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Mechanisms for Low-Light Therapy VI

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Cryptococcus neoformans capsule protects cell from oxygen reactive species generated by antimicrobial photodynamic inactivation

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ABSTRACT

Antimicrobial photodynamic inactivation (APDI) is based on the utilization of substances that can photosensitize biological tissues and are capable of being activated in the presence of light. *Cryptococcus neoformans* is an yeast surrounded by a capsule composed primarily of glucoronoxylomannan that plays an important role in its virulence. This yeast causes infection on skin, lungs and brain that can be associated with neurological sequelae and neurosurgical interventions, and its conventional treatment requires prolonged antifungal therapy, which presents important adverse effects. The aim of this study was to evaluate the protective effect of *Cryptococcus neoformans* capsule against reactive oxygen species generated by APDI. *Cryptococcus neoformans* KN99α, which is a strain able to produce capsule, and CAP59 that does not present capsule production were submitted to APDI using methylene blue (MB), rose bengal (RB), and pL-ce6 as photosensitizers (PS). Then microbial inactivation was evaluated by counting colony form units following APDI and confocal laser scanning microscopy (CLSM) illustrated localization as well as the preferential accumulation of PS into the fungal cells. *C. neoformans* KN99α was more resistant to APDI than CAP59 for all PSs tested. CLSM showed incorporation of MB and RB into the cytoplasm and a preferential uptake in mitochondria. A nuclear accumulation of MB was also observed. Contrarily, pL-ce6 appears accumulated in cell wall and cell membrane and minimal florescence was observed inside the fungal cells. In conclusion, the ability of *C. neoformans* to form capsule enhances survival following APDI.

Keywords: Antimicrobial photodynamic chemotherapy; Conjugate Chlorine; Methylene Blue; *Cryptococcus neoformans*; Photodynamic therapy; Photosensitizer; Rose Bengal.

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1. INTRODUCTION

Infections caused by fungi can be serious especially in immunocompromised and debilitated patients (HIV infection, transplantation, corticosteroid therapy and lymphoma). The incidence of invasive mycoses has increased significantly over the last 3 decades and now represents an exponentially growing threat for human health due to a combination of slow diagnosis and relatively few classes of available and effective antifungal drugs. Thus, systemic fungal infections result in high attributable mortality [1]. Cryptococcosis is an infection caused by the encapsulated yeasts, *Cryptococcus neoformans*, which is unique among pathogenic fungi because it produces a polysaccharide capsule to enclose its body. The polysaccharide contributes mostly to the overall virulence phenotype [2] and it is believed to protect against dehydration and other stress conditions [3,4]. In addition, the capsular polysaccharide is also released to the extracellular environment as exopolysaccharide. During infection, the exopolysaccaride has numerous toxic effects on the host immune response [5]. Furthermore, it has been proposed that the capsule of *C. neoformans* may protect the cell from reactive oxygen species (ROS) challenge [6]. The major causes of death by this yeast are related to brain and meningeal infection [7]. Furthermore, the infection requires prolonged antifungal therapy and it is associated with neurological sequelae and neurosurgical interventions [3,8]. Cranial nerves paresthesia may occur due to fungi invasion and compression secondary to cerebral edema, and paralysis can persist as permanent sequelae of the disease and involves one or more cranial nerves [5,9-12].

Antimicrobial photodynamic inactivation (APDI) combines a nontoxic photoactivatable dye or photosensitizer (PS) with harmless visible light of the correct wavelength to excite the dye to its reactive singlet state, which will go to triplet state and then generate reactive oxygen species, such as singlet oxygen and hydroxyl radicals that are toxic to cells [13-15]. APDI has been successfully employed in clinics as a treatment for cancer [16], age-related macular degeneration [17], and its killing effect on microorganisms was discovered more than 100 years ago [18]. The exponentially increasing threat of microbial multidrug resistance has placed APDI as a highly promising alternative treatment for localized infections [19,20]. The cells that are considered therapeutical targets are stained with the photosensitizing agent and irradiated with light [21-24]. The photodynamic process rapidly generates reactive oxygen species (ROS) as for instance peroxides, hydroxyl radicals, superoxide ions and singlet oxygen, the last one has being implicated as the major causative agent of cellular damage in photodynamic process [25]. In addition, this technique has been shown to have effects against a range of pathogens and also against drug-resistant microorganisms [26-28]. The aim of this study was to evaluate the protective effect of *C. neoformans* capsule against reactive oxygen species generated from APDI.

