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Anticandidal effect of multiple sessions of erythrosine and potassium iodide-mediated photodynamic therapy

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ABSTRACT

Background: Erythrosine+potassium iodide-mediated photodynamic therapy has shown an anticandidal effect. Single session, however, has inadequate fungal inhibition.

Objectives: We aimed to examine the effects of multiple aPDT sessions on *Candida albicans* inhibition and singlet oxygen formation.

Methods: 220 µM erythrosine +/-100 mM potassium iodide was applied to *C. albicans* biofilms for 1 min prior to irradiation at 530±10 nm using a 250 mW/cm^2 light-emitting diode. Negative and positive controls were phosphate buffer saline and nystatin, respectively. Single, double and triple irradiation sessions with a 5 min resting time between sessions were performed. Post-treatment candidal counts were done at 0, 1 6 and 24 hr while $\log_{10}$ colony forming unit/ml was calculated and compared using a Kruskal-Wallis with Dunn's post hoc test at a $p<0.05$ - Singlet oxygen amount was compared using one-way ANOVA with a post hoc test at a $p< 0.05$.

Results: Two and three irradiation sessions to erythrosine+potassium iodide could inhibit *Candida albicans* at 7.92 $\log_{10}$CFU/ml ($p < 0.001$) . Singlet oxygen from a combination groups was significantly higher than for erythrosine (positive control). Moreover, the correlation coefficient (r) between singlet oxygen production and decreased *Candida albicans* counts was equal to 1.

Conclusion: Multiple sessions PDT of 220 µM erythrosine+100 mM potassium iodide effectively inhibited a *Candida* biofilm.

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Anticandidiasis; erythrosine; multiple sessions; potassium iodide; photodynamic therapy; singlet oxygen

Introduction

Candida albicans (C. albicans), the most common commensal fungal species in the oral cavity, is a dimorphic opportunistic pathogen in vulnerable or immunocompromised individuals [1]. This species has the capability to foster optimal conditions for the survival and colonization of other bacteria. Such co-infections usually lead to increased disease severity as well as drug resistance [2]. Basically, *C. albicans* employs four survival strategies including 1) changing their cell wall structure to prevent identification and being killed by host cells, 2) activating their stress response pathways to prevent inactivation by immune cells, 3) alteration of cell size and shape to evade host phagocytic cells, and 4) induction of host cell death [3]. Altogether, these lead to the chronic or recurrent nature of this type of infection. An increased number and virulence of *C. albicans* was reported to correlate with many diseases, for instance periodontitis [4], pneumonia [5], and cancer [6]. Thus, efforts aimed at rapidly addressing candidal infections are essential measures for maintaining the quality of life, as well as preserving the lives of patients.

It is generally known that antifungal resistance is a long-term unresolved problem for clinicians. Fortunately, first-line antifungal drugs for oral candidiasis, *e.g.*, amphotericin and nystatin, are reported to have relatively lower drug resistance [7]. Nevertheless, an increasing number of immunocompromised individuals with systemic fungal infections necessitates prescription of standard systemic antifungal agents with greater resistance, especially the azole group. Azole group resistance in invasive candidiasis is a massive clinical threat [8]. Endeavors aimed at developing a more efficacious targeted strategy for mitigating *Candida* infections with minimal adverse effects/reactions hold a significant clinical value.

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Antimicrobial photodynamic therapy (aPDT) has gained interest in recent years due to its specificity, minimal adverse reactions, and relatively promising results [9,10]. The mechanism of aPDT relies on generation of reactive oxygen species (ROS) which in turn can result in irreversible photodamage to fungi [11]. Several photosensitizers can be used in aPDT, *e.g.*, porphyrin and stains including methylene blue and erythrosine. Recently, mesotetra (3-N-methylpyridyl)porphyrin, at concentrations of 4.68–75 μM, demonstrated the capability of inhibiting *Candida tropicalis, Candida glabrata and Candida parapsilosis* by nearly 100% when exposed to white LED light (370–800 nm) for 900 seconds with a power density of 50 mW/cm^2, energy density of 10.6 J/cm^2 [12]. 400 μM erythrosine irradiated at 532 nm using a light-emitting diode having a power of 90 mW/cm^2 or 16.2 J/cm^2 for 180 s could inhibit 0.74 $\log_{10}$ CFU of candidal biofilm [13]. Methylene blue-loaded polymeric micelles at a concentration of 780 μM with a preincubation time of 30 min and irradiation with 660 nm red light (19 mW/cm^2, energy density of 15 J/cm^2) for 796 s could reduce a *C. albicans* biofilm by 4 $\log_{10}$ CFU/ml compared to conventional methylene blue under the same light parameters. Current aPDT methods involve relatively long irradiation times and are hence impractical for clinical use.

