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In vitro aminolevulinic acid mediated-antimicrobial photodynamic therapy inactivates growth of *Prototheca wickerhamii* but does not change antibacterial and antifungal drug susceptibiltity profile

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ABSTRACT

Background: Antimicrobial photodynamic therapy(aPDT) has been used to treat localized cutaneous fungal infections that have an enhanced antifungal susceptibility profile. The aim of this study was to evaluate the effect of ALA aPDT on both the growth and the antimicrobial and antifungal susceptibility of *Prototheca wickerhamii*.
Methods: Six isolates of *P. wickerhamii* were used in the present study. The inocula in sterile 6-well microtiter plates were irradiated with narrow band LED (633 ± 10 nm) at the light intensity of 100 mW/cm^2 and at a distance of 1 cm for 900 s. The ALA was tested at concentrations of 1, 5, and 10 mmol/l, while 10-μl aliquots of suspensions from each group were inoculated on Sabouraud dextrose agar to test the photoinactivation. Antibiotic susceptibility was investigated by the disc-diffusion method.
Results: Our study shows ALA aPDT induced 46% ± 24.23% reduction of the growth of all tested *P. wickerhamii* strains in T1 group. ALA aPDT induced 50.39% ± 19.88% reduction of the growth of all tested *P. wickerhamii* strains in T2 group. ALA aPDT induced 52.68 ± 20.22% reduction of the growth of all tested *P. wickerhamii* strains inT3 group. Single ALA aPDT induced 32.97% ± 1.6% growith reduction of three tested strains(O23d, O23e and 62,207), while repeated ALA aPDT induced 51.65 ± 2.91% reduction of the growth(P value = 0.000). There were no significant difference of the inhibitory zone diameter of both antibacterial and antifungal agents before and after ALA aPDT.
Conclusions: ALA aPDT can inactivate the growth of *P. wickerhamii*, and repeated aPDT has more photoinactivation of *P. wickerhamii.* ALA aPDT does not change antibacterial agents and antifungal drugs susceptibility profile of *P. wickerhamii.*

1. Introduction

Protothecosis is a rare infection caused by members of the genus *Prototheca*, which are generally considered to be achlorophyllic algae and ubiquitous in nature [1]. *Prototheca* infection is indolent, with no tendency toward self-healing. Most reported cases have been treated with antifungal agents and surgical approaches, including amphotericin B, fluconazole, voriconazole and itraconazole. Intravenous amphotericin B has been shown to be effective, but has been often associated with intolerable side effects [2].

Aminolevulinic acid(ALA) photodynamic therapy has considerable potential as an antimicrobial treatment, especially in the treatment of cutaneous and mucocutaneous infections [3,4]. In veterinary medicine, *P. zopfii* is considered to be a major pathogen, responsible for a disaproportional number of bovine mastitis. In contrast, *P. wickerhamii* is the most prominent cause of such infections in humans. Methylene blue mediated antimicrobial photodynamic therapy(aPDT) has demonstrated to inactivate *P. zopfii* [8]. Antifungal miconazole combination with aPDT has the potential to increase the efficacy against *Candia albicans* [5]. Minimal inhibitory concentration ranges have decreased significantly with photoinactivation. Photodynamic therapy improved both inactivation rates and the antifungal susceptibility profile of *Scedosporium* and *Lomentospora* species [6]. Several successful clinical studies on recalcitrant chromoblastomycosis [7–9] have shown that combination of PDT and antifungal drugsoffers an attractive alternative to the current antifungal strategies.

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Table 1
Effect of ALA antimicrobial photodynamic treatment with *Prototheca wickerhamii* isolates($\times 10^5$ cfu/mL).

strains	C1	C2	C3	T1	T2	T3
O23a	3.06 ± 0.05	3.01 ± 0.04	3.73 ± 0.21	1.4 ± 0.11*	1.32 ± 0.07*	1.5 ± 0.1*
O23b	1.02 ± 0.11	0.85 ± 0.23	0.81 ± 0.06	0.52 ± 0.11*	0.38 ± 0.08*	0.32 ± 0.1*
O23c	0.52 ± 0.04	0.32 ± 0.13	0.66 ± 0.13	0.05 ± 0.03*	0.11 ± 0.09*	0.07 ± 0.03*
O23d	4.03 ± 0.1	4.02 ± 0.1	3.84 ± 0.14	3.02 ± 0.04*	3.03 ± 0.04*	2.83 ± 0.15*
O23e	2.99 ± 0.03	2.63 ± 0.07	4.05 ± 1.7	1.96 ± 0.04*	1.64 ± 0.06*	1.73 ± 0.09*
622207	3.74 ± 0.15	3.74 ± 0.12	3.69 ± 0.06	2.74 ± 0.22*	2.09 ± 0.22*	2.26 ± 0.14*

C1: spore suspensions in saline without irradiation or ALA, C2: spore suspensions in saline with 5 mmol/l ALA without irradiation C3: spore suspensions in saline with irradiation without ALA, T1:1 mmol/l ALA aPDT, T2: 5 mmol/l ALA aPDT T3: 10 mmol/l ALA aPDT.
* Reduction in growth of *P. wickerhamii* was statistically significant (p value < 0.05).

