REVISTA SAÚDE MULTIDISCIPLINAR
Revista Eletrônica Saúde Multidisciplinar da Faculdade Mineirense – Vol. III
ISSN 2318 – 3780

# PDT REDUCES GLYCOLYSIS ACTIVITY AND INDUCES *CANDIDA ALBICANS* DEATH

*Pdt reduz a atividade de glicólise e induz a morte de Candida albicans*

Renato V. Oliveira[a#], Wellington F. Rodrigues[a,b,c#], Camila Botelho Miguel[c#], André B. Vasconcelos[c], Ana L.F. Sobrinho[c], Marcia L. L. Silveira[a], and Tony P.Paulino[*, a]

[a] CEFORES, Universidade Federal do Triângulo Mineiro, 38015-050, Uberaba, MG, Brasil.
[b] Faculdade Mineirense – FAMA, 75830-000, Mineiros, GO, Brasil.
[c] Curso de Pós-graduação em Ciências da Saúde, Universidade Federal do Triângulo Mineiro, 38061-500, Uberaba, MG, Brasil.
[d] Universidade de Uberaba, 38050-501, Uberaba, MG, Brasil.

[*] Corresponding author: tppfarmacia@icloud.com
[#] Contributed equally to this work.

Revista Saúde Multidisciplinar Vol. III – agosto à dezembro de 2015 / Faculdade Mineirense

REVISTA SAÚDE MULTIDISCIPLINAR
Revista Eletrônica Saúde Multidisciplinar da Faculdade Mineirense – Vol. III
ISSN 2318 – 3780

**ABSTRACT**

Introduction: Candida species species can cause superficial damage to the mucosal, as well as systemic infections and is most commonly found in immunocompromised patients. The use of PDT has become promising. Objectives: To evaluate the effect of PDT doses on the bioenergetic metabolism. Methods: A suspension of C. albicans with the following experimental groups were prepared: L+P+ exposed to the LED irradiation in the presence of the photosensitizer; L+ P- exposed only LED irradiation; L-P+ only treated with the photosensitizer; L-P- without exposure to laser or photosensitizer. For determining the growth curve a suspension of *C. albicans* was adjusted, and inoculated in BHI. The growth was monitored in the times 0, 5, 8, 10 and 12 hours. For measurement of glucose was performed using an enzymatic glucose kit (Biotecnica). Germ tube formation was evaluated in Neubauer chamber and expressed in percentage. The conduction microcalorimeter too was evaluation. Results: The applied of PDT 15, 30 and 60 j/cm$^2$ shows differences when compared with the control group in relation the number of colony forming units. The growth kinetics was affected as well as the consumption of glucose was proportional the capacity of cell consumption, affected by treatment with PDT. The release of energy was reduced dose dependent manner to treatment. Conclusion: our results suggest that PDT cause damages on cell metabolism after generation of ROS that inhibit the activity of enzymes from biochemistry pathways, leading cell death.

**Keywords:** *Candida albicans,* photodynamic therapy.

REVISTA SAÚDE MULTIDISCIPLINAR
Revista Eletrônica Saúde Multidisciplinar da Faculdade Mineirense – Vol. III
ISSN 2318 – 3780