Apart from the aforementioned studies, the concept of combining photosensitizers with an inorganic salt emerges as a more powerful tool for antifungal treatment [14]. Among the inorganic salts, potassium iodide (KI) is a candidate for use with photosensitizers due to its safety, affordability, rapid reaction, and, most importantly, its ability to generate iodine and iodide derivatives that are highly toxic to microorganisms when stimulated by singlet oxygen [14], for instance, from erythrosine. Exposure to 510 nM of Rose Bengal or eosin combined with 100 mM KI, to a 530 ± 40 nm LED light at 6 J/cm^2 resulted in a greater than 1000-fold reduction (equivalent to 3 $\log_{10}$) in *Staphylococcus aureus* and *Salmonella typhimurium* compared to staining alone [15]. To the best of our knowledge, profound anticandidal effects mediated by erythrosine+KI-mediated PDT have never been reported.

Generally, a common, single application of PDT showed a relatively low capability to diminish microorganisms including *C. albicans* [16]. Recently, a comparison of single and dual applications of PDT using 750 ng/ml of methylene blue zinc chloride double salt irradiated with either protocol 1: a single dose of 20 J/cm^2 (41.2 mW/cm^2) at 630 nm from an LED, or protocol 2: twice application of protocol 1 done 5 min apart, and protocol 3: twice application of protocol 1, 24 hr apart [17]. An interesting finding is that double application of PDT in a single visit with a 5 min interval (protocol 2) yielded the most *C. albicans* biofilm inhibition, 4 $\log_{10}$ CFU/ml. We hypothesize that applying multiple sessions of PDT using erythrosine+KI, irradiated with a green LED, and including relatively short light-off intersession times, might be beneficial for inhibiting candidal biofilms.

The present study aimed to investigate the effects of multiple session photodynamic therapy using erythrosine and potassium iodide irradiated using a green LED to develop anticandidiasis. We also aimed to study whether these effects are mediated by singlet oxygen and if there is a correlation between the number of sessions and anticandidiasis.

Erythrosine, a member of the xanthene group, is the most common stain for dental biofilms due to its specificity for intra-oral microorganisms, including *Candida* spp. Interestingly, this stain can absorb light in the green region with a peak absorption wavelength of 532 nm [18]. Notably, erythrosine can generate large quantities of singlet oxygen when exposed to green light [19], making it a good choice for antimicrobial PDT in dentistry. As shown in a 2011 study [13], erythrosine as a single photosensitizer even in a relatively high concentration could subtly inhibit a *C. albicans* biofilm [20]. Efforts were directed toward combining photosensitizers to improve candidal inhibitory effects. Erythrosine at 440 μM+cyanidin at 404 μM with a low energy density setting of a 532 nm laser could significantly reduce a periodontopathic bacteria biofilm [19]. Combining 220 μM of erythrosine with 20 μM of curcumin derivatives in the presence of 10% (w/w) nano-titanium dioxide with irradiation using a blue LED dental curing light at 72 J/cm^2, resulted in a reduction of approximately 1.1 $\log_{10}$ CFU of a *C. albicans* biofilm [21]. However, these study results are still far from the generally accepted value for effective antimicrobial activity, which typically requires a reduction of at least 3 $\log_{10}$ CFU.

Materials and methods

Biofilm formation

Candida albicans (ATCC 10231) was used in the present study. It was cultured in Sabouraud dextrose broth, adjusted to a spectrophotometric (Varioskan, Thermo Scientific, USA) optical density of 0.38 at a wavelength of 520 nm. This is equivalent to 1×10^7 cells/ml. One milliliter of this *Candida* suspension was then transferred to an 18 × 18 mm pre-weight glass slide that was placed into a 6-well plate. The plate was shaken at 75 rpm for 90 min. Subsequently, 2 ml of the solution containing 1× of yeast nitrogen base supplemented with 50 mM of glucose was added to

each well. The plate was further shaken at 75 rpm for 48 hr to facilitate mature candidal biofilm formation. Finally, the mature biofilm was weighed.

Photosensitizer preparation group allocations

Erythrosine and KI in the present study were purchased from Sigma-Aldrich®, Darmstadt, Germany) and QRëC™, New Zealand, respectively. To prepare the stock solutions, both substances were dissolved in deionized water, and then underwent two-fold serial dilutions using phosphate buffer saline. There are six groups as follows:

(1) 220 μM erythrosine with green LED irradiation
(2) 100 mM potassium iodide with green LED irradiation
(3) 220 μM erythrosine and 100 mM potassium iodide with green LED irradiation
(4) Nystatin oral suspension 100,000 U/ml (positive control)
(5) Phosphate buffer saline (PBS) solution (negative control)
(6) Phosphate buffer saline solution with green LED irradiation (light control)

Light parameters

LED irradiation generated using a green LED with wavelengths between 520 and 530 nm was used in the present study. The distance from the light source to the well plate bottom is 36 mm to achieve a power density of 250 mW/cm^2. The diameter of the LED is approximately 36 mm thus each LED fit into a single well of a 6-well plate. Prior to irradiation each day, a light power calibration test was performed using a light power meter (PM160T HP Thorlabs, Newton, NJ, USA).

Photodynamic therapy procedure

Mature *Candida* biofilm on each glass slide was treated with 2 ml of each substance for 1 min, followed by irradiation for 80 s, resulting in a total exposure of 20 J/cm^2 per session. In the case of two or three sessions of irradiation, a 5-min resting (light-off) period was employed between sessions. All irradiation procedures were conducted under dim lighting conditions, as the experimental room was kept dark, except for when a corridor light was turned on. The distance from the light source in the corridor to the experimental area was approximately 8 m, room temperature was set at 25°C and humidity was 55–60%, with each group occupying three wells. All procedures were performed in triplicate.