Based on previous work, which suggests that aPDT can cause severe disruptions and deformations of both microconidia and mycelium [10], as well as enhanced antifungal susceptibility profile [6], we hypothesized that ALA aPDT may also render inactivate the growth of *P. wickerhamii* and enhanced the antimicrobial and/or antifungal susceptibility to *P. wickerhamii*.

2. Methods

2.1. Isolates

Six isolates of *P. wickerhamii* were used in the present study. Three isolates O23a, O23b, O23c, O23d and O23e were obtained from China Medical Fungi Culture Collection Center(CMCCC). *P. wickerhamii* (62,207) was kindly provided by Dr. Zhan Ping from Jiangxi Province Dermatology Hospital. All strains were identified using nucleotide sequence of its 26S/28S large subunit(LSU) D1/D2.

2.2. Photodynamic therapy

The photodynamic therapy was applied as previously described by Liu et al. [9]. The inocula were irradiated in sterile 6-well microtiter plates with a narrow band LED (633 ± 10 nm, LED-IB, Wuhan Yage Ltd, China) at the light intensity of 100 mW/cm^2 and at a distance of 1 cm for 900 s.

2.3. Photoinactivation of P. wickerhamii

For inoculum standardization, spore from cultures which had been grown for seven days on Sabouraud dextrose agar were suspended in saline and diluted to a concentration of 0.5–5 × 10^5 cells/ml. 5-aminolevulinic acid(ALA) (Sigma, America) stock solutions(50 mmol/l) was prepared and sterile filtered(0.2 μm, Whatman, UK) according to previous literature [4]. The ALA was tested at concentrations of 1(T1), 5(T2), and 10 mmol/l (T3), and then mixed with standardized inocula preliquoted into sterile 6-well microtiter plates. The suspensions were incubated for 0.5 h at 37 °C and protected from light. The following controls were included as previous study [11]: spore suspensions in saline without irradiation or ALA(C1), spore suspensions in saline with 5 mmol/l ALA without irradiation(C2), and spore suspensions in saline with irradiation without ALA(C3). Repeated irradiation was conducted after incubation at 37 °C for 24 h in three *P. wickerhamii* strains(O23d, O23e and 62,207). After irradiation, 10-μl aliquots of suspensions from each group were inoculated using Sabouraud dextrose agar, and were then uniformly coated using a sterile glass coating rod, which was incubated at 37 °C for 48–96 h, at which point the numbers of developing colonies could be counted. Control group C1 was considered as 100% of growth for each set, and growth reduction was calculated base on this.

2.4. Drug susceptibility

The *P. wickerhamii* were cultured in Sabouraud dextrose agar and incubated at 37 °C for 48 h under aerobic conditions. Spore suspensions were prepared with an optical density of one on the McFarland scale. Antibiotic susceptibility was investigated by the disc-diffusion method on Mueller-Hinton II agar (bioMerieux, France), as previously described [12]. The following antifungal chemotherapeutic agents in disc were used (Rosco, Denmark):, amphothericin B(20 μg), fluconazole(25 μg), itraconazole(1 μg), clotrimazole(10 μg), voriconazole(1 μg), nystatin (1 μg). The following antibacterial agents in disc were used (Oxoid, England): amikacin(30 μg), cephazolin(30 μg), minocycline(30 μg), ciprofloxacin(5 μg), ampicillin(10U) and chloramphenicol(30 μg). Results were verified after 48 and 72 h of incubation at 37 °C for all antimicrobials. The inhibitory zone diameters were measured and interpreted according to Clinical and Laboratory Standard Institute (CLSI) [13] and EUCAST standards [14].

2.5. Statistics

Data were expressed as both mean and standard deviation (SD). Differences between groups were assessed using *t*-test and ANOVA analysis. Statistical significance was considered to be reached at $P < 0.05$.