**RESUMO**

Introdução: Espécies de *Cândida* espécies podem causar danos superficiais à mucosa superficial, bem como infecções sistêmicas e é mais comumente encontrada em pacientes imunocomprometidos. A utilização de PDT tem se tornado promissora. Objetivos: Avaliar o efeito de doses PDT sobre o metabolismo bioenergético. Métodos: Uma suspensão de *C. albicans* com os seguintes grupos experimentais foram preparados: L+P+, expostos à irradiação LED na presença do fotossensibilizador; L+ P-, expostas apenas a irradiação LED; L-P +, tratados com apenas o fotossensibilizador ; L-P-, sem exposição a laser ou fotossensibilizador. Para a determinação da curva de crescimento, foi ajustada uma suspensão de *C. albicans*, e inoculadas em BHI. O crescimento foi monitorizado nos tempos 0, 5, 8, 10 e 12 horas. Para a medição da glicose utilizamos um kit glicose enzimática (Biotecnica). A formação do tubo do germe foi avaliada em câmara de Neubauer e expressa em percentagem. A condução de microcalorimetria também foi avaliada. Resultados: A aplicação de PDT 15, 30 e 60 j/cm$^2$ apresentou diferenças quando comparada com grupo controle em relação ao número de unidades formadoras de colônia. A cinética de crescimento foi afetada, assim como o consumo de glicose foi proporcional à capacidade do consumo celular, sendo afetada pelo tratamento com PDT. A Libertação de Energia foi reduzida de forma dose dependente. Conclusão: nossos resultados sugerem que PDT causam danos no metabolismo celular após geração de ROS que inibem a atividade de enzimas de vias bioquímicas, levando a morte celular.

**Palavras-chave:** *Candida albicans*, terapia fotodinâmica.

REVISTA SAÚDE MULTIDISCIPLINAR
Revista Eletrônica Saúde Multidisciplinar da Faculdade Mineirense – Vol. III
ISSN 2318 – 3780



## INTRODUCTION

*Candida* species are part of commensal microbiota in the oral cavity, also commonly found in the gastrointestinal tract, genital tract, and on human skin. *Candida albicans* is mostly isolated in the yeast form [1]. These species may cause injury to the superficial mucosa as well as systemic infections and is more commonly founded in immunocompromissed patients [2].

The indiscriminate use of antifungal drugs for HIV patients has favored the acquisition of resistance by pathogenic fungi, including *Candida* species [3]. Due to this increasing resistance to antifungal agents by *Candida albicans*, the potential of photodynamic therapy (PDT) has been widely studied [3, 4].

Several studies have reported that various microorganisms such as bacteria, viruses and fungi are destroyed by PDT, which is also used in cancer treatment [1, 3, 5]. PDT is a therapeutically modality that utilizes a combination of a sensitizing agent and a light source, in the presence of oxygen / nitrogen, which produces reactive species such as free radicals in the cell, causing cell damage and death [3].

One sensitizing agent widely used in various studies is Methylene blue [2, 6-8] is one dye widely used in histology, which belongs to phenothiazinium class [8]. This dye has a wavelength absorption band among 550—700 nm, presenting an optimal fluorescence quantum yield, besides some works suggests this dye for PDT [2, 6, 8].

The glycolysis is an important metabolic pathway that cleavage the glucose inside the cell and convert to sub products like ATP and compounds essentials for obtain energy to vital function of cell. ROS generated by PDT can bind with a lot of biomolecules inhibiting enzymes that act in metabolic pathways. Dosselli et al. [9] observed that in methicillin-resistant strain of *Staphylococcus aureus* after PDT, some photoxidative effects were observed decreasing or inhibiting the expression of proteins and enzymes. When enzymes/proteins are eradicated, damage in the cell can occur like: lipid peroxidation, inhibition of metabolism and consequently inhibition of glycolytic pathways. To measuring the produced energy as heat by the cell, the microcalorimeter can be used as an analytic technique for this study [10]. Here we propose to evaluate the effect of PDT doses over the bioenergetic metabolism.

REVISTA SAÚDE MULTIDISCIPLINAR
Revista Eletrônica Saúde Multidisciplinar da Faculdade Mineirense – Vol. III
ISSN 2318 – 3780



## METHODS

Microorganism preparation

A suspension of *C. albicans* (strain CEC 1293, provided by Biologie et Pathogenicité Fongigues Pasteur Institute, France) containing $10^7$ viable cells $mL^{-1}$ was prepared, inoculated onto Sabouraud dextrose agar (SDA; Difco, Detroit, MI, USA), and incubated for 24 h at 37°C. The growth was then transferred into brain heart infusion (BHI) broth (Difco) and incubated for 24 h at 37°C. The fungal culture was then centrifuged at 1,300 ×*g* for 10 min and the supernatant was discarded. This procedure was repeated and the pellet was suspended in 5 mL sterile water. The number of viable cells was determined using a spectrophotometer (B582, Micronal, São Paulo, Brazil) (λ 530 nm, OD = 0.680).

