



Effects of Photodynamic Inactivation on the Growth and Antifungal Susceptibility of *Rhizopus oryzae*

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Abstract Mucormycosis is an aggressive and high-mortality opportunistic fungal infection, especially in immunocompromised patients. Conventional antifungals or surgery showed a limited effect on this disease. The antimicrobial photodynamic therapy (aPDT) has been proven to be a promising therapeutic choice against multiple pathogenic fungi. We evaluated the effect of aPDT by using methylene blue (MB) combined with a light emitting diode (LED) on the viability of *Rhizopus oryzae*, as well as the antifungal susceptibility after aPDT treatment in vitro. A total of six strains were included in this study; MB (8, 16, and 32 µg/ml) was chosen for the photosensitizer, and a

light source of LED (635 ± 10 nm, 12 J/cm²) device was used to activate it. aPDT with MB (32 µg/ml) and LED was highly effective in cell growth inhibition and exhibited colony-forming unit reductions of up to $4.3 \log_{10}$. The minimal inhibitory concentration ranges of itraconazole, posaconazole, and amphotericin B decreased from > 32 µg/ml to 4–8 µg/ml, 8–16 µg/ml to 0.5–2 µg/ml, and 2–4 µg/ml to 0.25–0.5 µg/ml, respectively, after pre-treatment with MB (8 µg/ml) and LED. In conclusion, aPDT with MB and LED was a promising therapeutic option against *R. oryzae* infections alone or combined with antifungal agents. However, further investigation is needed to determine the potential for clinic therapy and to elucidate the underlying mechanism.

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Introduction

Mucormycosis is an aggressive and frequently fatal opportunistic fungal infection that has emerged in patients with severe underlying immunosuppression, especially those with hematological malignancies or recipients of allogeneic hematopoietic stem cell transplantation [1]. *Rhizopus oryzae* is by far the most common cause of mucormycosis, which accounts for approximately 60% of all disease manifestation and

90% of all rhinocerebral cases [2]. *Mucorales* are resistant to most triazoles, except for posaconazole [3, 4]. Treatment strategies are based on high doses of amphotericin B (occasionally used in combination with echinocandins) and posaconazole, associated with surgical resections when possible. However, even with aggressive antifungal treatment, the estimated attributable mortality rate for mucormycosis exceeds 50% [2]. In the absence of surgical debridement of the infected tissue, antifungal agents alone are rarely curative, resulting in 100% mortality rate for patients with disseminated disease [5]. Therefore, there is an urgent need for alternative new treatment strategies.

In recent years, antimicrobial photodynamic therapy (aPDT) alone or in combination with antifungals has arisen as a promising approach for mycoses [6, 7]. Previous studies have demonstrated the efficacy of aPDT against a variety of pathogenic fungi in vitro, including *Candida* spp., *Fusarium* spp., *Fonsecaea* spp., *Exophiala dermatitidis*, *Sporothrix schenckii*, *Trichophyton rubrum*, *Scedosporium*, and *Lomentospora* spp., ect [8–13]. In addition, aPDT has also been successfully applied in vivo to treat chromoblastomycosis and onychomycosis [14–16]. However, little is known about the effects of aPDT on the viability and antifungal susceptibility of *R. oryzae*. In the present study, the growth inhibition effects of aPDT mediated by methylene blue and light emitting diode (LED) on *R. oryzae* and the impacts of pretreatment of aPDT on antifungal susceptibilities were evaluated.

Materials and Methods

Samples

A total of six strains of *R. oryzae* were studied, including the sequencing strain RA99880 (purchased from FGSC), ATCC56536, ATCC44170, and three clinical strains isolated from rhinocerebral mucormycosis patients. All isolates were identified by standard morphological criteria and confirmed by molecular sequencing of the internal transcribed spacer (ITS) ribosomal DNA (rDNA) as required. The quality control strain for the in vitro antifungal susceptibility test is ATCC22019.

Inoculum Preparation and Photodynamic Treatment

Conidia were freshly harvested from cultures grown for 2 days on Sabouraud dextrose agar (SDA) by sterile distilled water and further diluted to a concentration of $1-3 \times 10^6$ CFU/ml. The methylene blue was applied as photosensitive agents and tested at concentrations of 8 µg/ml (T1), 16 µg/ml (T2), and 32 µg/ml (T3), with 100 µl of each concentration mixed with 100 µl of the prepared inocula pre-aliquoted into sterile 96-well microtiter plates. After incubation in dark at 37 °C for 2 h, the plates were then irradiated with LED (Type: Carnation-86C, Lifotronic Technology Co., Ltd., Shenzhen, China) at a wavelength of 635 ± 10 nm and at a distance of 1 cm for 120 s (irradiance: 100 mW/cm², 12 J/cm²). The control groups included conidial suspension in saline without LED irradiation (C1), conidial suspension with 32 µg/ml of methylene blue and without LED irradiation (C2), and conidial suspension in saline and irradiated (C3). After aPDT treatment, 10 µL aliquots of each well was diluted with sterile saline to a total volume of 1L, subsequently 100 µL of the mixture were inoculated on SDA and incubated at 37 °C in 24 h before counting colony-forming unit (CFU).