2. MATERIAL AND METHODS

2.1 Strain and culture conditions

Cryptococcus neoformans KN99α (capsule positive strain) and CAP59 (capsule deficient strain) were sub-cultured from -80°C vial stock in yeast extract peptone dextrose (YPD) broth at incubation conditions of air atmosphere for 48h at 30°C. The cells were collected by centrifugation (3000 rpm for 10 min) and washed twice in phosphate buffered saline (PBS) pH 7.4. Inocula of approximately $5x10^6$ colony form units (cfu)/mL were prepared for each strain.

2.2 Photosensitizers and Irradiation source

Chloride salt of Methylene blue (MB), Rose Bengal (RB) (Sigma-Aldrich St. Louis, MO), and poly-L-lysine chlorin (e6) conjugate (pL-ce6) were used as antimicrobial photosensitizers [29]. Stock solutions were prepared in water and stored at 4°C in the dark before use. A non-coherent lamp (LumaCare LC122, MBG Technologies, Inc.) was used with interchangable fiber optic probes containing band pass filters. Light characteristics are presented in table 1.

Table 1 – Irradiation parameters for each photosensitizer

	pL-ce6	**MB**	**RB**
wavelengh (nm)	650	665	530
Out put power (mW)	700	700	450
Fluence-rate (mW/cm^2)	40	40	30

Yeast strains were irradiated from the top of a well microtitulation plaque and the laser beam passed through all the suspensions at 17cm^2 spot size, which was coincident for all groups.

2.3 Photodynamic therapy studies and colony-forming units (CFU) determination

Photosensitizer was added to yeast suspensions (10^6 CFU/mL) in a final concentration of 10μM for 30 min in dark conditions, and then suspensions were washed twice in PBS. Aliquots of 1 mL of yeast suspension with PS were putted in a 24-well plate and subsequently irradiated with light, resulting fluence-rates of 30mW/cm² and 40mW/cm² (table 1). Aliquots were collected before and during the irradiation, and serially diluted in PBS to generate dilutions of 10^{-1} to 10^{-5} times the original concentration [30]. Ten micro liters aliquots of each dilution were streaked onto a sabouraud plaque in triplicate and incubated for 48h at 30°C to allow colony formation [31].

The control group was untreated by either light or photosensitizer, and in the light groups the yeast suspensions were irradiated with the maximum fluence of each wavelength in the absence of the photosensitizer.

2.4 Yeast up-take of photosensitizers

Inoculum of *C. neoformans* was stained by photosensitizers for 30 min and the yeast cells were washed twice in PBS. One mL aliquot was centrifuged and the pellet suspended in 1 mL 0.1 M NaOH and 1% SDS for 24 hours to dissolve the cells. The fluorescence was measured in a spectrophotometer (Spectra MAX Gemini EM, Molecular Devices, USA) at excitation and emission mode. Uptake values were calculated by dividing the number of nmol of photosensitizer in the dissolved pellet by the number of CFU obtained by serial dilution, and the number of PS molecules per cell was calculated using Avagadro's number.