Using a standard *C. albicans* suspension ($10^7$ CFU/mL), the following experimental groups were prepared: L+P+, exposed to LED irradiation in the presence of the photosensitizer (n =10); L+P−, exposed to LED irradiation only (n =10); L−P+, treated with the photosensitizer only (n=10); L−P−, no exposure to Laser or photosensitizer (n=10).

### Photosensitizer and Light source

Methylene blue (MB) (Synth, São Paulo, Brazil) was used for sensitization of *C. albicans*. A stock solution of 2 mg $ml^{-1}$ was prepared The photosensitizer was prepared by dissolving the dye in distilled water, filtered through a sterile 0.22-μm Millipore membrane (Merck - Barueri - SP, Brazil). This stock solution was diluted 20-fold in the experiments

The light source used was a laser Coherent Cube (Coherent Corporate, Santa Clara, CA, USA) with wavelength of 660 nm linked to a computer with a software to regulate the parameters. The laser potency applied was 50 mW adjusted thought the software (Coherent Corporate). The fluency applied in the treatments was 15, 30 and 60 $J/cm^2$.

### Planktonic photosensitization of *C.albicans* with MB

As described above, 1.9 mL *C. albicans* suspension in a microtube (Eppendorf®) was used for each group and 100 μL methylene blue was added for groups L+P+ and L−P+, whereas 100 μL

distilled water was added for groups L+P− and L−P−. The tubes were incubated for 10 min at 37°C. Subsequently, groups L+P+ and L+P− were irradiated.

After exposure to irradiation, the suspensions were serially diluted in phosphate buffered saline (PBS) to give dilutions of $10^{-2}$ to $10^{-4}$ times from the original concentration. 20 µL of solution was added to the Sabouraud dextrose agar (SDA) medium in petri dishes. Each test consisted of ten replicates. After incubation in the dark at 24 hours at 37°C, the numbers of colony-forming units per milliliter were counted and the results were expressed in Log of CFU $ml^{-1}$.



**Evaluation of optical density and glucose uptake during the growth of *C. albicans***

For determination of growth curve, it was adjusted a suspension of *C. albicans* using a spectrophotometer (λ = 530 nm. O.D = 0.01), and inoculated in a BHI broth for 12 hours. The growth was monitored using a spectrophotometer (UV-1600 Angstron) in the times 0, 5, 8, 10 and 12 hours. For measurement of glucose, 100 µL of growth medium was removed at the times: 0, 5, 8, 10 and 12 hours, and it were frozen at -80°C to stop the fungal growth. The glucose quantification was performed was carried out using a enzymatic glucose kit (Biotecnica) adding 1 mL of reagent for glucose detection plus 10 µL of sample, and incubated for 15 min at 37°C.

After this, the samples were read in a spectrophotometer (λ = 505 nm). and the absorbance were determined and the absorbance were determined and the results were expressed in mg/dL.

**Germ tube formation**

We evaluated the ability of *C. albicans* to form germ tubes (GT) after PDT treatment. After exposure to PDT, a cell suspension of approximately $10^7$ CFU $mL^{-1}$ was incubated with FBS for 2 h at 37°C, after which 20 µL yeast suspension was placed in a Neubauer chamber. Germ tube formation was expressed as the percentage of cells forming germ tubes in a total of one hundred viable cells counted.

**Microcalorimetric assay**

The conduction microcalorimeter [11, 12] consisted of two glass vessels, each serving as a reference for the other. The vessels were 35 mm in diameter and 5 to 7 mm in thickness, located

between two Seebeck thermopiles. The reference vessel was connected opposite to the sample vessel. Each vessel had two chambers (5 mL in total). The smaller chamber held 0.5 to 1.0 mL, while the larger chamber could hold 1.0 to 2.0 mL sample. The instrument had a calorimetric constant close to 7 μW/μvolt output signal. The production of total heat was calculated starting from the integrated area of the curve. Amplifiers transformed signals of 1 μVolt into 7 μW. The calorimeter was placed in a constant temperature box under isothermal conditions at a temperature of 30°C ± 1°C(13, 14). Calibration was performed by neutralization of Tris (hydroxymethyl) aminomethane by HCl, with heat generation of (-) 47234 J/mole of neutralized H+[11]. The data were collected for 20 min and the vessels were washed with 1 M HCl once and 6 times with distilled water before the next assay.