Candida drop plate assay

After irradiation, the glass slide was thrice washed with phosphate buffer saline. Then, 1.2 ml of fresh PBS was poured into each well, and all wells were subjected to ultrasonication for 15 min. The solution was collected and centrifuged at 8,000 rpm for 5 min. Finally, the supernatant, approximately 950 μl was discarded, resulting in 150 μl of a solution that was transferred to a ten-fold serial dilution until 10^{-3} was achieved. Three drops (10 μl/drop) of each concentration (total of four concentrations) were dropped onto SDA plates and cultured in a humidified incubator at 37°C and 90% humidity for 48 hr. At 0, 1, 6, and 24 hr, the candidal count was measured and a logarithm transformation was performed to the units of $\log_{10}$ CFU/ml/biofilm weight.

Singlet oxygen formation

All test substances (1–3 in Section 2.2) were prepared in 10× concentrations. About 100 μl of each test substance was mixed with 10 μl of 1 mM 9,10-dimethyl anthracene (DMA) in dimethyl sulfoxide (DMSO). Then, the volume of the test substance was adjusted to 1000 μl. The final concentration of the probe was 10 μM, and the concentration of DMSO was <1% by volume. The negative control was a probe with photosensitizers not exposed to light, while the positive control was 10 μM erythrosine with light exposure. Three wells per group in a 96-well plate were used and each plate was subjected to different light energies as follows. The 1st group was exposed to no light, the 2nd had one light session, the 3rd had two light sessions, and the 4th had three light sessions. Immediately after irradiation, each plate well was read using a microplate reader at excitation and emission wavelengths of 375 and 430 nm, respectively. The fluorescence intensity was measured at 0, 1, 3 and 5 min and the average was calculated using the following formula.

Quantity of relative singlet oxygen = $100 - (100 * [FI\_DMA]_t/[FI\_DMA]_{t=0})$

Where $[FI\_DMA]_t$ = fluorescence intensity of DMA at time = t

$[FI\_DMA]_{t=0}$ = fluorescence intensity of DMA at time = 0 (baseline)

Statistical analyses

The reduction of candidal numbers in terms of $\log_{10}$ CFU/ml/biofilm weight was reported using a median with its interquartile range (IQR). Comparison of candidal reduction among different sessions was performed using a Kruskal–Wallis test with Dunn's *post hoc* analysis. Singlet oxygen production was expressed as its mean and standard deviation (SD). Repeated-measures ANOVA was performed to compare the

mean percentage of singlet oxygen production between the one, two, and three light sessions. Correlation between the candidal count reduction and number of irradiation sessions was assessed using Spearman's rank correlation coefficient. A level of $p < 0.05$ indicated statistical significance

Results

Anticandidal effects

Figure 1(a) demonstrates the immediate effects (0 hr) of photodynamic therapy on the inhibition of a candidal biofilm. It is evident that an increased number of PDT sessions can enhance the inhibitory effect on *Candida*. In the case of erythrosine, belonging to the single photosensitizer (PS) group, irradiation with a single light energy dose of 20 J/cm^2 resulted in a median candidal reduction equal to 0.678 (IQR = 0.21) $\log_{10}$ CFU/ml, which is significantly reduced compared to PBS or a negative control ($p = 0.02$). However, when the number of irradiation sessions was increased to two and three, the candidal inhibition increased to 1.32 (IQR = 0.65, $p = 0.003$), 1.62 (IQR = 0.91) $\log_{10}$ CFU/ml ($p = 0.004$), respectively. For the KI-only group, irradiating for one and two sessions could not inhibit *C. albicans* biofilm, while three sessions of green light (total energy of 60 J/cm^2) inhibited at a level of 0.96 (IQR = 0.86) $\log_{10}$ CFU/ml ($p = 0.0168$). Apart from a single photosensitizer, the use of erythrosine and KI after two and three sessions of green light (with a total light energy density of 40 and 60 J/cm^2, respectively) could reduce *C. albicans* colony counts by 7.92 $\log_{10}$ CFU/ml or the total number microorganisms in the biofilm could be inactivated by nystatin. This is significantly different from the negative control ($p < 0.001$, in all cases), while a single session of light irradiation in this combination group could reduce the Candida colonies by only 0.56 (IQR = 0.48) $\log_{10}$ CFU/ml.