2.5 Confocal Laser Scanning Microscopy (CLSM)

C. neoformans cells were incubated with each photosensitizer for 30min. Then rhodamine123 (R123; Eastman Kodak, Rochester, NY), a mitochondrial localizing dye, was added into sample tubes in a final concentration of 10μM. Suspensions were washed twice with PBS and fixed in 2% formaldehyde. Four-μL aliquots were taken from the pellet and placed on a slide and covership for analysis. A confocal laser microscope (Leica TCS NT, Leica Mikroskopie und System GmBH, Wetzlar, Germany) with excitation at 488nm from an argon laser was used. The cells were observed with 100x oil immersion objective and images at 512 x 512 pixels resolution were recorded. Two channels collected fluorescence signals in either the green range (580nm dichroic mirror plus 525/50nm bandpass filter) from R123, and in red range (580nm dichroic mirror plus 665nm longpass filter) from PS. The false output color green and red images were superimposed for the figures.

2.6 Statistics

The yeast colonies were counted and converted into colony forming units (CFU) for analysis. All samples were submitted to this process and statistical analysis of the experimental data was performed using one-way analysis of variance (ANOVA) and the Tukey's test means comparison. For all tests, significance was set at $\alpha = 0.05$[32].

3. RESULTS AND DISCUSSION

Neither light nor photosensitizer alone presented any effect on yeast cells inactivation (fig. 1). No statistically significant differences were observed among control group, laser group and photosensitizer group before irradiation. The photosensitizers had no toxicity effect on these samples for 30 min of cell contact, as well as light irradiation alone did not change the number of viable yeasts.

C. neoformans KN99α was less inactivated than the capsule deficient strain CAP59 with all photosensitizers used in this study. The ability of KN99α in resist ROS challenge seems to be related to its ability in producing capsule.

Methylene blue presented moderated effect against the tested *C. neoformans* strains. The effect was dependent on the exposure time; thus, the lowers fluences were less effective than higher ones. Following 24 min of irradiation (60J/cm², 40mW/cm²) there was less than 1 log reduction of cell viability for both strains (fig.1A). The use of rose Bengal under

irradiation presented a considerable difference between the strains. CAP59 had 1.5 logs reduction after 40 J/cm² of green light irradiation (24 min.; 30mW/cm²), and KN99α presented only 0.5 log of cell reduction. However, RB had also a moderate photoinactivation effect on *C. neoformans* (fig.1B).

On the other hand, pL-ce 6 conjugate was the most effective photosensitizer on this yeast. Following 20J/cm² (8 min.; 40mW/cm²), KN99α had 2 logs reduction on cell viability and CAP59 was completely eradicated with more than 6 logs of cell inactivation. In addition, 40 J/cm² (16 min.; 40mW/cm²) were able to completely kill KN99α, which presented about 6 logs of cell reduction (fig.1C).