In vitro Antifungal Susceptibility Testing

The effects of aPDT on antifungal susceptibility were evaluated via determination of the minimal inhibitory concentrations (MICs) of itraconazole (ITC), posaconazole (POS), and amphotericin B (AMB) (all purchased from Sigma Chemical Co., St. Louis, MO, USA) against photodynamic treated (T1) and untreated planktonic cells (C1). The working concentrations of all tested agents were 0.06–32 µg/ml. According to the broth microdilution method M38-A2 of the Clinical and Laboratory Standards Institute [17], the 96-well plate was inoculated with 100 µL of conidial suspensions of T1 or C1 and 100 µL of the serially diluted drugs. After 24 h of incubation at 35 °C, the MICs were determined visually as the lowest drug concentrations resulting in complete growth inhibition [17]. All experiments were conducted in triplicate.

Results

Effects of Photodynamic Treatment on *R. oryzae*

As shown in Table 1, all aPDT treatment groups resulted in reduction in CFU counts. Notably, aPDT with 32 µg/ml methylene blue in T3 group inhibited all cell growth and exhibited CFU reductions of up to 4.3 log₁₀, revealing biologically relevant antimicrobial activity (Fig. 1). As for T1 and T2 group, the average CFU reductions were 1.1 log₁₀ and 2.2 log₁₀, respectively. For each particular treatment group, there were no significance differences in response to aPDT treatment among individual tested strains.

Photodynamic Effects on Antifungal Susceptibilities

The MIC results presented in Table 2 revealed that both ITC (> 32 µg/ml) and POS (8–16 µg/ml) were inactive against all strains, while AMB exhibited moderate activity with a MIC range of 2–4 µg/ml. However, with aPDT pre-treatment, the MIC ranges of ITC, POS, and AMB decreased to 4–8 µg/ml, 0.5–2 µg/ml, and 0.25–0.5 µg/ml, respectively, resulting a fourfold to eightfold reduction in MICs.

Statistical Analysis

All the data presented in the Tables are expressed as mean value of three independent experiments. Analysis of variance (ANOVA) and Bonferroni post hoc test were carried out by SPSS V.13 to assess values obtained between different groups. All tests were two-

tailed, and $P \leq 0.05$ was considered statistically significant.

Discussions

Rhizopus oryzae is the primary cause of mucormycosis, especially the most frequently reported rhinocerebral form [18]. The infection always starts in the nasal tissues and spreads into the paranasal sinuses and deep organs. The characteristic rapid angioinvasive growth nature leads to thrombosis and tissue ischemia. Timely control of the infections prevents dissemination of the infection. However, surgical debridement may result in disfiguration and available antifungals showed limited activity against *Mucorales*. In contrast, aPDT with methylene blue and LED, which allows maximal tissue transmission with low invasive character, is very convenient and inexpensive. The antimicrobial effects of PDT are based on the combination of a non-toxic photosensitizer (PS) and a specific wavelength of visible light, which can promote a phototoxic reaction and produce reactive oxygen species (ROS) at the presence of oxygen. ROS were highly cytotoxic to microorganisms through inducing oxidation of cellular structures, modifying the plasma membrane structures or the DNA, and inhibiting enzymatic systems. However, the effect of PDT is selective since ROS were only produced in microorganisms or diseased tissue accumulated with PS. And PDT is a noninvasive procedure which could be repeatedly applied without causing cumulative toxicity or damage of normal tissue [19, 20]. From the present study, the results have proved the efficacy of aPDT in both inhibiting the cell

Table 1 Photodynamic effect on the growth of *R. oryzae*

Strain	T1	T2	T3	C1	C2	C3
RA99880	1.5×10^3	1×10^2	0	1.9×10^4	2×10^4	1.8×10^4
ATCC56536	2.2×10^3	1.5×10^2	0	2.1×10^4	2.3×10^4	2.5×10^4
ATCC44170	1.2×10^3	2×10^2	0	1.8×10^4	2.3×10^4	1.8×10^4
RO1	2.2×10^3	1.2×10^2	0	2.2×10^4	1.9×10^4	2×10^4
RO2	9×10^2	1×10^2	0	2.3×10^4	2.2×10^4	1.9×10^4
RO3	2.2×10^3	1.3×10^2	0	2.2×10^4	2.1×10^4	1.9×10^4