At 1 hr post-irradiation, inhibition of the *Candida* biofilm from all groups showed the same trend, except for erythrosine, when irradiated with a single session of light (Figure 1(b)). Compared to 0 hr, erythrosine in a single session of light after 1 hr could not reduce the *Candida* biofilm. In contrast, two and three sessions of light showed an inhibition capability, reducing it by 1.03 (IQR = 0.59) and 1.42 (IQR = 0.14) $\log_{10}$ CFU/ml, respectively. This is significantly different from the negative control (1 hr) with $p = 0.0067$ and 0.0049, respectively. For the KI-only group, only three sessions of light energy could significantly reduce candidal colonies by 1.05 (IQR = 0.59) $\log_{10}$ CFU/ml ($p = 0.0168$). Although all the above mentioned groups could significantly inhibit *Candida* biofilm, they all remained below the 3 $\log_{10}$ level. Interestingly, the combination group exhibited a 100% reduction in *Candida* counts with two and

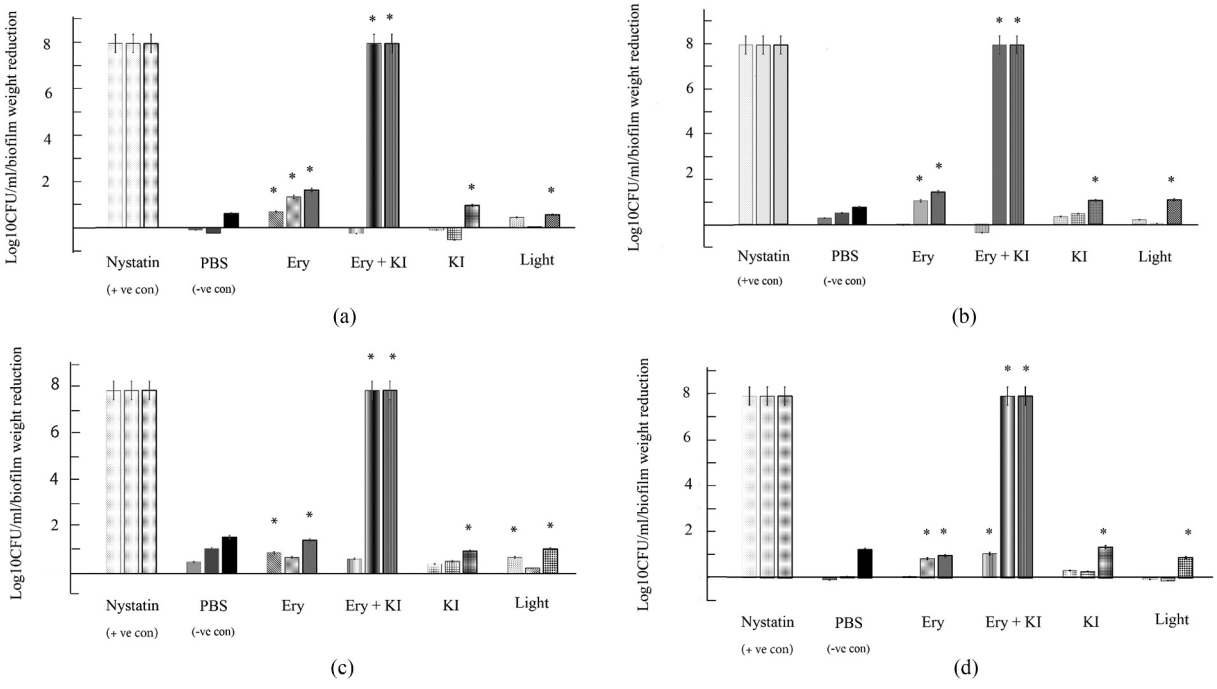

Figure 1. Candidal reduction (a) at 0 hr, (b) at 1 hr, (c) at 6 hr, and (d) at 24 hr, among groups after PDT using green LED (520–530 nm) with 250 mW/cm^2 for one (left bar), two (middle bar) or three (right bar) sessions (energy density 20 J/cm^2 per session) with a negative control (phosphate buffer saline) and a positive control (nystatin 1:100,000 unit/ml) (n = 6) *= statistically significant difference when compared with a negative control in the same irradiation session(s) at $p < 0.05$.

Abbreviations: PBS = phosphate buffer saline, Ery = erythrosine 220 μM, KI = potassium iodide 100 mM, Light = green light without photosensitizers

three sessions, and this reduction was statistically significant ($p < 0.001$) under both conditions. Notably, the three sessions of light-only also reduced the *Candida* population to the same level as those with erythrosine.

When comparing the effect of PDT after 6 hr using the results presented in Figure 1(c), erythrosine+one session of green light could maintain candidal biofilm inhibition by 0.9 (IQR = 0.76) $\log_{10}$ CFU/ml, which was significantly greater than the negative control at $p = 0.0157$. Interestingly, a single session of a light-only group could inhibit candidal biofilm by 0.7 (IQR = 0.63) $\log_{10}$ CFU/ml, which was also significantly different from the negative control ($p = 0.0192$). The reduction of *Candida* counts in both groups, however, did not reach the clinically acceptable level of 3 $\log_{10}$ CFU. On the contrary, the combination group still maintained a superior inhibitory effect, up to 100% inhibition or 7.92 $\log_{10}$ CFU/ml, by irradiating for two or three sessions ($p < 0.001$).

After 24 hr, as shown in Figure 1(d), erythrosine with two sessions of light energy could inhibit by 0.83 (IQR = 0.23) $\log_{10}$ CFU/ml, which was significantly different from the negative control at $p = 0.008$. Surprisingly, the combination group, with only one session of light energy, could inhibit candidal biofilm by 1.06, 0.4 $\log_{10}$ CFU/ml ($p = 0.003$). For two and three sessions, 100% inhibition was achieved at this time ($p < 0.001$).