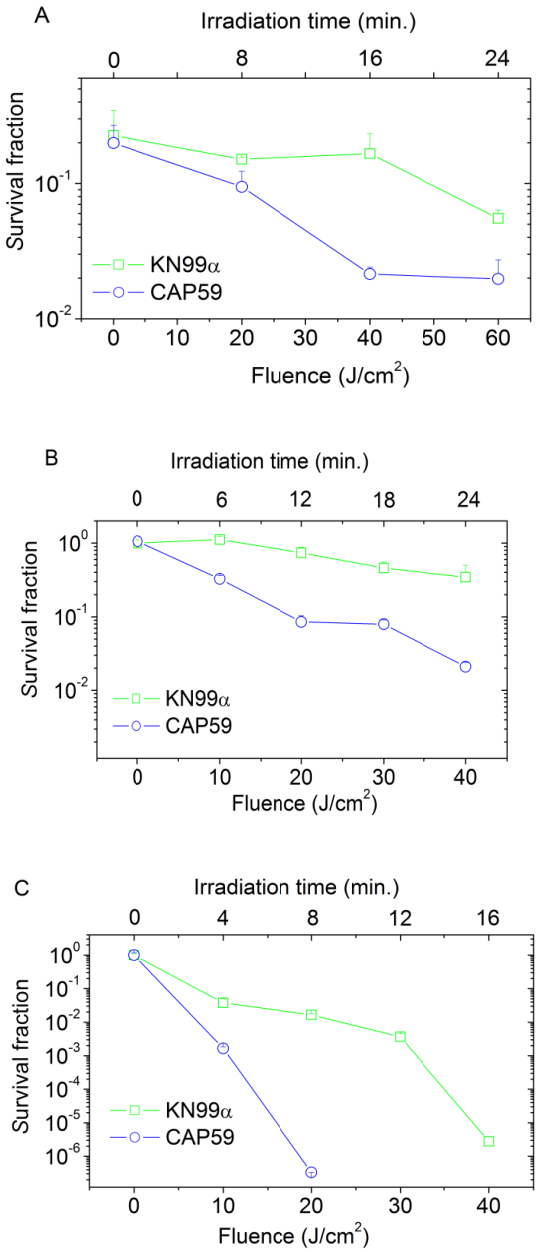

Figure 1- Photodynamic inactivation of *C. neoformans* KN99α (capsule positive strain) and CAP59 (capsule deficient strain). (A) Methylene blue, (B) Rose bengal and (C) pL-ce 6 conjugate. Data are means of survival fraction, and bars are standard deviation. (A)

The up-take of photosensitizers was measured for the two strains used in this study. The pL-ce6 presented the highest level of cell accumulation. Furthermore, there was no statistical difference between KN99α and CAP59 regarding PS up-take. Rose Bengal also does not presented statistical difference between the strains; nevertheless, it was less accumulated than pL-ce6 inside yeast cells. Methylene blue presented similar results in accumulation inside *C. neoformans* to Rose Bengal.

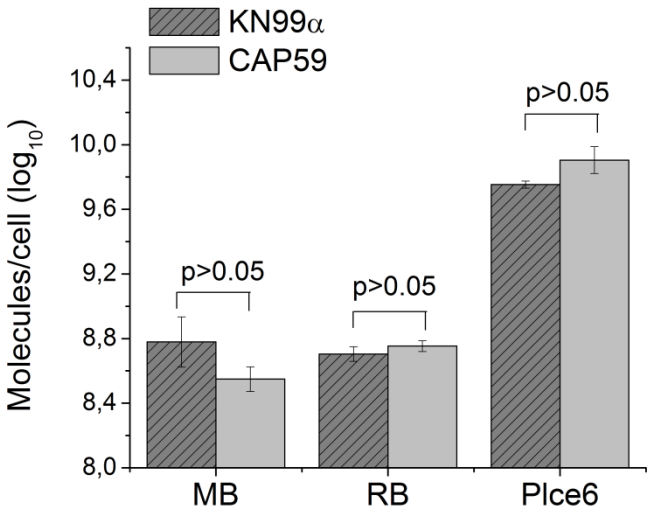

Figure 2 – Up-take of photosensitizer by *C. neoformans* strains

Confocal microscopy was used to determine where the photosensitizer might attach or accumulate inside yeast cells. Transmittance and green/red overleap fluorescence image were plotted for both strains with the photosensitizers used. The main result is that each photosensitizer has different accumulation in *C. neoformans* (fig.3).

Methylene blue presented a preferential accumulation inside the cell; it was possible to note an intensity fluorescence coming from cytoplasm. In addition, there was some fluorescence signal that came from sites related to membrane as well as nucleus. CAP59 cells presented a more pronounced overlap on green/red image than KN99α (fig.3 MB). Rose Bengal accumulated inside the yeast cell preferentially in mitochondria. In addition, it was distributed in cytoplasm; however, there was no signal from nucleus (fig.3 RB). Chlorine conjugated pl-ce6 presented an intense red fluorescence in the cell wall and membrane, and it showed accumulation inside cytoplasm and in nucleus as well (fig.3 pl-ce6).