In the control experiment, 1 mL yeast suspension was added to the larger chamber and 0.5 mL water with 6 mM glucose (Sigma®) (final concentration) was added to the smaller chamber in the conduction microcalorimeter. The other vessel contained 1 mL distilled water in the larger chamber and 0.5 mL water with 6 mM glucose (Sigma®) (final concentration) in the smaller chamber.

Total heat production was calculated starting from the integrated area of the curve. The result was expressed in time versus value of emitted tension. Regression curves obtained from the experiments with yeast and from calibration of the calorimeter were calculated using the software Origin® version 7.0 (Originlab – Scientific Graphing and Analysis Software). In order to avoid the different energy yield results from different viable cell number, all the samples was counted and normalized previously.



## RESULTS

### Planktonic photosensitization of *C.albicans* with MB

In order to determine the lethal dose of light for a better efficacy of PDT-MB mediated, 15, 30 and 60 J/cm$^2$ of light were applied. MB did not show toxicity for yeast cells after incubation for 10 min in the dark (data no show), and the groups that were applied PDT 15 and 30 j/cm$^2$ did not show significative difference ($P$= 0.4961), but when compared all groups with the control (L-P-) signficative difference were observed ($P$ <0.0001).

REVISTA SAÚDE MULTIDISCIPLINAR
Revista Eletrônica Saúde Multidisciplinar da Faculdade Mineirense – Vol. III
ISSN 2318 – 3780

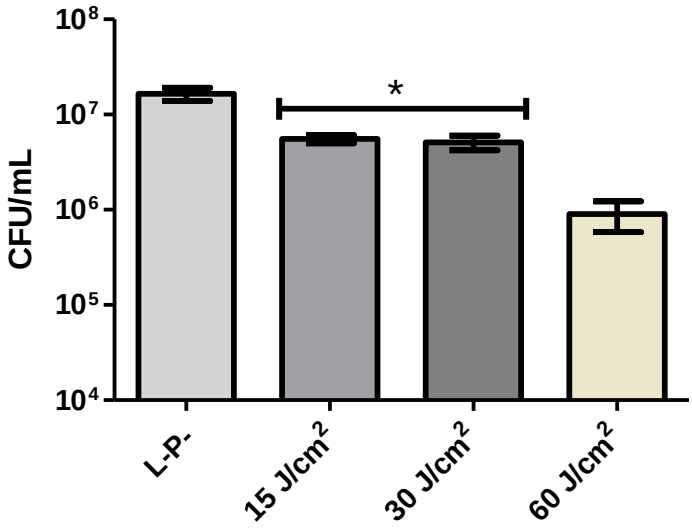


Figure 1. Colony forming unity per milliliter (CFU $mL^{-1}$) of yeast after PDT exposure with the energy doses, 15, 30 and 60 $J/cm^2$ and control group (L-P-). All groups have significant difference when compared with the control group (L-P-) ($P$<0.0001). (*) No significant difference was observed among them ($P$= 0.4961). Error bars are standard deviations of three independent experiments.

**PDT delays *C. albicans* growth and glucose up take**

After an exposure of PDT-MB mediated, growth kinetics of *C.albicans* and glucose up take was analyzed. Analysing the growth kinetics, it is possible to observed that *C.albicans* exposed to 15, 30 and 60 $J/cm^2$ of PDT-MB mediated remained in the lag phase longer, and this alteration was time dependent when compared with the control group (L-P-) (Fig.2-A). The glucose up take is directly related to growth kinetics, and data presented at (Fig.2-B) it is possible to observed that glucose up take levels of groups irradiated with 30 and 60 $J/cm^2$ were less than the control group (L-P-)(51.68 and 67.90 mg/dL respectively) may this effect caused by a dose-dependent effect. In the group irradiated with 15 $j/cm^2$ it was measured a glucose concentration of 12.83 mg/dL.