Singlet oxygen quantification using a fluorescent probe method

Quantification of generated singlet oxygen was accomplished using a dimethyl anthracene probe. This was achieved by promptly measuring the fluorescence intensity after the final irradiation procedure was completed. The percentage of relative fluorescence intensity was calculated as demonstrated in Figure 2. Collectively, it was observed that an increased number of irradiation sessions led to a proportional increase in singlet oxygen production.

The erythrosine-only group could produce a high amount of singlet oxygen with one, two, and three sessions, with average singlet oxygen levels of 79.67 ± 6.89%, 82.4 ± 6.44%, and 85.66 ± 5.7%, respectively. These were significantly higher than the negative control at $p = 0.003$, 0.003 and 0.001, for one, two, and three sessions, respectively. Conversely, KI induced a relatively low amount of singlet oxygen. After one, two, and three sessions of light exposure, KI was stimulated to produce singlet oxygen at 10.14 ± 30.45%, 19.57 ± 29.31%, and 28.28 ± 28.49%, respectively. As anticipated, a combination of erythrosine and KI exhibited the highest singlet oxygen production among all the groups subjected to the same levels of light energy. With one, two, and three light sessions, singlet oxygen generation reached levels of 90.65 ± 3.17%, 92.74 ± 2.64%, and 94.19 ± 2.31%, respectively. The amount of generated singlet oxygen of these groups was significantly greater than that of the positive control (erythrosine 10 μM) at $p = 0.003$, 0.004, and 0.01 for one, two, and three sessions of irradiation, respectively.

Correlation between irradiation sessions and candida biofilm reduction with time

Figure 3(a-d) demonstrated the relationship between the number of irradiation sessions and the reduction of *Candida* counts in the units of $\log_{10}$ CFU/ml using a Spearman rank's correlation test.

Immediately after the PDT reaction (Figure 3(a)), a direct correlation between an increased number of irradiation sessions and a reduction in the candidal

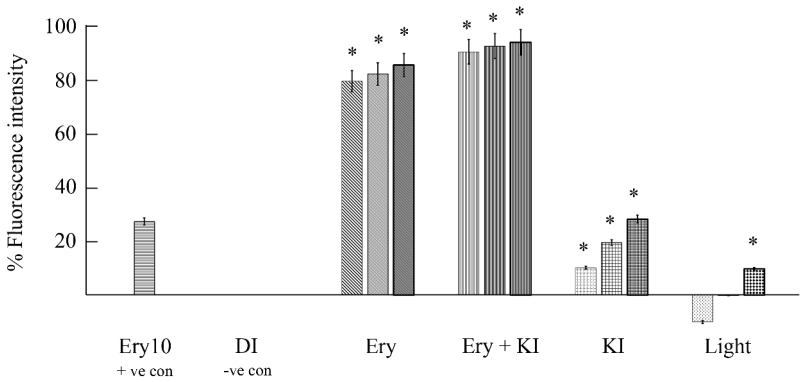

Figure 2.Percentage of relative fluorescence intensity (relative singlet oxygen amount) measured by DMA (final concentration 1 μM) fluorescence probe among groups after PDT using green LED (520–530 nm) with 250 mW/cm^2 for one (left bar), two (middle bar) or three (right bar) sessions (energy density 20 J/cm^2 per session) with a positive control (10 μM erythrosine) and a negative control (deionized water) (n = 6) *= statistically significant difference when compared with the negative control at p < 0.05.

Abbreviations: DI = deionized water, Ery = erythrosine 220 μM, KI = potassium iodide 100 mM, Light = green light without photosensitizers

