PDI) had low effectiveness and, therefore, the effect was not enough to promote a significant bacterial inactivation even when combining the therapies.

5. Conclusion

The concentration of antibiotics and/or PDI that are not/very efficient in monotherapy tends to present antagonistic results. The most strongly synergistic conditions were identified for GEN, which did not show any antagonistic results.

Disclosures if required

The authors declare that there is no conflict of interest.

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Antimicrobial photodynamic response in *Rhyzopus oryzae*

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Mucormycosis is an extremely aggressive fungal disease that has a high mortality rate. Conventional treatment consists of high doses of antibiotics and antifungal agents associated with surgical resections, but even with this aggressive treatment, the mortality rate is still high. The antimicrobial photodynamic therapy (aPDT), also called photodynamic inactivation (PDI), it is an interesting alternative to antifungal therapy because it has non-specific biological site action and can also be an additional method of treatment in conjunction with surgery and antifungal therapy. In this work we studied the evaluation of the photodynamic antimicrobial response in different phases of fungus growth (conidium and hyphae in the white and black phases) and for different protocols of PDI with PDZ, varying the concentration of photosensitizer, the incubation time, the association with surfactant and the fluence of light at 660 nm. In this work, PDI showed great potential in the in vitro study of *R. oryzae*.

Keywords: Mucormycosis. *Rhizopus oryzae*. Photodynamic inactivation. Microbial control

1. Introduction and Background

During the COVID-19 pandemic, several secondary infections appeared in patients, one of them being mucormycosis, which is an extremely aggressive fungal disease with a high mortality rate. In October 2022, the WHO released the first list of fungi that pose a great risk to public health due to the invasive way these fungal diseases affect debilitated patients, one of the fungi cited is Rhizopus spp. which causes mucormycosis [1]. PDI has been presented as an alternative treatment to the conventional one in the treatment of mycoses and may be a potential coadjuvant alternative for microbial control of *Rhizopus spp.*

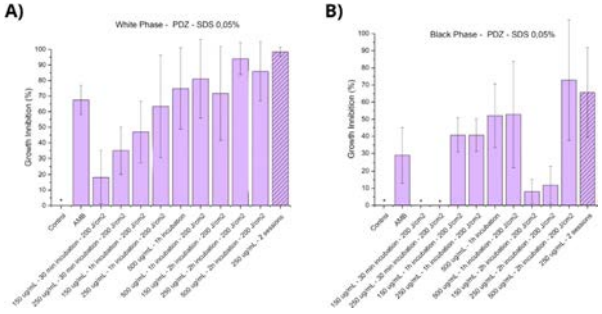
2. Aims

The purpose of this study is to evaluate the efficiency of photodynamic inactivation in the growth of *R. oryzae*.

3. Methods

Eight mm diameter discs were removed from 24-hours (light phase) and 48-hours (dark phase) cultured plates using a sterile syringe. Then, the disks were inserted into a well of a 24-well plate. For the PDI protocol PDZ was used, several protocols were tested varying incubation times from 20 minutes to 2 hours, PDZ concentrations, and association with the surfactant Sodium Dodecyl Sulfate (SDS). PDZ was tested in concentrations ranging from 150 μg/mL to 2.6 mg/mL. A light device at 660 nm and fluence between 100 and 200 J/cm^2 was used to perform the PDI irradiation. After treatment, the growth inhibition rate was calculated. The light and black phases samples were analyzed with Confocal Fluorescence Microscopy to obtain images of the fungus autofluorescence, bright field images, to observe cell structures, PDZ internalization and damage after aPDT. Samples were also analyzed by Transmission Electron Microscopy to study subcellular structures of *R. oryzae.* PDI experiments were performed on conidium suspension, using PDZ, varying the concentration from 25 to 150 μg/mL and fluence 100 J/cm^2.

4. Results



The combination of PDZ and 0.05% SDS showed a positive PDI response in *R. oryzae* light phase samples. A 94 % inhibition in the 1 session protocol and for the 2 sessions protocol, 98% growth inhibition was achieved. A 72 % inhibition was observed in the 1 session PDI protocol, while for the 2 sessions protocol 65 % growth inhibition was obtained. Using confocal microscopy, it was noted that for the white phase of growth of *R. oryzae*, the PDZ was distributed heterogeneously in some regions and homogeneously in others, in the hyphae, while in the black phase the PDZ is distributed completely heterogeneously. After the PDI it was observed that the hyphae showed a change in their structure, presenting an irregularity in the structure of their cell wall, showing the damage induced in the cell wall, it is also noted that there was a rupture of some hyphae, and extravasation and deposition of amorphous

material after the PDI. For the experiments on conidium it was noted that there was a reduction of 1.7 $\log_{10}$.

5. Conclusion

In the studies using only PDZ, for the hyphae, in the light and black phases, we observed an ineffective photodynamic response, when adding SDS 0.01% we noticed an improvement in the response only in the withe phase, and with the combination of PDZ and SDS 0.05% we obtained a growth inhibition rate of 98% for 2 sessions of PDI in the light phase and 72% inhibition in the 1 session protocol for the black phase. In the conidium study we observed a 1.7 $\log_{10}$ reduction. In this present work, PDI showed great potential in the in vitro study of R. oryzae.

