Oxidative stress of photodynamic antimicrobial chemotherapy inhibits *Candida albicans* virulence

Ilka Tiemy Kato[1*], Renato Araujo Prates[1], George P. Tegos[2,3,4,], Michael R. Hamblin [3,4,5], Martha Simões Ribeiro[1]

[1] Center for Lasers and Applications, IPEN-CNEN/SP, Av. Prof. Lineu Prestes, 2242 - Cidade Universitária - São Paulo - SP - 05508-000 – Brazil

[2] Department of Pathology School of Medicine & Center for Molecular Discovery, University of New Mexico UNM Health Sciences Center, 2325 Camino de Salud MSC - 074025 Albuquerque, NM – 87131 – USA

[3] Wellman Center for Photomedicine, Massachusetts General Hospital, 40 Blossom Street - 02114, Boston, Massachusetts, USA

[4] Department of Dermatology, Harvard Medical School, Boston, Massachusetts, USA

[5] Harvard-MIT Division of Health Sciences and Technology, Cambridge, Massachusetts, USA

ABSTRACT

Photodynamic antimicrobial chemotherapy (PACT) is based on the principal that microorganisms will be inactivated using a light source combined to a photosensitizing agent in the presence of oxygen. Oxidative damage of cell components occurs by the action of reactive oxygen species leading to cell death for microbial species. It has been demonstrated that PACT is highly efficient *in vitro* against a wide range of pathogens, however, there is limited information for its *in vivo* potential. In addition, it has been demonstrated that sublethal photodynamic inactivation may alter the virulence determinants of microorganisms. In this study, we explored the effect of sublethal photodynamic inactivation to the virulence factors of *Candida albicans*. Methylene Blue (MB) was used as photosensitizer for sublethal photodynamic challenge on *C. albicans* associated with a diode laser irradiation (λ=660nm). The parameters of irradiation were selected in causing no reduction of viable cells. The potential effects of PACT on virulence determinants of *C. albicans* cells were investigated by analysis of germ tube formation and *in vivo* pathogenicity assays. Systemic infection was induced in mice by the injection of fungal suspension in the lateral caudal vein. *C. albicans* exposed to sublethal photodynamic inactivation formed significantly less germ tube than untreated cells. In addition, mice infected with *C. albicans* submitted to sublethal PACT survived for a longer period of time than mice infected with untreated cells. The oxidative damage promoted by sublethal photodynamic inactivation inhibited virulence determinants and reduced *in vivo* pathogenicity of *C. albicans*.

Keywords: Methylene blue, photodynamic therapy, germ tube, systemic candidiasis, mice, pathogenicity

1. INTRODUCTION

Antifungal therapy is a challenging problem in management of infections caused by clinically relevant fungal pathogens due to a high incidence of resistance developed during therapy, specially in immunocompromised individuals[1]. *Candida albicans* is a commensal fungi of the human gastrointestinal and genitourinary tract that frequently causes superficial infections of mucosa and skin[2, 3]. The infection depends on imbalances between *Candida albicans* virulence attributes and impaired host defense. In immunocompromised induviduals, however, *C. albicans* may invade deeper tissues, penetrate the blood vessels, and cause life-threatening systemic infections[3, 4].

* itkato@usp.br, phone + 55 11 3133-9197, fax + 55 11 3132-9374

Mechanisms for Low-Light Therapy VI, edited by Michael R. Hamblin, Ronald W. Waynant, Juanita Anders, Proc. of SPIE Vol. 7887, 78870B · © 2011 SPIE · CCC code: 1605-7422/11/$18 · doi: 10.1117/12.876598

Photodynamic antimicrobial chemotherapy (PACT) combines a nontoxic photosensitizer (PS) with a harmless light of ressonant wavelenght to generate reactive oxygen species (ROS) that are toxic to microrganisms. The high killing effect has placed this therapy as a promising alternative treatment for localized infections[5]. Although the production of reactive oxygen species from PDT has been implicated in changes of virulence determinants expression in yeasts [6, 7], the impact of this therapy on survival microorganisms, as well as the consequences of these alterations on infection is uncertain.

The photodynamic effect depends on photosensitizer type and concentration, as well as the irradiation parameters applied on cell suspension or biofilm[6, 8, 9]. Literature reports an efficient inactivation on yeast and bacteria following light delivered over the cells[8, 10, 11]. However, the cell death is achieved just after some time of irradiation. Thus, it is plausible that an infected tissue could not receive an homogeneous irradiation *in vivo* and some target cells may survive after treatment. The damaged cells that survival from oxidative stress caused by PACT may present differences in their behavior in adhesion [7] and germ tube expression[6]. This responsive differences is caused by the massive toxicity produced by reactive oxygen species generated by photodynamic action [6, 7]. On the other hand, some types of photosensitizers are able to penetrate the cell and it can bind to cytoplasm components and nuclear material. Methylene blue (MB) has been widely studied as a photosensitizer and it has an affinity to guanine base of deoxyribonucleic acid (DNA)[12]. Consequently, generation of ROS activity nearby DNA may occur and it can induce mutations in a random form, since ROS can interact with organic compounds.

In this study, we evaluated if *Candida albicans* exposed to sublethal conditions of photodynamic inactivation may exhibit altered virulence characteristics that could change an infection process.

2. MATERIALS AND METHODS

This study was composed by evaluation of irradiation parameters for sublethal MB-mediated PACT of *C. albicans*, analysis of germ tube formation, and *in vivo* pathogenicity of *C. albicans* exposed to sublethal PACT.