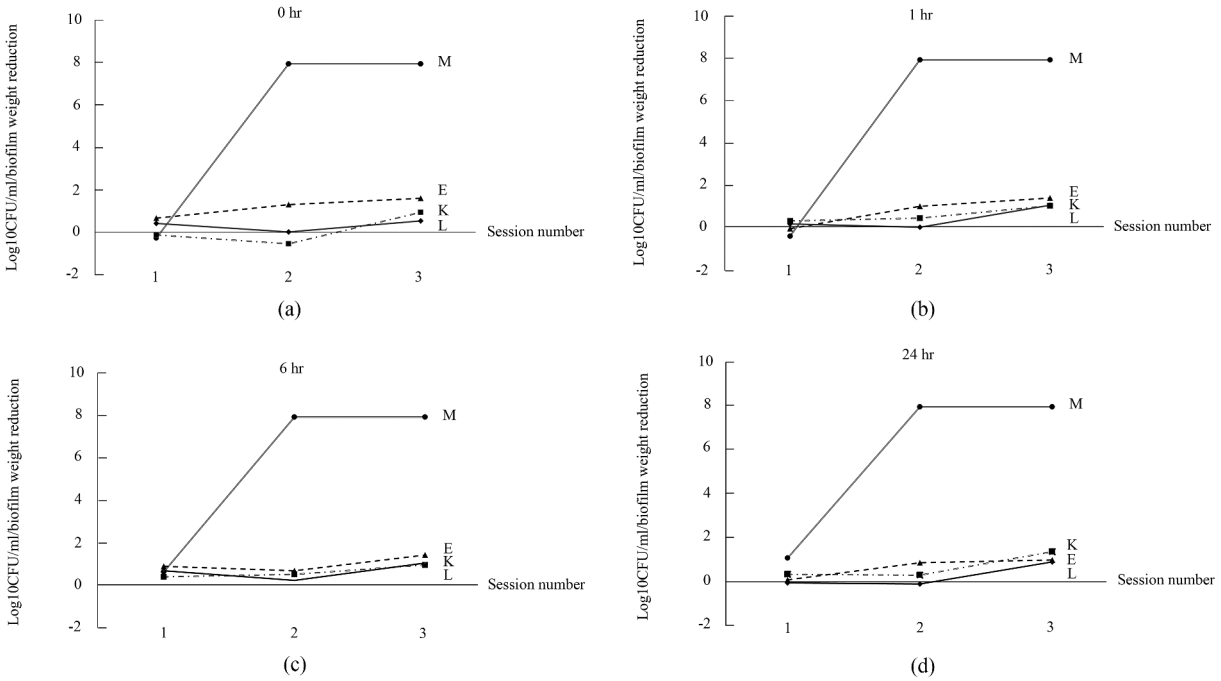

Figure 3. Correlations between candidal reduction and irradiation sessions at (a) 0 hr, (b) 1 hr, (c) 6 hr, and (d) at 24 hr, among groups after PDT using green LED (520–530 nm) with 250 mW/cm^2 after one session (left), two sessions (middle), and three sessions (right) M = combination of erythrosine and KI, E = erythrosine, K = KI, L = light.

count was observed only in the group using a combination of substances, with a strong correlation coefficient of 0.84. The same pattern of correlation was also observed at other times, 1 hr (Figure 3(b)), 6 hr (Figure 3(c)), and 24 hr (Figure 3(d)).

Correlation between singlet oxygen from different irradiation sessions and candidal biofilm reduction

Correlation of singlet oxygen generation from each substance when irradiated with different numbers of

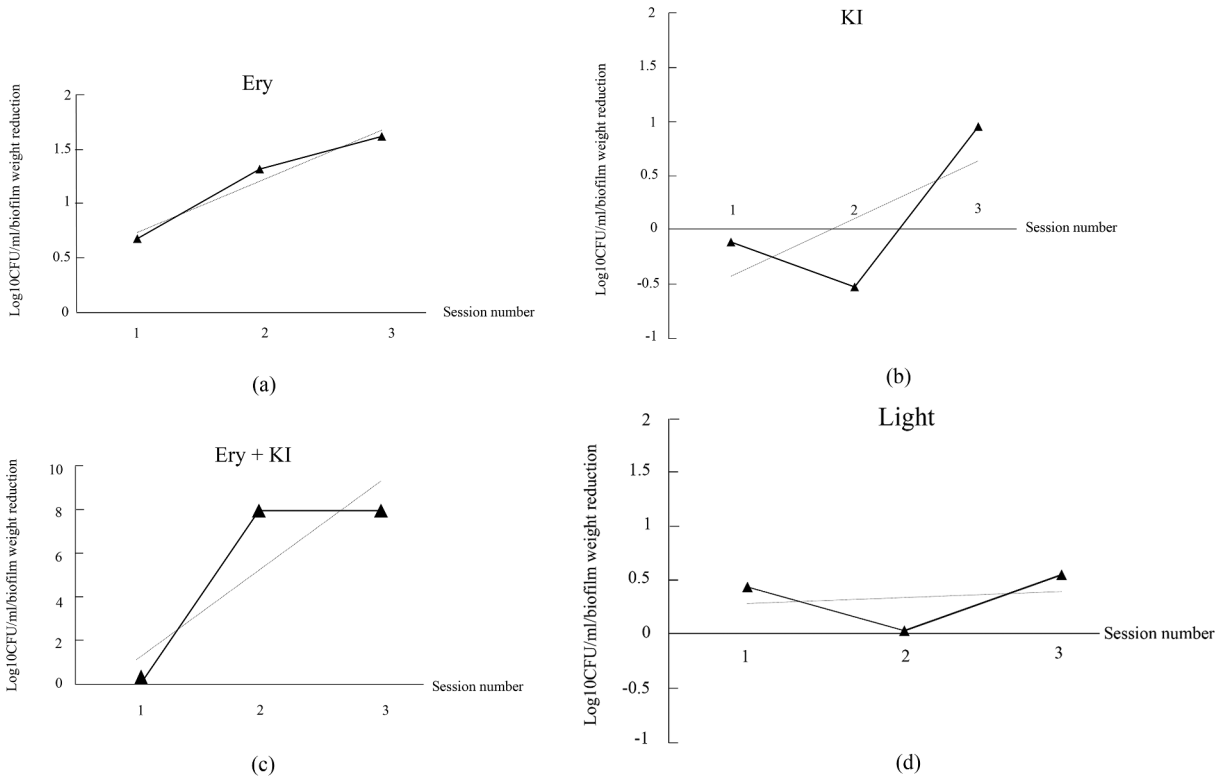

Figure 4. Correlations between singlet oxygen formation (from different irradiation sessions) and Candida reduction using (a) erythrosine, (b) combination of erythrosine and potassium iodide, (c) potassium iodide, and (d) light only after PDT using green LED (520–530 nm) with 250 mW/cm^2 after one, two and three sessions.

light sessions and the reduction in candidal biofilm is shown in Figure 4(a-d).

