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Erythrosine-Mediated Photodynamic Inactivation of Bacteria and Yeast Using Green Light-Emitting Diode Light

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ABSTRACT

Erythrosine, also known as FD&C Red No. 3, is a synthetic dye that has been used for food coloring and dental plaque detection. The objective of this study was to evaluate erythrosine as a viable photosensitizer in photodynamic inactivation (PDI) of bacteria and yeast. Cells were treated with erythrosine with or without acetic acid in the presence and absence of light. Erythrosine in the absence of light did not show any activity against the microorganisms. When treated with light, erythrosine was efficient in eradicating non-adherent (planktonic) Gram (+) bacteria and yeast. However, Gram (-) bacteria were less responsive to PDI. Addition of acetic acid was found to enhance the activity of erythrosine and significantly lower the survival of Gram (-) bacteria. This study demonstrated the potential applications of erythrosine in PDI and the potentiating effects of acetic acid.

Key words: erythrosine, acetic acid, photodynamic inactivation (PDI), bacteria, yeast, biofilm

INTRODUCTION

Dental caries and periodontitis are common plaque-related diseases occurring in human mouth. Control of these diseases has relied on removing plaque periodically and antimicrobial agents. The development and widespread use of antibiotics to treat bacterial infections however, has led to a rapid increase in antibiotic resistance amongst pathogenic bacteria[1].

In recent years, the growing resistance to antibiotics among pathogens rendered antimicrobial photodynamic inactivation (PDI) an alternative anti-infection treatment modality[1]. PDI is a platform technology in which a combination of photosensitizers (PSs) and visible light produces a phototoxic response that results in the oxidative damage to a variety of targets, including pathogenic and resistant microorganisms[2].

Erythrosine is commonly used as a dental plaque disclosing agent[3]. It also belongs to a class of cyclic compounds called xanthenes, which absorb light in the visible region and can initiate a photochemical reaction[4,5]. The medical applications of erythrosine and its reactivity with light give it an advantage over other PS in development.

In a previous study, erythrosine was evaluated for its PDI effect, where a 400 W tungsten filament lamp was employed as the light source. This required the use of a heat dissipating water bath between the lamp and the biofilm samples[6].

In this study, a green light-emitting diode (LED) device was utilized as an alternative and a more efficient light source. The aim of this study was to investigate the photodynamic inactivation effect of erythrosine on microorganisms, including Gram (+), Gram(-) bacteria and *Candida albicans*. The susceptibility of both planktonic and biofilm forms was evaluated. The enhancement effect of acetic acid on PDI was also determined.

MATERIALS AND METHODS

I. *Materials*

Erythrosine and glacial acetic acid were purchased from Sigma (St. Louis, MO, USA). A 20 mM stock solution of erythrosine in phosphate buffered saline (PBS) was foil-covered and stored at 4°C. After filtration through a 0.2 μm polycarbonate membrane, the dye was appropriately diluted with PBS to obtain the working solutions of desired concentrations. Double distilled water was used for chemical and medium preparation and dilution. Microbial culture media Tryptic soy broth (TSB) and Yeast Extract Peptone Dextrose

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broth (YPD) were from Difco (Detroit, MI, USA).

II. *Microbial Strains and Culture Conditions*

Staphylococcus aureus (BCRC 10780) was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Other microbial strains used in this study including *Streptococcus mutans* (ATCC25175), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia .coli* (ATCC 25922) and *Candida albicans* (SC5314) were from ATCC (Manassas, VA, USA). TSB was used as the liquid medium for *S. aureus , S. mutans, P. aeruginosa* and *E. coli* cultures whereas YPD was used for *C. albicans*. A 0.1% medium was used for batch cultures, while a 0.01% medium for continuous biofilm cultures.

III. *Irradiation Dose Parameters*

A noncoherent light source was used to activate erythrosine. Green LED light with a wavelength of 540 ± 5 nm was used and the output power was set at 22 mW/cm^2. Light dose was calculated by multiplying the output power by the irradiation time as given in the following equation:

$$\text{Light dose (J/cm}^2\text{)} = \frac{\text{output power}\left(\frac{\text{mW}}{\text{cm}^2}\right) \times \text{irradiation time(sec)}}{1000}$$

IV. *Biofilm Preparation*

Biofilms were cultured on a rotating disk reactor modified from the design of Pitts *et al*.[7]. The reactor consisted of a 500 mL polypropylene container, a Teflon rotor, and 24 removable 316 L stainless steel disks inserted on the rotor. One mililiter of each bacterial suspension seed culture was inoculated into 150 mL 0.1% medium in the reactor and incubated at room temperature for 24 h. After incubation, the medium was moved. The reactor was then continuously fed with a 0.01% medium at a rate of 240 mL/h. Biofilms reached a steady state after 24 h for the bacteria and 48 h for *C. albicans*.

