic acid hydrochloride (Sigma Chemical Company, St Louis, MO), was dissolved in Dulbecco's phosphate-buffered saline (Hyclone, USA) to a concentration of 100 mg/mL and then filtered through a 0.2 μm filter before use.

A custom-made LED was used as the light source. The LED array was created in a cylindrical shape with a diameter of 5Φ for exposure in test tubes. The 630 nm LED array measured 120 mm horizontally by 180 mm vertically and contained a total of 384 LEDs (16×24). The LED circuit connections were made in both series and parallel, and direct current power was used. To extend the LED's lifespan and prevent damage, a stable resistance of 150 Ω was used, and the light intensity of the LED array was adjusted to 10.0 mW/cm^2 by controlling the current from the power supply unit (Hanil, Korea). The distance between the LED array and the test tube was set to 100 mm and the bacterial suspension was evenly exposed by tilting the Petri dish to one side. A 630 nm LED with a voltage of 12.0 V, a current of 0.4 A, and an energy density of 10.0 mW/cm^2 was applied for 30 minutes. The final energy delivered was 18 J/cm^2.

2.3. Measurement of *P. aeruginosa* colony forming units (CFU)

P. aeruginosa strain subcultured on MacConkey agar was adjusted to McFarland No. 0.5 using a turbidimeter (DensiCHEK™ Plus, bioMérieux, USA) with Muller-Hintone broth to 1.5×10^4 cells/mL, and the strain suspension was made in 12×75 mm polyethylene cap tubes (SPL Life Sciences, Korea). ALA was diluted in the strain suspension to final concentrations of 10, 5, 2.5, and 1.25 mg/mL. The mixture was then incubated for 30 minutes in a 37°C incubator (Thermo Forma, 47502-3362), with light blocked using aluminum foil. Subsequently, the samples were exposed to light at 10 mW/cm^2 for 30 min using a 630 nm LED light source. After LED exposure, the samples were mixed and 50 μL from each group was evenly spread onto MacConkey agar plates. These plates were incubated for 18 hours, and the number of colonies formed was counted. Six measurements were taken for each group, and for colonies exceeding 100, additional dilution with sterilized saline was performed, and colony counts were recorded. Mean values and standard deviations were calculated.

2.4. Measurement of *E. faecalis* colony forming units (CFU)

E. faecalis strain subcultured on trypticase soy agar was adjusted to McFarland No. 0.5 using a turbidimeter (DensiCHEK™ Plus, bioMérieux, USA) with Enterococcosel broth to 1.5×10^5 cells/mL, and the strain suspension was made in 12×75 mm polyethylene cap tubes (SPL Life Sciences, Korea). ALA was diluted in the strain suspension to final concentrations of 5, 2.5, and 1.25, 0.625 mg/mL. The mixture was then incubated for 30 minutes in a 37°C incubator (Thermo Forma, 47502-3362), with light blocked using aluminum foil. Subsequently, the samples were exposed to light at 10 mW/cm^2 for 30 minutes using a 630 nm LED light source. After LED exposure, the samples were mixed and 50 μL from each group was evenly spread onto trypticase soy agar plates. These plates were incubated for 18 hours, and the number of colonies formed was counted. Four measurements were taken for each group, and for colonies exceeding 100, additional dilution with sterilized saline was performed, and colony counts were recorded. Mean values and standard deviations were calculated.

3. Result

3.1. Measurement of *P. aeruginosa* CFU

P. aeruginosa formed an average of 415 and 245 colonies in the control and LED irradiated samples, respectively. In the PDT group with ALA at concentrations of 10, 5, 2.5, and 1.25 mg/mL, a total of 109, 225, 297, and 285 colonies were formed, respectively, as shown in **Figures 1** and **2**.

3.2. Measurement of *E. faecalis* CFU

E. faecalis formed an average of 8,750 and 8,000 colonies in the control and LED irradiated samples, respectively. In contrast, in the PDT group with ALA at a concentration of 5 mg/mL, no colony formation was observed. At ALA concentrations of 2.5, 1.25, and 0.625 mg/mL, a total of 2,350, 4,825, and 7,475 colonies were formed, as shown in **Figures 3** and **4**.