Figure 4(a) shows that an increased number of irradiation sessions with 220 μM erythrosine resulted in a greater inhibition of candidal biofilm, with a correlation coefficient, r, of 0.96. However, 100 mM KI, when irradiated with light for one, two, and three sessions, could generate singlet oxygen with a medium to low correlation coefficient of $r = 0.49$ (Figure 4(b)). As anticipated, the combination of 200 μM erythrosine and 100 mM KI produced singlet oxygen in a session-dependent manner, with an increased number of sessions strongly correlated with candidal biofilm inhibition (correlation coefficient $r = 1$, Figure 4(c)). Surprisingly, at two and three sessions of this combination group, complete candidal inhibition was observed. The light-only group exhibited almost no singlet oxygen production (Figure 4(d)).

Discussion

The present study demonstrated that an increased number of light irradiation sessions led to a dramatic and effective inhibition of candidal biofilm *in vitro*. The rationale for using erythrosine is based on its widespread availability in dental clinics and its generally recognized safety, even at very high concentrations in tablet or solution form, for clinically staining intra-oral microorganisms in biofilms. There have been reports suggesting that erythrosine could serve as a novel photosensitizer due to its capability to generate great amounts of singlet oxygen molecules that are aggressive in attacking microorganisms through apoptosis or necrosis upon exposure to specific light conditions [19,22]. Additionally, erythrosine exhibits peak light absorption at 530–550 nm [18,23]. Within this wavelength range, light can generally penetrate to the location of *C. albicans*.

In a previous report by Costa *et al.* in 2011, it was demonstrated that while erythrosine, as a sole photosensitizer, effectively inhibited the planktonic form of *C. albicans*, it was not capable of inhibiting the biofilm form as only 0.74 $\log_{10}$ CFU/ml of biofilm weight was inhibited after 532 nm LED irradiation at 16.2 J/cm^2 with 400 μM erythrosine [13]. Additionally, many attempts have been made to use erythrosine combined with other substances, *e.g.*, erythrosine with curcumin derivatives and nano-titanium dioxide [21,24], but this combination still yielded low biofilm inhibition. It was demonstrated that 220 μM erythrosine with 20 μM bisdemethoxycurcumin and 10% w/w nano-titanium dioxide under 72 J/cm^2 of a blue dental LED could reduce candidal biofilm by only 1.1 $\log_{10}$ CFU/ml [21]. The rationale for choosing a 220 μM dose of erythrosine was based on preliminary measurements indicating that the amount of singlet oxygen produced from erythrosine was significantly higher at greater concentrations (data not shown).

Additionally, erythrosine was selected due to its widespread use as an oral microbial stain in dental practice. Moreover, our study highlights erythrosine's superior efficacy against candidiasis compared to methylene blue, although a direct comparative analysis was not feasible. Our findings demonstrate that a single application of 220 μM of erythrosine, supplemented with 100 mM KI in two sessions of light irradiation, effectively suppressed candidal biofilm formation to a degree comparable to nystatin. In contrast, a recent study by de Souto Medeiros *et al.* (2023) reported that a double application of 3100 μM (0.1% w/v) of methylene blue was successfully used to treat patients with erythematous candidiasis [25]. In essence, achieving equivalent therapeutic efficacy necessitates employing methylene blue at a 15-times higher dose than that of erythrosine. Pre-irradiation is an important factor in effective application of erythrosine. Unlike methylene blue, which requires a 15-min pre-irradiation, erythrosine achieves comparable results with just 1 min of pre-irradiation. This practical advantage makes erythrosine a more feasible option than methylene blue in the context of antimicrobial photodynamic therapy.

The factor in PDT that influences microbial inhibition is not limited to the use of photosensitizers, but also involves an adequate light energy. Most previous studies relied on a single, continuous irradiation session. Our group, however, recognized that using a single session might not produce adequate energy or could lead to hyperthermia which, in turn, could negatively affect adjacent normal human tissue. Therefore, we designed our approach to use a low light energy density (fluency) and with a 5 min light-off time for a re-oxygenation process. This is because it has been demonstrated that using fractional mode irradiation can generate higher ROS levels, facilitating candidal biofilm inhibition [26].

Compared to a previous study, after one light session (20 J/cm^2) in the present protocol, which is close to 16.2 J/cm^2 irradiation with 400 μM erythrosine, we were able to inhibit approximately 0.68 $\log_{10}$ CFU/ml, as opposed to 0.74 $\log_{10}$ CFU/ml [13]. Notably, when combined erythrosine with KI and subjected to two and three repeated irradiation sessions, the inhibitory capability of our protocol increased to 7.9 $\log_{10}$ CFU/ml, which is equivalent to the standard antifungal nystatin and significantly superior to the previous study. Furthermore, the effect of our protocol, with the half concentration of erythrosine used, was sustained for at least 24 hr.