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Correlation of *in vitro* cell viability and cumulative singlet oxygen luminescence from protoporphyrin IX in mitochondria and plasma membrane

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Significance: Photodynamic therapy (PDT) can be targeted toward different subcellular localizations and it is proposed that different subcellular targets vary in their sensitivity to photobiological damage. Since singlet oxygen ($^1O_2$) has a very short lifetime with a limited diffusion length in cellular environments, measurement of cumulative $^1O_2$ luminescence is the most direct approach to compare the PDT sensitivity of mitochondria and plasma membrane.
Approach: PDT-generated near-infrared $^1O_2$ luminescence at 1270 nm was measured together with cell viability for 5-aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX) and exogenous PpIX, at different incubation times. Confocal fluorescence microscopy indicated that ALA-induced PpIX (2 h) localized in the mitochondria, whereas exogenous PpIX (1 h) mainly localized to the plasma membrane. Cell viability was determined at several time points during PDT treatments using colony-forming assays, and the surviving fraction correlated well with cumulative $^1O_2$ luminescence counts from PpIX in mitochondria and plasmas membrane, respectively.
Results: The mitochondria are more sensitive than the plasma membrane by a factor of 1.7.
Conclusions: The potential value of direct $^1O_2$ luminescence dosimetry for comparing the PDT sensitivity of different subcellular organelles was demonstrated, which could be useful for developing subcellular targeted-novel photosensitizers to enhance PDT efficiency.

Keywords: Photodynamic therapy, 5-aminolevulinic acid, protoporphyrin IX, Subcellular localization, Singlet oxygen luminescence, Cell viability

1. Introduction and Background

PpIX is an efficient photosensitizer for PDT, and it may be generated endogenously in cells by administration of the nonphototoxic prodrug 5-aminolevulinic acid (ALA) or be administered exogenously. Tabata *et al.* firstly observed that ALA-induced PpIX was photodynamically more efficient than exogenous PpIX in HeLa cells [1]. Moreover, Ji *et al.* also demonstrated that PDT with ALA- induced PpIX was significantly more efficient than exogenous PpIX at a similar level of intracellular PpIX accumulation [2]. These observations suggest that the mitochondria are more sensitive than the plasma membrane for photodamage, since PpIX synthesis takes place in the mitochondria following ALA administered. Here, PDT-generated singlet oxygen ($^1O_2$) luminescence was measured directly to correlate with the cell viability for ALA-induced, mitochondrially-localized PpIX (2 h), and the plasma membrane-localized exogenous PpIX (1 h) in OCI-AML5 leukemia cells as an *in vitro* model.

2. Aims

To compare the PDT sensitivity of ALA-induced PpIX localized in the mitochondria and exogenous PpIX in plasma membrane by using the previously established direct $^1O_2$ luminescence dosimetry, and to demonstrates the potential value of direct $^1O_2$ luminescence dosimetry for comparing the PDT sensitivity of different subcellular organelles.

3. Methods

Measurement of accumulated PpIX. The intracellular accumulation of ALA-induced PpIX and exogenous PpIX was determined by spectrofluorometry following a well-established protocol [3]. Briefly, cells were incubated with ALA (1 mM) for 2 h or various concentration of exogenous PpIX (0.1-0.4 μM) for 1 h in 6-well tissue-culture plates. The cells from each well were pelleted by centrifugation at 200 *g* for 10 min, washed twice with phosphate buffered saline to remove extracellular PpIX and re-centrifuged. The cell pellet was then resuspended in serum-free alpha- MEM medium with 1 M perchloric acid and methanol (1:1) to lyse the cells and extract PpIX.
Subcellular localization of ALA-induced PpIX and exogenous PpIX. The subcellular localization of ALA-induced PpIX and exogenous PpIX was characterized using confocal laser- scanning fluorescence microscopy (LSM510, Carl Zeiss, Inc., Thornwood, NY). The excitation wavelength was 488 nm from an argon ion laser, and 585 nm long-pass and 505-550 nm band- pass filters were used for acquiring PpIX and MitoTracker fluorescence images, respectively.
Measurement of $^1O_2$ luminescence. The apparatus used to measure $^1O_2$ luminescence for cell suspensions has been described in detail elsewhere [4-5]. with the exception that the pervious NIR- PMT R5509 has been replaced by a new NIR-PMT module (H9170-45, Hamamatsu Corp., Bridgewater, NJ, USA) with thermoelectric cooling, and the optical collection geometry has been re-optimized. Light at 523 nm from a diode-pumped, Q-switched, frequency-doubled Nd:YLF laser (QG-523-500; Crystalaser Inc., Reno, NV) was used as the light source. The laser output was adjusted to produce a uniform fluence rate of 35 mW cm^{-2} and a treatment fluence of 25.2 J cm^{-2} was used. The pulse duration was 10 ns and the pulse repetition rate was 10 kHz.

4. Results

As shown in Fig.1, no significant difference was found before and after adding NaN_3 in case of ALA-induced PpIX, while a significant decrease was observed for exogenous PpIX. The intracellular fractions of $^1O_2$ luminescence counts for ALA-induced PpIX and exogenous PpIX were 95±4 % and 75±5 %, respectively, *i.e.* only 75% of the $^1O_2$ generated from plasma membrane- localized exogenous PpIX would have resulted in cellular damage. The survival curves as a function of the cumulative $^1O_2$ luminescence counts after corrected to τ_D and the in-