4. Discussion

In the context of the recent COVID-19 pandemic, PDT has been proposed as a potential treatment for respiratory infections related to COVID-19, using methylene blue-mediated therapy [20]. PDT represents a promising and innovative alternative therapy for treating microbial infections and is particularly

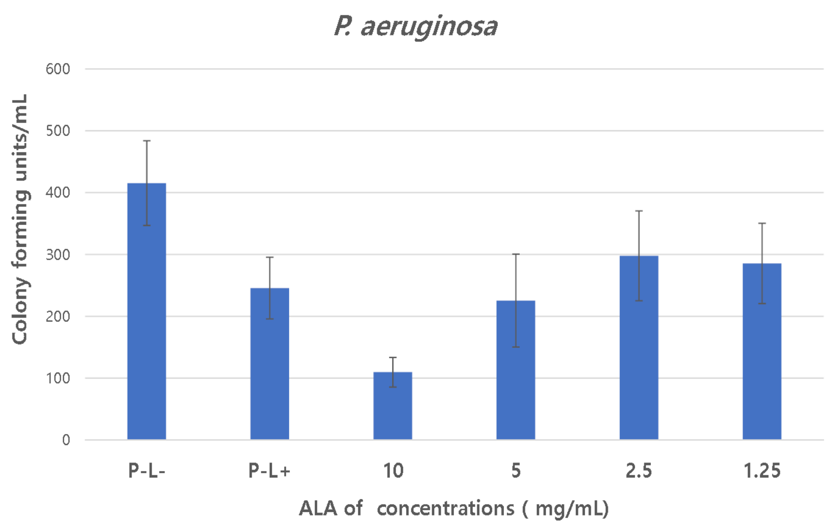

Figure 1. Colonies of *P. aeruginosa* were counted after photodynamic therapy (PDT) with 5-aminolevulinic acid (ALA) concentrations of 10, 5, 2.5, and 1.25 mg/mL, using 630 nm LED irradiation on bacterial suspensions. Abbreviations: P-L-, Bacterial suspensions not treated; P-L+, LED irradiation only; 10~1.25, ALA concentrations of 10~1.25 mg/mL with LED irradiation on bacterial suspensions.

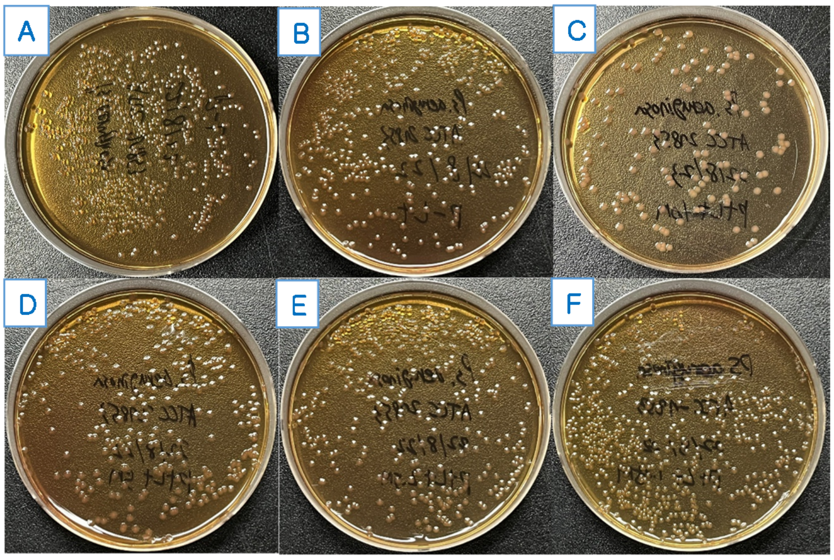

Figure 2. The colony-forming images of *P. aeruginosa* on MacConkey agar in the photodynamic therapy (PDT) and control groups. **(A)** No treatment; **(B)** LED only; **(C~F)** 5-aminolevulinic acid (ALA) concentrations of 10, 5, 2.5, and 1.25 mg/mL with irradiation using a 630 nm LED.

important for the management of infectious skin lesions, such as skin ulcers, abscesses, and cellulitis. While significant progress has been made in the treatment of infectious skin wounds, the exact mechanism through which PDT promotes healing remains unclear.