The effect of multiple irradiation sessions on *Candida* reduction was clearly demonstrated in the present study. We have shown that even a single

irradiation session with erythrosine and KI could not inhibit *Candida* at a clinically acceptable level. This result is consistent with a previous study [16], who reported that repeated irradiation using a 532 nm laser with a total energy density of 42.63 J/cm^2 applied to erythrosine every 24 hr for four sessions could reduce *C. glabrata* by 5.94 $\log_{10}$ CFU, while a single session resulted in only a 1.3 $\log_{10}$ CFU reduction. Repeating irradiation every 24 hr is effective, but not practical. An intersession time of 5 min between sessions is more practical, both from a clinical procedure perspective and in terms of oxygen replenishment. Further study regarding optimization of the irradiation intersession time is crucial. Notably, two and three irradiation sessions with erythrosine and KI always resulted in the same anticandidal effects. From a clinical perspective, a protocol of two irradiation sessions is clinically acceptable.

The reason that multiple sessions were more effective than a single session can be partially explained by the higher accumulated energy, which can induce more reactive oxygen species. However, at early times (0–1 hr) with a single irradiation session, we observed that *Candida* counts could increase. This may have resulted from an inadequate amount of ROS, where residual and low amounts of ROS could potentially stimulate candidal activity. Additionally, erythrosine, even with three light irradiation sessions, could not effectively reduce *Candida* biofilm, even though this produced a high amount of singlet oxygen. Therefore, other molecules may synergistically interact to form a final product that inhibits *Candida*.

In addition to the singlet oxygen's previously elucidated role, as explained by Hamblin, MR (2017) [14], singlet oxygen exhibits the capability to catalyze the formation of peroxy iodide and iodide derivatives from iodine molecules. These reactive species are further implicated in the inhibition or eradication of *Candida*. Increased numbers of exposure sessions not only amplify production of singlet oxygen but also enhance the yield of these compounds, culminating in a heightened anticandidal efficacy. Quantitative assessment of iodide derivative levels shows the potential to refine our understanding of whether singlet oxygen or iodide derivatives assume a more dominant role in the anticandidal effect. Our results in the KI combined group are consistent with a previous report demonstrating that 0.3 μM of methylene blue with 100 mM KI or 0.05 μM of Rose Bengal with 100 mM KI irradiated with green light at 9 J/cm^2 could inhibit 4 $\log_{10}$ CFU/ml more *E. fecalis* compared to groups with no KI [27]. Based on our results, the KI-only group could also induce singlet oxygen formation, but in relatively low amounts (18–26%). KI itself could not reduce *Candida* counts, even with an increased energy density to 100 J/cm^2 (data not shown). The plausible reason is that KI typically absorbs UVA wavelength light [28], but not the green light used in the present study. However, light energy can stimulate dissociation of KI into iodine or iodide, and these molecules, in turn, inhibit *Candida*.

We used water to dissolve erythrosine. It was found that water reduced the lifetime of erythrosine to 89 ps [29]. This leads to rapid dissociation of erythrosine molecules, while simultaneously accelerating singlet oxygen production. Moreover, KI was found to reduce the lifetime of erythrosine. It has been observed that higher concentrations of KI resulted in shorter lifetimes of erythrosine, with a reduction to 51 ± 9 ps using 1.51 M KI [23]. In the present study, we used a relatively low concentration of KI, thus, the lifetime of erythrosine is only subtly affected.

In the current study, the total irradiation time ranged from 80 to 240 s. However, from a practical perspective, this time is still relatively long. Therefore, in future research, investigating the anticandidal effects at higher energy densities to reduce the total irradiation time may be useful. Additionally, considering the presence of multiple microbial species in the normal oral environment should be examined, which may strengthen their tolerance. Soares *et al.*, 2023 demonstrated the methylene-loaded polymeric micelle could effectively inhibit a multispecies (*S. mutans and C. albicans*) biofilm [30]. Additionally, another limitation of our study is that only a single dosage of erythrosine was used. In our ongoing study, however, lower concentrations, especially 100 and 50 μM, are being tested to minimize the erythrosine dose while maintaining a superior, nystatin-equivalent antifungal effect. Testing our PDT regimen in a multispecies model could contribute to clinically relevant and successful anticandidiasis.

Conclusion

Using two sessions of green LED light (520–530 nm) at 20 J/cm^2 per session irradiating 220 μM erythrosine and 100 mM KI together in photodynamic therapy could effectively inhibit *C. albicans*, reducing it by approximately 7.92 $\log_{10}$ CFU/ml. This effect is equivalent to nystatin and mainly occurs *via* singlet oxygen formation.

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Author Contributions

TD contributed to resources, conceptualization, review and editing. PP, NM, and PS performed data curation and wrote the draft paper SP and PrS performed formal data analysis All authors have been involved in revising the manuscript critically and have approved the final version for publication.

Data availability statement

The data that support the findings of this study are openly available.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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