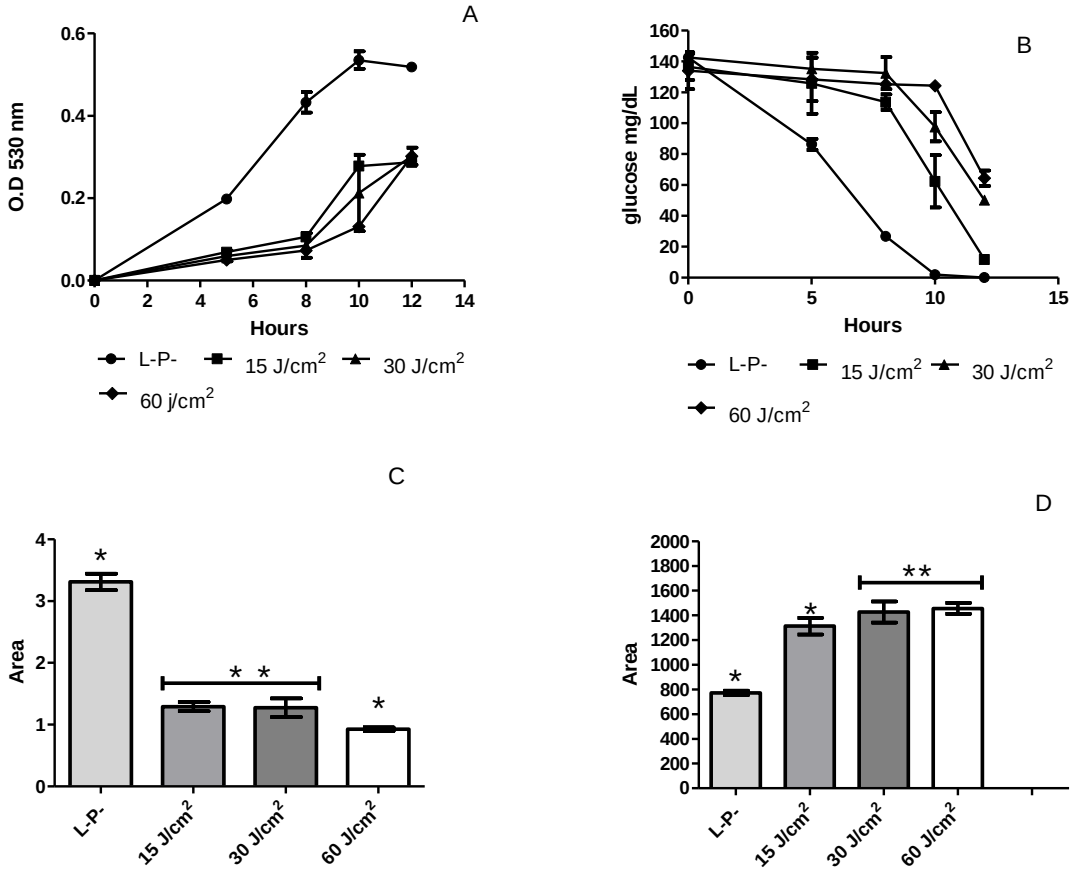


**Figure 2.** Graph A represents a growth kinetic of *C.albicans* after PDT exposure with the energy doses, 15, 30 and 60 J/cm$^2$ and control group (L-P-). All groups tested showed significant difference when compared with the control group (L-P-) (*P*= 0.0003). Graph B represent glucose up take during the growth kinetic after PDT exposure with the energy doses, 15, 30 and 60 J/cm$^2$ and control group (L-P-). For 30 and 60 J/cm$^2$, a significative difference was observed when compared with the control group (L-P-) (*P*= 0.0003).Graph C represent normalized areas under the curves of growth kinetic of *C.albicans.* Graph D represent normalized areas under the curves of glucose up take during the growth kinetic after PDT treatment. Symbols represent statistically significant differences compared to the other groups. (*) represent significant difference (P < 0.05). (**) Represent no significant difference (*P* > 0.05). Error bars are standard deviations of three independent experiments.