V. *PDI in Planktonic Cells*

All strains were grown aerobically overnight at 37°C. Bacterial cells were first harvested by centrifugation at 11,500 ×g for 5 min and then resuspended in PBS to produce a cell suspension containing 10^8 CFU/mL. Suspensions containing 10^8 CFU per mL were treated with erythrosine and kept in the dark for 10 min. Treated samples were then centrifuged at 17,500 ×g for 1 min after which the supernatant was remove and the pellet was resuspended in 1 mL of PBS. The resultant suspension was split into a number of equivalent portions in a 96-well plate (0.2 mL/well), each of which was irradiated with green light for various periods of time. After irradiation, 0.1 mL samples were taken from each well and serially diluted (10-fold) with PBS. Aliquots were spread over agar plates made from appropriate media. Plates were incubated aerobically overnight at 37°C (48 h for *S.mutans*) after which colony count was determined.

VI. *PDI in Biofilm Cells*

Disks with biofilms were placed into a sterile 48-well microtiter plate. Each biofilm was first treated with 300 µL of erythrosine and stored in the dark. After 10 min, disks were moved to a new dish and irradiated with green light for various periods of time. Following irradiation, three biofilms per time point were first disrupted from the plugs by vortexing in 1 mL PBS for 1 min and then serially diluted with PBS. Aliquots were spread over agar plates made from appropriate media and incubated aerobically overnight at 37°C, followed by colony counting.

VII. *Bacterial Cell Survival Assay*

CFU of a bacterial suspension was counted using the following standard protocol: aliquots (10 µL) of appropriate dilutions (from 10^{-1} to 10^{-5}) were plated on TSB agar plates and incubated at 37°C in darkness for 18 h. The survival fraction was calculated as the number of CFU per mL after photodynamic inactivation and devided by the number of CFU per mL in the initial sample. The intrinsic toxicity of the compounds in the absence of light, was monitored by evaluating the survival fraction of incubated but non-illuminated microbial samples, and calculated from the number of CFU per mL of the non-illuminated samples compared to that of the initial sample.

VIII. *Statistics*

Survival values were expressed as means ± standard deviation (SD). Differences between two means were assessed for significance by the two-tailed *Student's t-* test and a value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

I. *Erythrosine-Mediated PDI in Planktonic Cells*

When planktonic bacterial cells were treated with PDI using 0.05 mM of erythrosine and irradiation light dose of 50 J/cm^2, no viable Gram (+) cell was detected (Figure 1A & 1B). However, Gram (-) bacteria were not significantly affected by the PDI treatment even after the erythrosine concentration was raised up to 20 mM (Figure 1C & 1D). Similar result with Gram (-) bacteria has been presented by Rodnei *et al*.[8] who reported that erythrosine under light illumination didn't reduce the number of CFUs per mL of *Enterobacter Cloacae*, *E. coli*, *Klebsiella pneumonia* and *K. oxytoca*. When the concentration of erythrosine was raised to 4 mM, no viable cells of *C. albicans* was detected (Figure 1E). Treatment of the studied planktonic cells with erythrosine