3. Results

3.1. aPDT inactivation of P. wickerhamii

Our results show that 5-aminolevulinic acid aPDT led to a reduction of the growth of all tested *P. wickerhamii* strains (Table 1). There were no significant difference of growth among the control groups(C1–C3). Importantly, there was a significantly reduction of growth between the treatment groups(T1–T3) and the control group(C1). Our study shows that ALA aPDT induced a 46% ± 24.23% reduction of the growth of all tested *P. wickerhamii* strains in T1 group. ALA aPDT induced a 50.39% ± 19.88% reduction of the growth of all tested *P. wickerhamii* strains in T2 group. ALA aPDT induced a 52.68 ± 20.22% reduction of the growth of all tested *P. wickerhamii* strains inT3 group. The higher concentration of ALA aPDT, the more reduction there was of *P. wickerhamii* (Fig. 1). However, there were no significant differences of growth reduction among the various 5-aminolevulinic acid concentration aPDT groups(T1, T2 and T3, $P = 0.64$).

3.2. Single and repeated aPDT inactivation of P. wickerhamii

Single ALA aPDT induced a 27.31% ± 1.82% reduction of the growth of O23d strains, while repeated ALA aPDT induced a 42.52% ± 4.21% reduction of the growth($P = 0.0044$, Fig. 2). Single ALA aPDT induced a 34.75% ± 3.29% reduction of the growth of O23e strains, while repeated ALA aPDT induced a 53.23% ± 3.31% reduction of the growth($P = 0.0011$, Fig. 2). Single ALA aPDT induced a 36.85% ± 2.12% reduction of the growth of 62,207 strains, while repeated ALA aPDT induced a 59.19 ± 6.03% reduction of the growth ($P = 0.003$, Fig. 2). Totally, single ALA aPDT induced a 32.97% ± 1.6%

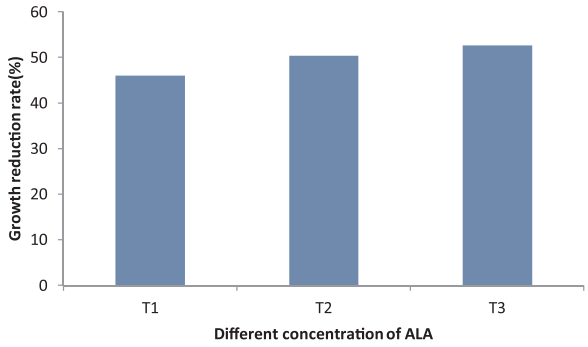

Fig. 1. Growth reduction rate of *Prototheca wickerhamii* among the different 5-aminolevulinic acid concentration aPDT groups. Reduction rate was not statistically significant(P value > 0.05).

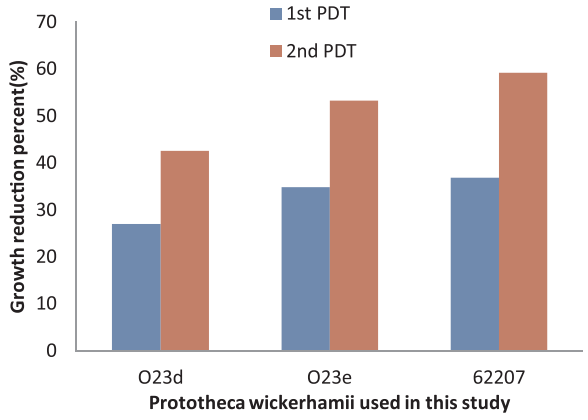

Fig. 2. Growth reduction rate of *Prototheca wickerhamii* after aPDT(red) and second aPDT(blue). The ALA concentration is 5 mmol/l. O23d, O23e and 62,207 *Prototheca wickerhamii* strain were tested. Reduction rate was ntatistically significant(P value < 0.05).

growth reduction of all three strains, while repeated ALA aPDT induced a 51.65% ± 2.91% growth reduction(P = 0.000).

3.3. Drug susceptibility before and after ALA aPDT

All six strains of *P. wickerhamii* were resistant to cephazolin, ciprofloxacin, ampicillin and chloramphenicol. Additionally, all six strains of *P. wickerhamii* treated with ALA-PDT were also resistant to cephazolin, ciprofloxacin, ampicillin and chloramphenicol ($P > 0.05$) (Table 2, Fig. 2a, b). Only two strains of *P. wickerhamii* were resistant to amikacin and minocycline before and after ALA aPDT. Four strains of *P. wickerhamii* were sensitive to minocycline before and after ALA aPDT, but no significant difference of the inhibitory zone diameter was found. All six strains of *P. wickerhamii* were sensitive to amikacin (Table 2, Fig. 2a, b), no significant difference was found the inhibitory zone diameter before and after ALA-aPDT treatment($P > 0.05$).