## Evaluation of heat produced by *C.albicans* after a PDT-MB mediated thought Microcalorimetry

Calibration was performed by neutralization of Tris (hydroxymethyl) aminomethane by HCl with heat generation of (-) 47234 J/mole of neutralized H+(13). Standard calibration plots were described as follows: A = 1.016 × q + 0.0355, where, A is the integrated area under the output envelope, and q is the amount of heat released. These results were also previously reported [12, 16], wherein calibration was performed by neutralization of Tris by HCl, with heat generation of (-) 47234 J/mole of neutralized $H^+$. In order to determine energy produced by *C. albicans* after a PDT

treatment, it was mensured the heat released by *C. albicans* through glucose consumption using a microcalorimeter. The energy production is according to glycolysis pathway that release energy after glucose broken. In our work we measure this energy using a microcalorimeter that measure energy of cell production in the scale of milijoule Here we evaluated the groups P+L+ (15 ,30 and 60 J/cm$^2$) and P-L- after a PDT treatment.

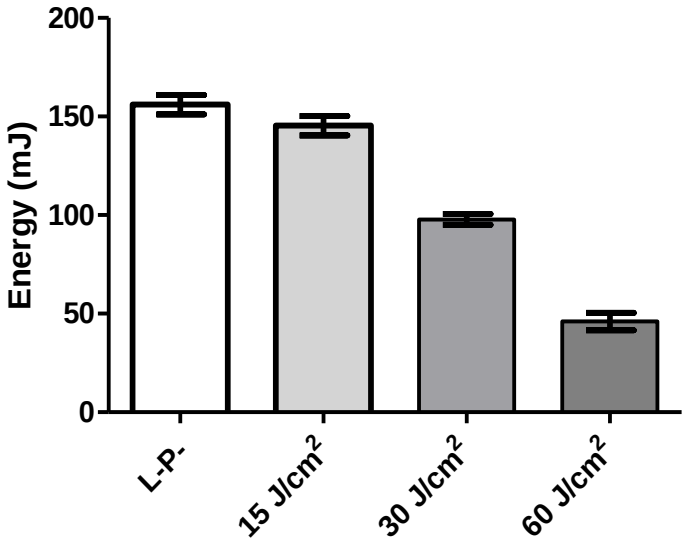


**Figure 3.** Heat response to glucose addiction after PDT exposure with the energy doses, 15, 30 and 60 J/cm$^2$ and control group (L-P-). The groups 15, 30 and 60 J/cm$^2$ and L-P- were analyzed by production of total heat during 3200 seconds. Error bars are standard deviations of three independent experiments.

**Inhibition of germ tube formation after PDT treatment**

The ability of *C.albicans* to form GT decrease after a PDT-MB mediated exposure. Light doses of 15, 30 and 60 J/cm$^2$, showed a difference on germ tube formation, indicating a dose-response effect. Significative differences was observed among the groups when compared with the control group (L-P+) ($P<0.0001$).