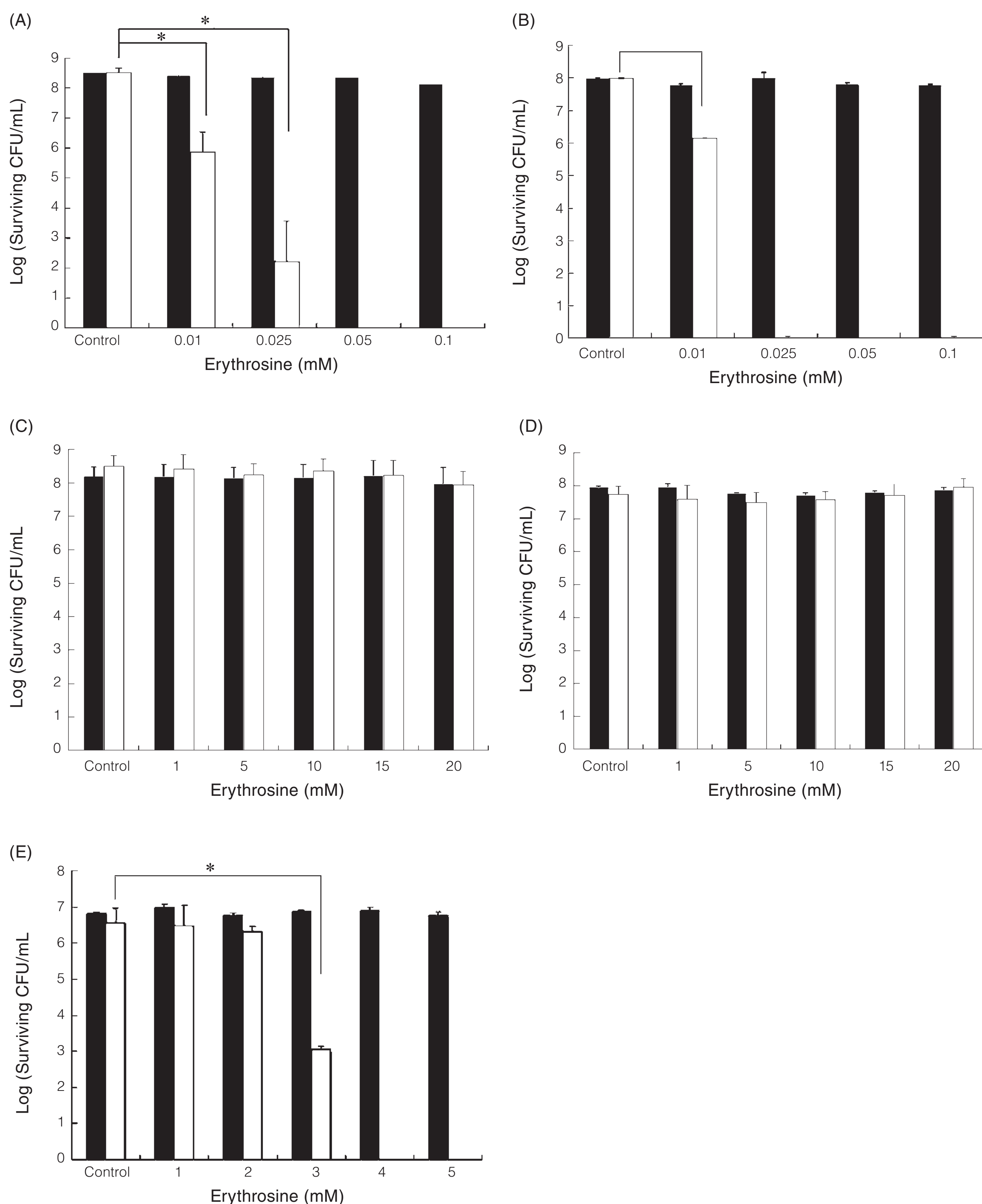

Figure 1. The survival of *S. aereus* (A), *S. mutans* (B), *P. aeruginosa* (C), E.coli (D), (10^8 CFU/mL) and *C. albicans* (E) (10^7 CFU/mL) treated with different concentrations of erythrosine in the presence or absence of light (Light dose: 0 (■) and 50 (□) J/cm^2). The CFU of *S. aereus* and *S. mutans* was reduced by almost 2-log when cells were concurrently treated with 0.01 mM erythrosine and 50 J/cm^2 light dose. Likewise, the CFU of *C. albicans* (10^7 CFU / mL) was reduced by almost 4-log when concurrently treated with 3 mM erythrosine and a light dose of 50 J/cm^2. Figure (A), (B), and (E) also show an increase in *S. aereus*, *S. mutans*, and *C. albicans* eradication with an increase in erythrosine concentration, respectively. No change in the survival rate of *P. aeruginosa* and *E.coli*, however, was observed after PDI treatment. *$p < 0.05$.