All six strains of *P. wickerhamii* were shown to be resistant to fulcytosine before and after ALA aPDT. However, only two strains of *P. wickerhamii* were resistant to fluconazole before and after ALA aPDT. One strain of *P. wickerhamii* were resistant to itraconazole before and after ALA aPDT, while all six strains of *P. wickerhamii* were sensitive to voriconazole, nystatin and amphotericin B (Table 3, Fig. 3c, d). The inhibitory zone diameter after ALA-PDT treatment was found to increase more than control group (Table 3, Fig. 3c, d). There were no significant difference of the inhibitory zone diameter between the two groups($P > 0.05$).

4. Discussion

Photodynamic therapy (PDT) utilizes a photosensitive substance activated by the light source of a specific wavelength. The photoactivation induces cascades of photochemicals and photobiological events which cause irreversible changes to the exposed cells [3]. The use of PDT for localized viruses, protozoa, Gram-positive and Gram-negative bacteria and fungi has been extensively explored in the literature [9,11,15,16]. Current research suggests that PDT hold great promise for combating certain types of cutaneous fungal pathogens.

Human protothecosis is an uncommon infection caused by *Prototheca* genus, which is primarily caused by *P. wickerhamii*, followed by *P. zopfii* [17]. Previous study has shown that methylene blue mediated antimicrobial photodynamic therapy inactivates growth of *P. zopfii* [18]. Since ALA was licensed in clinics in China, we choose ALA aPDT to test the photoinactivation of *P. wickerhamii*. Our study shows ALA-aPDT induced about 50% reduction of the growth of all tested *P. wickerhamii* strains, which was similar to 50–80% growth reduction in pathogenic fungi [16].Our study demonstrated that repeated ALA aPDT has a highly significant photoinactivation effect of *P. wickerhamii*, which indicates that several sessions of aPDT is necessary in clinic practice.

To date, despite the testing of various treatment regimens, there is no effective treatment for human protothecosis. There were reports of large-scale screening of the in vitro antibiotics susceptibility of *P. zopfii* isolated from dairy-associated environments [19–21]. There are only a few case report studies are available on in vitro susceptibility of antibiotics to the *Prototheca* species that has been isolated from human. However, there is low susceptibility of antimicrobials currently employed in veterinary and human medicine. Several studies demonstrated that aPDT could increases cell permeability, which led to improvement of antifungal agents for some fungal species [5,6,22]. Clinical studies on unmanageable chromoblastomycosis have shown that clinical response resulted from combination of aPDT and antifungal drugs [8,9].

We ask whether ALA aPDT can enhance the antimicrobial and/or antifungal susceptibility to *P. wickerhamii* using disc-diffusion method. Our results show that if the *P. wickerhamii* was resistant to some antibacterial and antifungal agents, it was still resistant even after ALA aPDT. If the strains were sensitive to some antibacterial and antifungal agents, the inhibitory zone diameter of antibacterial and antifungal agents after ALA aPDT treatment were all slightly increased than

Table 2
Comparison of inhibitory zone diameter of antibacterial agents to *Prototheca wickerhamii* before(control) and after ALA-aPDT.

isolate	Chloramphenicol		Ampicillin		Cephazolin		Ciprofloxacin		Minocycline		Amikacin	
	control	PDT	control	PDT	control	PDT	control	PDT	control	PDT	control	PDT
O23a	6	6	6	6	6	6	6	6	6	6	14	18
O23b	6	6	6	6	6	6	6	6	24	26	10	13
O23c	6	6	6	6	6	6	6	6	6	6	18	20
O23d	6	6	6	6	6	6	6	6	18	20	16	18
O23e	6	6	6	6	6	6	6	6	21	21	15	15
62,207	6	6	6	6	6	6	6	6	18	18	10	10

Table 3
Comparison of inhibitory zone diameter of antifungal agents to *Prototheca wickerhamii* before(control) and after ALA-aPDT.

isolate	Flucytosine		Fluconazole		Itraconazole		Voriconazole		Nystatin		Amphotericin B	
	control	PDT	control	PDT	control	PDT	control	PDT	control	PDT	control	PDT
O23a	9	9	9	9	9	9	32	33	30	33	28	30
O23b	9	9	17	21	16	19	22	30	40	42	34	36
O23c	9	9	9	9	22	27	14	20	29	32	24	28
O23d	9	9	16	20	17	21	18	23	38	40	34	37
O23e	9	9	22	24	18	22	36	40	38	40	40	42
62,207	9	9	14	20	12	16	18	21	38	40	34	36