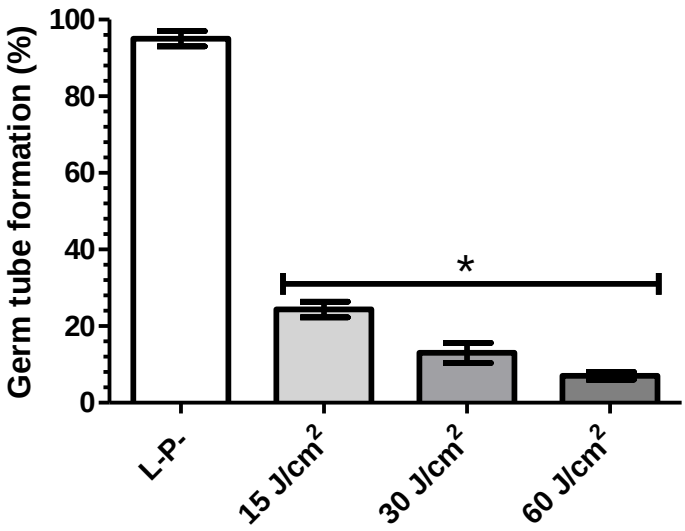


**Figure 4**. Effect after PDT exposure with the energy doses, 15, 30 and 60 J/cm$^2$ and control group (L-P-) over inactivation of GT formation expressed in percentage. (*) represent significant differences compared to the control group (L-P-) ($P$<0.0001). Error bars are standard deviations of three independent experiments.

Revista Saúde Multidisciplinar Vol. III – agosto à dezembro de 2015 / Faculdade Mineirense

## DISCUSSION

The increase in fungal infections and resistance to antifungal drugs in addition to the rise in drug-resistant strains has led to the investigation of new antifungal approaches by several research groups [1, 3]. Photodynamic therapy has emerged a new modality that is potentially effective against resistant microorganisms [3].

The effect of PDT over *C.albicans* with energy-dose of 15, 30 and 60 J/cm$^2$ reduced the number log of cells. Our result is agreement with various studies that also showed reduction of cells after PDT inactivation [1, 2, 15, 16]. In our study we observed that the energy dose of 60 J/cm$^2$ presented 1 log reduction of cells and it was the best lethal – dose. The formation of ROS after a light exposure promotes damages on cell, when $^{-}$OH is produced, this type of ROS binds with a wide range of molecules inhibiting the biological effect of them. Another type of ROS is $H_2O_2$ that may also cause peroxidation in the lipids of cell wall [3]. But low doses of light (15 and 30 J/cm$^2$) did not show significative difference with the control group, and this may be associated with rescue responses of cell to oxidative stress [17].

In the growth kinetics of *C.albicans* we observed that after PDT exposure, the cell growth was different from the control group, as observed in the figure 2, graph C, the area of the curve of energy dose of 60 J/cm$^2$ was lower when compared with L-P- group. After PDT, cells presents

lesions in the metabolism due to ROS formation, and for some energy – doses, rescue mechanism decrease their activity, making the cells reduce the growth speed [1].

This fungistatic effect can be observed thought the observation of glucose levels, and calorimetric rates. These biochemistry parameters indicate how the cell metabolism is working [18]. Glucose levels is correlated with glycolysis, a biochemistry pathway that converts glucose in energy to the cell [18]. In our study we observed that a significate reduction on the glucose levels, and the measurement of total heat produced by the cell using a calorimeter showed that energy production has been compromised. This data suggest that the PDT cause damage on the enzymes that are part of biochemistry pathway inhibiting the glycolysis and subsequently the energy production.

An study performed by Souza *et al* [19] showed that after a PDT treatment there was a significant reduction of germ tube formation. Germ tube formation is the transition phase of *C. albicans* from yeast to filamentous form, this transition is an important virulence factor to be analysed once, the formation of the germ tube is the initial phase of its accession in the tissue [20]. In our study we observe similar results to Souza *et al* [19], were the author suggest that the decrease on the germ tube formation may be associated to phototoxic responses after the PDT.

In conclusion, our results suggest that PDT cause damages on cell metabolism after generation of ROS that inhibit the activity of enzymes from biochemistry pathways, leading cell death.

**Acknowledgments**

The authors thank CEFORES/UFTM for financial support, and Murielle Chauvel from Unité Biologie et Pathogénicité Fongiques, Institut Pasteur France.

**REFERENCES**

1. Kato IT, Prates RA, Sabino CP, Fuchs BB, Tegos GP, Mylonakis E, et al. Antimicrobial photodynamic inactivation inhibits Candida albicans virulence factors and reduces in vivo pathogenicity. Antimicrobial agents and chemotherapy. 2013;57(1):445-51.