concentrations of 0.1 mM for Gram (+), 20 mM for Gram (-) and 5 mM for the yeast in the dark did not result in any toxic effect. Similarly, the exposure of these planktonic cells to the LED light with the highest studied dose alone did not cause any change in cell survival compared to the survival rates of untreated controls. From these results, a fundamental difference in the susceptibility to PDI between Gram (+) and Gram (-) bacteria was observed. The high susceptibility of Gram (+) bacteria could be explained by their physiology, as their cytoplasmic membrane is surrounded by a relatively porous layer of peptidoglycan and lipoteichoic acid that allows PS to cross[9]. The cell envelope of Gram (-) bacteria however, consists of an inner cytoplasmic membrane and an outer membrane that are separated by a peptidoglycan-containing periplasm. The outer membrane forms a physical and functional barrier between the cell and its environment. Furthermore, different proteins are present in the outer membrane; some function as pores to allow passage of nutrients, whereas others have an enzymatic function or are involved in maintaining the structural integrity of the outer membrane and the shape of the bacteria[10]. Gram (-) bacteria are known to be resistant to many of the commonly used PS in PDI that otherwise readily lead to phototoxicity in Gram (+) bacteria[2,10,11]. Nonetheless, it is expected that PS bearing a cationic charge or the use of agents that increase the permeability of the outer membrane will enhance the efficacy of PDI against Gram (-) microorganisms.

II. *The Effect of Acetic Acid on the Erythrosine-Mediated PDI*

When cells were treated with 0.1% acetic acid, no change in the survival of Gram (-) bacteria was observed when compared to the survival rates of untreated controls (Figure 2). Similarly, no change in toxicity and survival of Gram (-) bacteria was observed when cells were concurrently treated with acetic acid (0.05 and 0.1%) and up to 10 mM of erythrosine in the dark for 10 min (Figure 3A & 3B). The pH's of 0.05 and 0.1% acetic acid are approximately 6.2 and 4.8, respectively. These results showed that erythrosine, even in 0.1% acetic acid, did not exert any toxic effect in the dark. However, when Gram (-) bacteria treated with 1, 5 and 10 mM erythrosine in 0.1% acetic acid were illuminated with a light dose of 50 J/cm^2, a significant enhancement in PDI effect was observed (Figure 4A & 4B). The survival rate of *P. aeruginosa* cells was reduced by ~3 log; and the survival rate of *E.coli* cells was reduced by ~2 log when compared to the survival of cells treated with erythrosine and light alone. At pH>4, erythrosine exists in the dianionic form (EB^{2-}) whereas at pH<3.5 it exists in the less negatively charged monoanionic form (HEB^{-})[12]. Since the cell membrane is negatively charged, it is assumed that monoanionic form of erythrosine would be more effective than the dianionic form

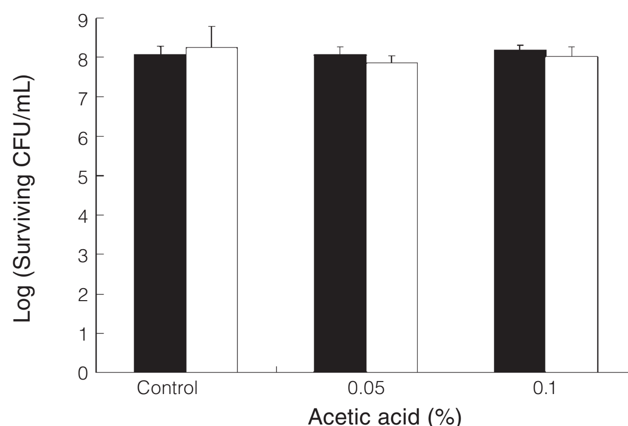

Figure 2. The survival of *E.coli*(■) and *P. aeruginosa*(□) (10^8 CFU/mL) treated with different concentrations of acetic acid for 10 min. This graph also shows that the survival of both microorganisms was not influenced by acetic acid. *$p < 0.05$.