T1, photodynamic treatment with 8 µg/ml of methylene blue; T2, photodynamic treatment with 16 µg/ml of methylene blue; T3, photodynamic treatment with 32 µg/ml of methylene blue. C1, growth in SDA without irradiation or methylene blue; C2, isolates exposed to 16 µg/ml of methylene blue without irradiation; C3 isolates exposed to irradiation without methylene blue

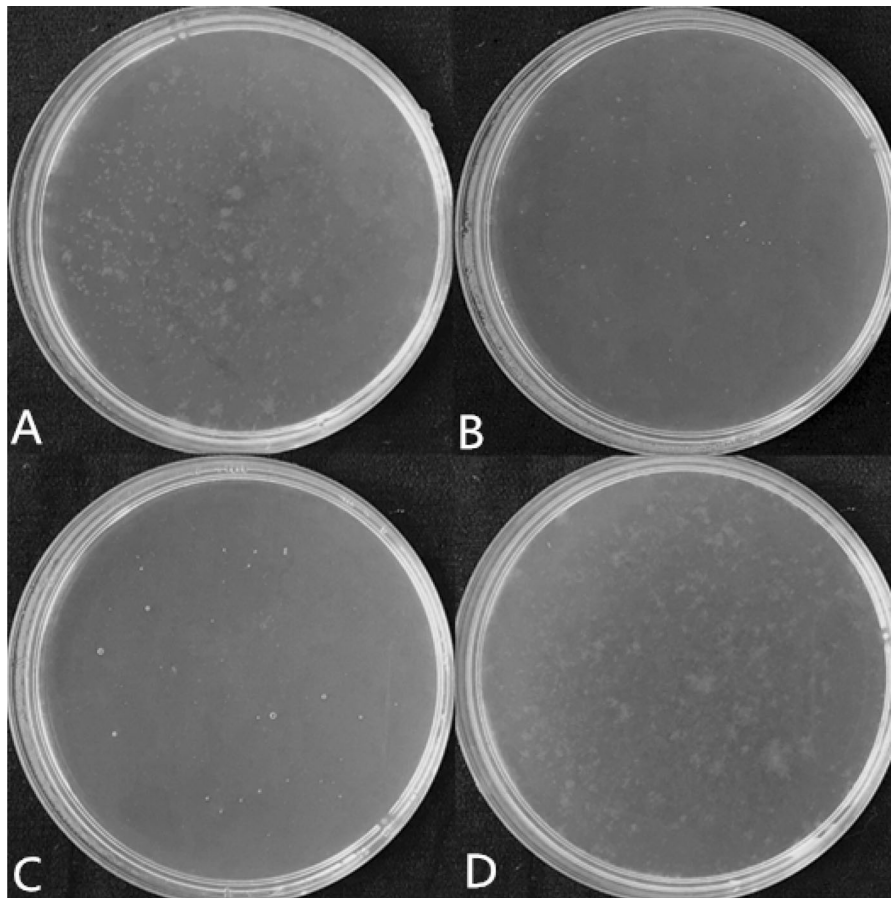


Fig. 1 Photodynamic effect on the growth of RA99880. A, photodynamic treatment with 8 ug/mL of methylene blue; B photodynamic treatment with 16 ug/mL of methylene blue; C photodynamic treatment with 32 ug/mL of methylene blue; D negative control

Table 2 Photodynamic effect on antifungal susceptibility of *R. oryzae*

aPDT antimicrobial photodynamic therapy, *MIC* minimal inhibitory concentrations, *ITC* itraconazole, *POS* posaconazole, *AMB* amphotericin B

Strain	MIC(μ g/ml)					
	Without aPDT			With aPDT (T1 treatment)		
	ITC	POS	AMB	ITC	POS	AMB
RA99880	≥ 32	8	2	4	1	0.5
ATCC56536	≥ 32	8	2	4	1	0.25
ATCC44170	≥ 32	8	2	8	0.5	0.5
RO1	≥ 32	16	2	4	1	0.5
RO2	≥ 32	16	4	4	2	0.5
RO3	≥ 32	8	2	8	1	0.5

growth of *R. oryzae* and significantly enhancing the effects of azoles and AMB against *R. oryzae*. These results suggest aPDT can be a therapeutic method against mucormycosis caused by *R. oryzae*, or aPDT can combine with antifungal agents, which may help to overcome the antifungal drug resistance, and has the

potential to decrease drug dosages, side effects, and improving patients' compliance. Nevertheless, further investigations including in vivo experimental and clinical studies are warranted to determine the safe and reliable application in clinical practice.

Conclusion

In summary, our results expand the knowledge regarding the photodynamic inactivation of *R. oryzae*. The aPDT mediated by methylene blue and LED revealed antimicrobial activity against *R. oryzae* and rendered *R. oryzae* more sensitive to azoles and AMB. The findings encourage further studies to evaluate the effectiveness of aPDT in mucormycosis.

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Compliance with Ethical Standards

Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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