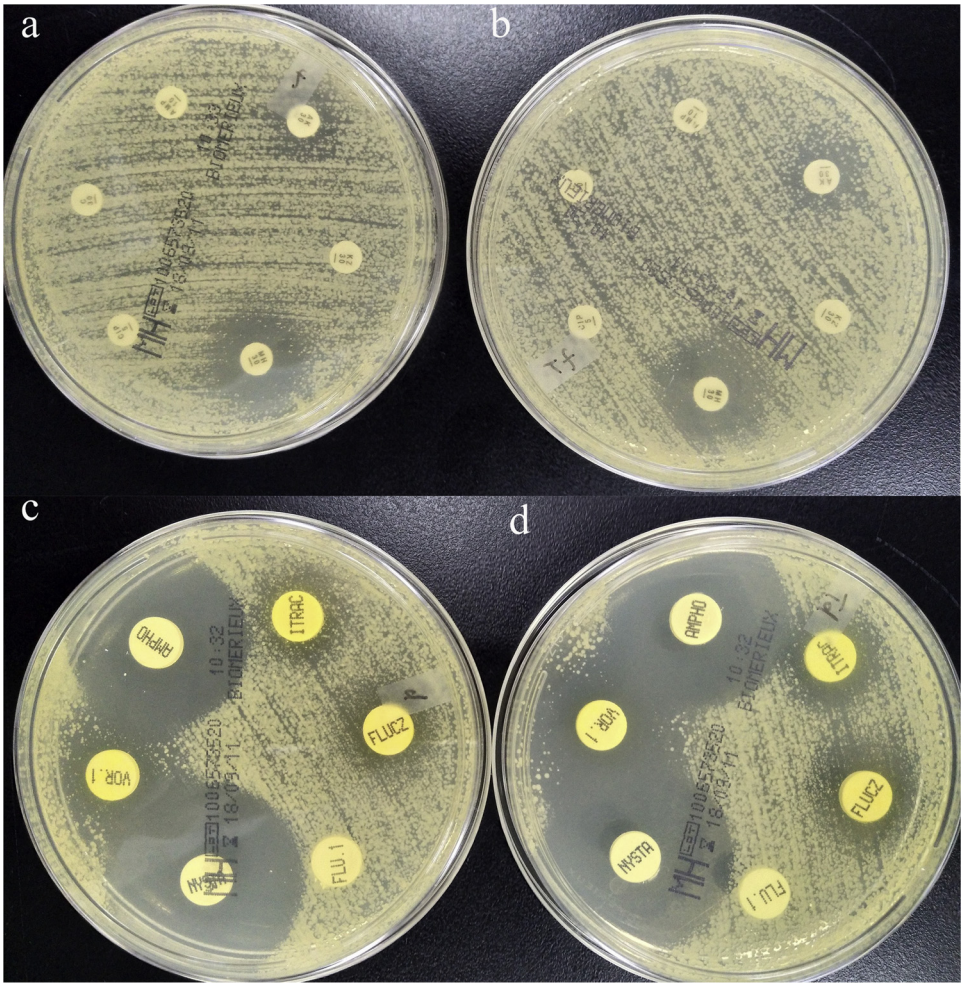

Fig. 3. a: Antibacterial drug susceptibility of *Prototheca wickerhamii* strain 62,207. b: Antibacterial drug susceptibility of *P. wickerhamii* strain 62,207 after aminolevulinic acid photodynamic treatment. c: Antifungal drug susceptibility of *P. wickerhamii* strain O23d. d: Antifungal drug susceptibility of *P. wickerhamii* strain O23d after aminolevulinic acid antimicrobial photodynamic therapy.

control group. However, our study shows that there were no significant difference of the inhibitory zone diameter between the two groups.

5. Conclusion

In conclusion, it has been demonstrated that aminolevulinic acid antimicrobial photodynamic therapy can render inactivate the growth of *P. wickerhamii*, and that repeated aPDT can led to further photoinactivation of *P. wickerhamii*. Aminolevulinic acid antimicrobial photodynamic therapy does not change antibacterial agents and antifungal drugs susceptibility profile of *P. wickerhamii*.

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Declaration of interest

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

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Prototheca wickerhamii strain 62,207. We would like to thank Dr Liu Wei-Da and Shen Yong-nian for kindly providing all the other strains of *Prototheca wickerhamii.*

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