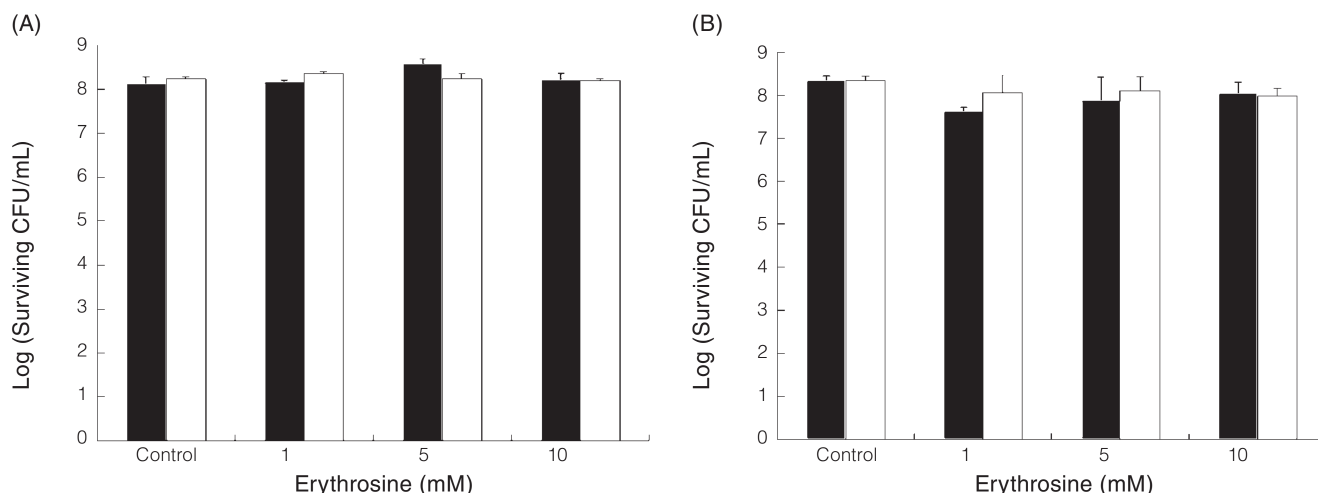

Figure 3. The survival of *P. aeruginosa* (A) and *E.coli* (B) (10^8 CFU/mL) treated with different concentrations of erythrosine in the dark in the presence or absence of acetic acid. No change in the survival rate of both microorganisms was observed. (Conentration of acetic acid: 0.05(■), and 0.1(□)%). *$p < 0.05$.

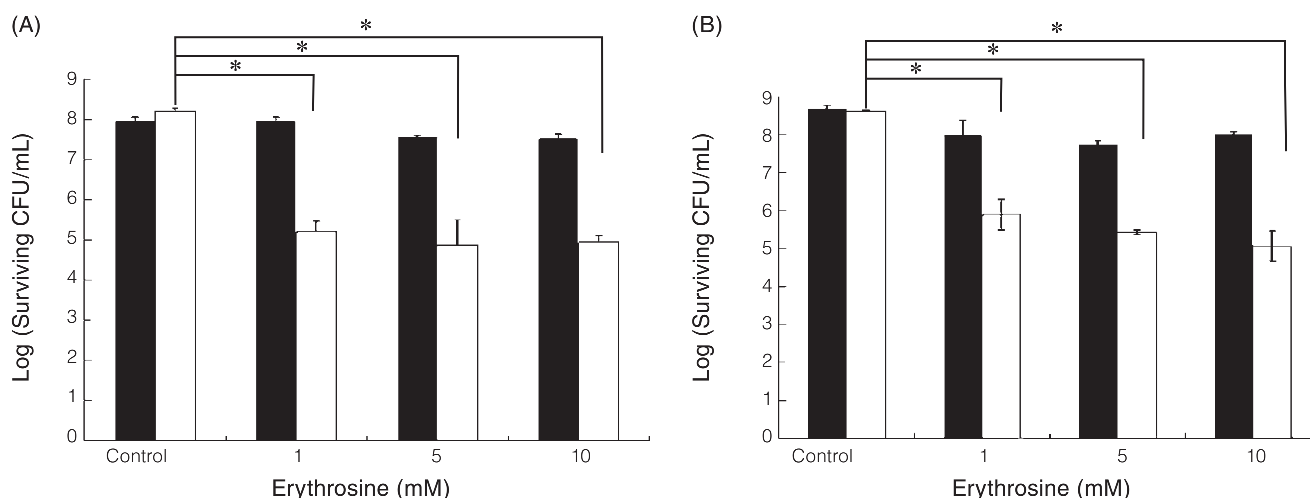

Figure 4. The survival of *P. aeruginosa* (A) and *E.coli* (B) (10^8 CFU/mL) concurrently treated with different concentrations of erythrosine and a light dose 50 J/cm^2 in the presence or absence of acetic acid. At 0.1% acetic acid, the CFU of *P. aeruginosa* was reduced by almost 3-log and those of *E.coli* by 2-log when cells were treated with 1, 5, and 10 mM of erythrosine and a light dose 50 J/cm^2. (Conentration of acetic acid: 0.05(■), and 0.1(□)%). *$p < 0.05$.