Infections caused by the Gram-negative bacterium *P. aeruginosa* are notoriously difficult to treat. *P. aeruginosa* is a common pathogen responsible for severe skin wounds, and urinary and respiratory tract infections. Tan *et al.* reported that PDT using ALA can effectively kill planktonic and viable biofilm-associated *P. aeruginosa* cells, disrupt biofilm structures, reduce virulence factor secretion, and influence gene expression [21]. Furthermore, the incidence of *P. aeruginosa* infections related to skin wounds is on the rise. Therefore, this study aimed to explore the feasibility of treatment and verify the inhibitory effects of ALA-based PDT against *P. aeruginosa*. Even when only red LED light at 630 nm was used for irradiation, it was observed that, in comparison to the control group with an average of 415 colonies, the PDT group with an ALA concentration of 5 mg/mL resulted in the formation of 225 colonies, indicating a significant antimicrobial effect. Future research should consider comparative experiments based on energy density

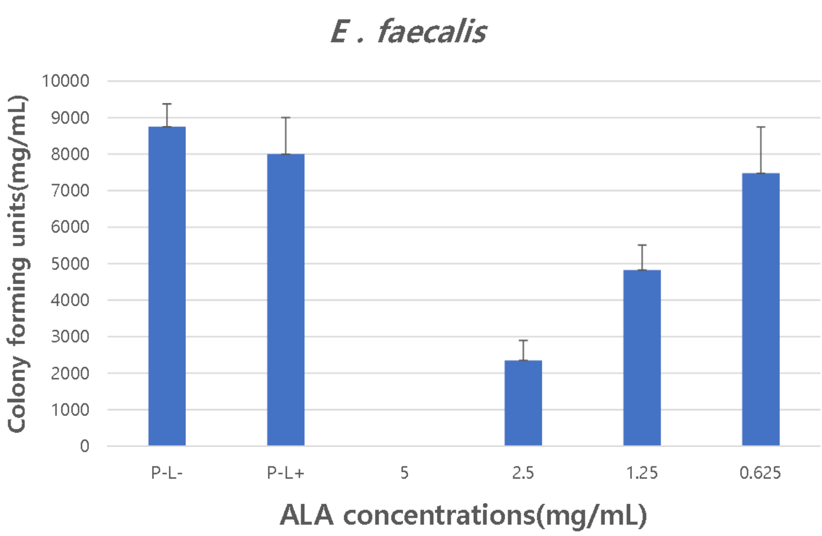

Figure 3. Colonies of *E. faecalis* were counted after photodynamic therapy (PDT) with 5-aminolevulinic acid (ALA) concentrations of 5, 2.5, 1.25, and 0.625 mg/mL, using 630 nm LED irradiation on bacterial suspensions. Abbreviations: P-L-, Bacterial suspensions not treated; P-L+, LED irradiation only; 5~0.625, ALA concentrations of 5~0.625 mg/mL with LED irradiation on bacterial suspensions.