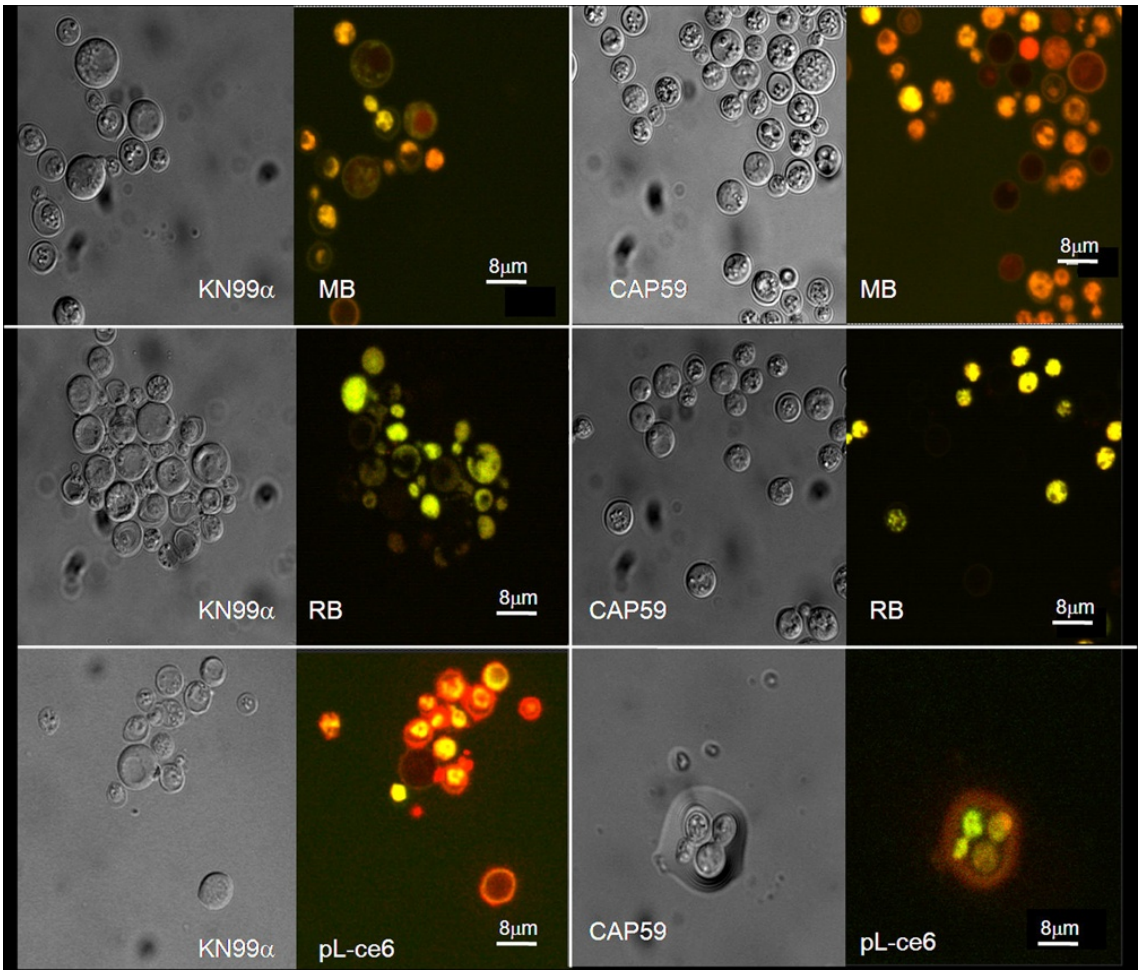

Figure 3 – Confocal images. The gray field is the transmission mode and colored field represents fluorescence mode. The cell fluorescence images were superimposed to observe coincidence in red/green signal and track PS accumulation inside the cells

In conclusion, the ROS formed by APDI can inactivate *C. neoformans* cells and the absence of capsule increased susceptibility of this microorganism to be killed by ROS. The higher APDI efficiency in capsule deficient *C. neoformans* was not dependent on PS uptake. The presence of capsule seams to play an important role on microbial protection against the challenge produced by APDI.

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