2. Souza RC, Junqueira JC, Rossoni RD, Pereira CA, Munin E, Jorge AO. Comparison of the photodynamic fungicidal efficacy of methylene blue, toluidine blue, malachite green and low-power laser irradiation alone against Candida albicans. Lasers in medical science. 2010;25(3):385-9.

3. Dai T, Fuchs BB, Coleman JJ, Prates RA, Astrakas C, Denis TGS, et al. Concepts and principles of photodynamic therapy as an alternative antifungal discovery platform. Frontiers in microbiology. 2012;3.

4. So CW, Tsang PWK, Lo PC, Seneviratne CJ, Samaranayake LP, Fong WP. Photodynamic inactivation of Candida albicans by BAM-SiPc. Mycoses. 2010;53(3):215-20.

5. Paulino TP, Ribeiro KF, Thedei Jr G, Tedesco AC, Ciancaglini P. Use of hand held photopolymerizer to photoinactivate< i> Streptococcus mutans</i>. Archives of Oral Biology. 2005;50(3):353-9.

6. Prates RA, Kato IT, Ribeiro MS, Tegos GP, Hamblin MR. Influence of multidrug efflux systems on methylene blue-mediated photodynamic inactivation of Candida albicans. Journal of antimicrobial chemotherapy. 2011;66(7):1525-32.

7. Carvalho GG, Felipe MP, Costa MS. The photodynamic effect of methylene blue and toluidine blue on Candida albicans is dependent on medium conditions. The Journal of Microbiology. 2009;47(5):619-23.

8. Teichert MC, Jones JW, Usacheva MN, Biel MA. Treatment of oral candidiasis with methylene blue-mediated photodynamic therapy in an immunodeficient murine model. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2002;93(2):155-60.

9. Dosselli R, Millioni R, Puricelli L, Tessari P, Arrigoni G, Franchin C, et al. Molecular targets of antimicrobial photodynamic therapy identified by a proteomic approach. Journal of proteomics. 2012;77:329-43.

10. Kong WJ, Zhao YL, Xiao XH, Li ZL, Jin C, Li HB. Investigation of the anti-fungal activity of coptisine on Candida albicans growth by microcalorimetry combined with principal component analysis. Journal of applied microbiology. 2009;107(4):1072-80.

11. Mares-Guia M, do Nascimento VV, Lovrien R, Melo MN. Microcalorimetric determination of glucose utilization by Leishmania. Thermochimica acta. 1990;172:203-11.

12. Kemp R. Calorimetric studies of heat flux in animal cells. Thermochimica acta. 1991;193:253-67.

13. Irving R, Wadsö I. Use of tris (hydroxymethyl) aminomethane as a test substance in reaction calorimetry. Acta Chem Scand. 1964;18:195-201.

14. Inskeep PB, Hammerstedt RH. A calorimetric method to assess endogenous metabolism and its application to the study of bovine sperm. Journal of Biochemical and Biophysical Methods. 1983;7(3):199-210.

15. Pereira Gonzales F, Maisch T. Photodynamic inactivation for controlling< i> Candida albicans</i> infections. Fungal biology. 2012;116(1):1-10.

16. Khan MSA, Ahmad I, Aqil F, Owais M, Shahid M, Musarrat J. Virulence and Pathogenicity of Fungal Pathogens with Special Reference to Candida albicans. Combating Fungal Infections: Problems and Remedy. 2010:21.

17. Mittler R. Oxidative stress, antioxidants and stress tolerance. Trends in plant science. 2002;7(9):405-10.

18. Lehninger A. Glicólise: a via central do catabolismo da glicose. Princípios de bioquímica São Paulo: Sarvier. 1984:288-310.

19. Munin E, Giroldo LM, Alves LP, Costa MS. Study of germ tube formation by< i> Candida albicans</i> after photodynamic antimicrobial chemotherapy (PACT). Journal of Photochemistry and Photobiology B: Biology. 2007;88(1):16-20.

20. Calderone RA, Fonzi WA. Virulence factors of< i> Candida albicans</i>. Trends in microbiology. 2001;9(7):327-35.