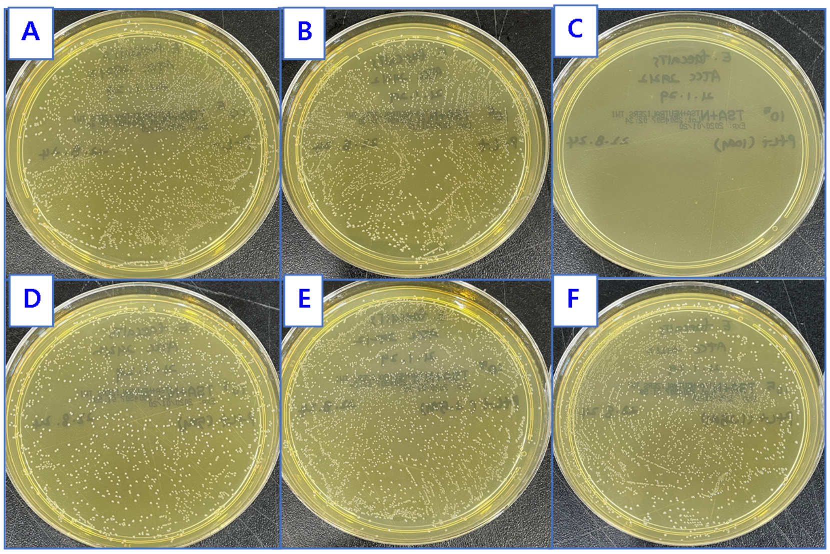

Figure 4. The colony-forming images of *E. faecalis* on trypticase soy agar in the photodynamic therapy (PDT) and control groups. **(A)** No treatment; **(B)** LED only; **(C~F)** ALA concentrations of 5, 2.5, 1.25, and 0.625 mg/mL with irradiation using a 630 nm LED.

and investigate the underlying mechanisms. On the other hand, the control group of *E. faecalis* exhibited an average of 8,750 colonies, and even with LED irradiation alone, 8,000 colonies were formed. While this indicated a slightly lower effect as compared to the *P. aeruginosa* samples, it suggests some inhibitory effect.

In 2013, Reena and Karthika indicated that PDT requires three key factors: a photosensitizer, a light source, and oxygen [22]. ALA is the predominant photosensitizer used in acne treatment, although new photosensitizers such as indocyanine green (ICG) and indole-3-acetic acid (IAA) have been introduced. Similarly, it is suggested that extensive experiments are needed for the treatment of *P. aeruginosa* and *E. faecalis* to apply various types of photosensitizers and light sources to achieve maximum effect.

In 2011, Kwon demonstrated 100% eradication of VRE using photogem from the porphyrin group and LED light [23]. Considering photogem at 50 µg/mL with the same light source, this study showed that PDT with ALA. even at a high concentration of 10 mg/mL, did not result in the complete eradication of *P. aeruginosa*. However, *E. faecalis* was completely eradicated at 5 mg/mL, and at 2.5 mg/mL, it showed an average of 4,075 colonies. Based on these results, it can be inferred that ALA-based PDT is less effective compared to photogem from the porphyrin group.

In the 1990s, PDT for bacterial infections demonstrated fundamental differences in sensitivity between Gram-positive and Gram-negative bacteria. Gram-positive bacteria were generally more susceptible to PDT, as they effectively bound to common neutral or negatively charged photosensitizer molecules, resulting in photodynamic inactivation. In contrast, Gram-negative bacteria were known to be more challenging to treat using PDT, primarily due to the limited binding of photosensitizers to their outer membranes.

As demonstrated in previous studies, such as the work of Hamblin *et al*. in 2004 [7], one key observation in attempts to photoinactivate bacteria using porphyrin-based photosensitizers was the relatively high sensitivity of Gram-positive bacteria to photodynamic inactivation, while Gram-negative bacterial strains exhibited significantly higher resistance. This difference in susceptibility was attributed to the fact that Gram-positive bacteria are surrounded by physiologically more permeable layers, such as peptidoglycan or lipoteichoic acid in their cell walls, making them more susceptible to PDT [24,25].

In the results of this study using ALA-based PDT, it was observed that PDT had a more favorable effect on *E. faecalis* than *P. aeruginosa*, a gram-negative bacterium. Although no specific examinations such as ROS measurements or photosensitizer accumulation tests were conducted to investigate the mechanism of PDT, the primary antimicrobial effect has been confirmed. In the future, further studies will be conducted to explore the therapeutic effects of various photosensitizers, the effects based on energy density, and the mechanisms involved.

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Disclosure statement

The author declares no conflict of interest.

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