Table 1. The survival of *S. aereus* and *S. mutans* in biofilms after PDT treatment (Log-unit)*

	Control	Light only	PS only	PS (0.05 mM)+Light
S. aureus	7.55 ± 0.12	7.32 ± 0.40	7.64 ± 0.04	0
S. mutans	7.54 ± 0.46	7.45 ± 0.58	7.45 ± 0.48	0

* This table shows the survival of *S. aureus* and *S. mutans* (10^7 CFU/mL) in biofilms after treatment with light only, erythrosine only, or erythrosine in the presence of light. The microorganisms in both biofilms were completely eradicated after treatment with 0.05 mM erythrosine and a light dose of 50 J/cm^2 for 37 min.

in cell contact and the resulting PDI. Therefore, to enhance the effect of erythrosine-mediated PDI against Gram (-) microorganisms, incubation process was carried out under acidic environment using acetic acid at a concentration range from 0 to 0.1%. It was also suggested that acetic acid possess antimicrobial effect[13-15], and the use of acetic acid in the treatment of wound infection can be traced back to 1916 when Taylor found that application of a 1% solution to war wounds led to the elimination of *Bacillus pyocyaneus* after 2 weeks[14]. The exact mechanism by which acetic acid exerts its effect is unknown, but a physical alteration of the bacterial cell wall is presumed[16]. In this study, since a much lower concentration of acetic acid was used and the incubation time was only 10 min, the enhanced erythrosine-mediated PDI on Gram (-) bacteria in the presence of acetic acid would be mainly due to the pH effect that favors the formation of erythrosine monoanionic form.

III. *Erythrosine-Mediated PDI in Biofilm Cells*

The biofilms of Gram (+) bacterial cells (*S. aureus* & *S.mutans*) were also sensitive to PDI treatment. As shown in Table 1, an increase in the concentration of erythrosine to 0.05 mM and the light dose to 50 J/cm^2 resulted in complete eradication of the bacteria. *C. albicans* biofilm, however, showed high tolerance to PDI treatment and was not significantly affected even when treated with 20 mM erythrosine in combination with 100 J/cm^2 light dose (Figure 5). The resistance to PDI in biofilms may be attributed to the slow or

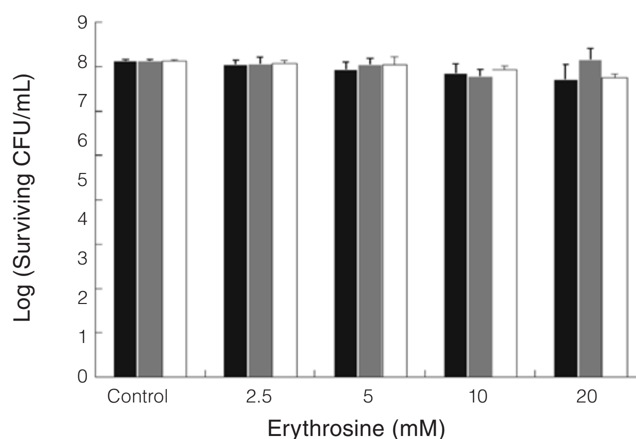

Figure 5. The survival of *C. albicans* biofilms (10^7 CFU/mL) treated with different concentrations of erythrosine in the absence or presence of different doses of light. This graph also shows that *C. albicans* biofilms have high tolerance to PDI (Light dose: 0 (■), 50(■) and 100(□) J/cm^2).

incomplete penetration of the PSs and light into the biofilms. It has been reported that cells growing in a biofilm differ from their planktonic counterparts in their wall composition, rate of growth, and presence of polysaccharide intercellular adhesion (PIA) matrix, which hinder the uptake of photosensitizers and penetration of light, thereby reducing the photosensitization effect[(17)]. Incomplete PSs penetration into biofilms has also been shown by confocal laser scanning microscopy in which S. mutans biofilm was monitored after exposure to HeNe laser or LED light in the presence of toluidine blue O[(18)]. Results demonstrated that photosensitization occurred predominantly in the outer layers of the biofilms, leaving some of the inner-most bacteria alive. This was attributed to the inability of the photosensitizer to diffuse into inner regions, probably due to the presence of PIA/extracellular polymeric substance in the biofilms. It can also be attributed to the stratification of light exposure within biofilms[(19)]. Therefore, to achieve the same PDI effect, a higher light dosage is required for biofilms than for planktonic cells. It is also possible that cells in the biofilm, when starved for a long term, may develop an adaptation mechanism that renders them resistant to PDI.

CONCLUSIONS

This study demonstrated that erythrosine is a potential photosensitizer for PDI against Gram (+) bacteria. An experimental approach involving a combination of 0.1% acetic acid and PDT in treating Gram (-) bacteria may be worth exploring. Data generated in this study could aid in the development of products for use to improve oral hygiene or in the prevention and/or eradication of oral infections.

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