2.1 Inoculum preparation

Candida albicans ATCC 90028 were sub-cultured from vial stock onto Sabouraud dextrose agar in aerobic conditions for 48h at 37°C. Turbidity of cell suspension was measured in an optical spectrophotometer in order to obtain suspensions of approximately $2x10^7$ cfu mL^{-1} (optical density of 0.8 at 540nm).

2.2 Irradiation source and photosensitizer

The solution of methylene blue (MB) was prepared by the dissolution of the powder (Sigma Ltd, Poole, UK) in distillate water in a concentration of 10mM, which was filtered through a sterile filter membrane (0.22 μm, Millipore, São Paulo, Brazil). This photosensitizer was added to the yeast suspension in proportion of 1/100 that resulted a final concentration of 100μM [8].

A diode laser (Photon Lase III, DMC, São Carlos, Brazil) with wavelength of 660nm and output power of 30-100 mW was used in this study. The laser probe was fixed on a holder keeping the beam spot size with the same diameter of the well from a 96-well microtiter plates (0.3 cm^2).

2.3 Sublethal conditions of PACT

Preliminar tests were conducted to determine the killing curve of MB-mediated PACT of *C. albicans* (table). A sublethal condition of irradiation that caused no reduction of the viable cells was used to investigate the effects of sublethal PACT on germ tube formation and *in vivo* pathogenicity.

C. albicans cells were incubated with 100μM MB for 10 min at room temperature and in the dark. Aliquots were placed in wells of a microtiter plate and then irradiated with paramenters decribed in Table 1. Yeast suspensions were serially diluted in PBS to give dilutions of 10^{-1} to 10^{-5} times the original concentration. Ten-μL aliquots of each dilution were streaked onto Sabouraud agar plate in triplicate and incubated at 37°C overnight [13]. The yeast colonies were counted and converted into cfu mL^{-1} for analysis. Two types of control conditions were used: without PS and irradiation, and with PS in the dark.

Table 1. Parameters of irradiation

Power (mW)	Fluence rate (mW/cm^2)	Area (cm^2)	Time (min)	Fluence (J/cm^2)
			2	12
30	100	0.3	4	24
			6	36

2.4 Germ tube formation

After sublethal PACT, yeast cells were incubated with 10% bovine fetal calf serum at concentration of approximately 10^5-10^6 cells mL^{-1} for 3 hours at 37°C [14]. After this period, 5 μL of the yeast suspension was placed on a microscope slide and then covered with a coverslip. The number of germ tube (GT) was determined by examining 100 yeast cells under a light microscope and percentage of GT formation was obtained. A control group with cells untreated either by irradiation or PS was also used. Three independent experiments were conducted with four analysis in each one.

2.5 *In vivo* pathogenicity assay

A mice model of hematogenously disseminated candidiasis was used to investigate the pathogenesis alterations caused by PACT. Fourteen female BALB/c with 9-11 weeks of age were injected in the lateral caudal vein with 0.1mL of *C. albicans* suspension, containg $2x10^6$ cells [15]. Animals from PACT group were infected with *C. albicans* pre-treated with sublethal PACT, and mice from control group were inoculated with cells untreated either by irradiation or PS. Animal survival was evaluated every day[16].

2.6 Statistics

Mean and standard deviation values were obtained for percentage of germ tube formation data. The difference between two means was compared by two-tailed unpaired t-test with significance level of 5%.

3. RESULTS AND DISCUSSION

In order to determine the parameters of irradiation for sublethal MB-mediated PACT, three different conditions of irradiation were evaluated. A hundred-μM MB did not show any toxicity on yeast cells after incubation for 10 minutes. The mean cfu/mL of *C. albicans* treated only with MB (7.25±0.17 log) did not present a significant difference ($p>0.05$) compared to the control cells (7.29±0.16 log). After two min of irradiation, no reduction of viable cells was observed, whereas 4 min and 6 min of irradiation produced less than 1 $\log_{10}$ of killing (Figure 1).

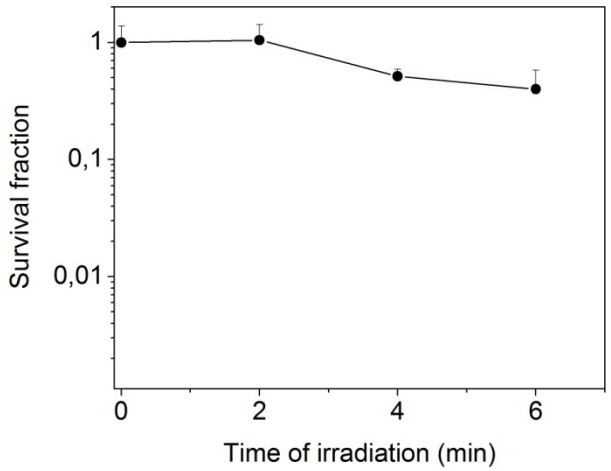

Figure 1. Reduction of viable *C. albicans* after MB-mediated PACT with different parameters of irradiation. Data represents mean value and bars are standard deviation. There were no reduction of viable cells after two min of irradiation. MB-mediated PACT with 4 min and 6 min of irradiation reduced 50% and 60% the number of viable cells

Methylene blue is frequently employed class of antimicrobial PS that has been largely used in association with a red light (λ=660nm) to kill a broad range of pathogens, including *C.albicans*. Different phototoxic efficiency has been reported according to MB concentration and irradiation parameters[6, 8, 17]. In the present study, we observed a lower killing effect than previously described by Prates *et al.*[8] due to the higher cell density used [10]. Part of the light applied to *C. albicans* suspension is scattered by the cells, which reduces light transmission through microbial suspension. Thus, in a inoculum with a higher cell density, less fotons are available to be absorbed by the photosensitizer, reducing the killing effect of PACT.

After determination of parameters of irradiation for sublethal PACT, we analysed germ tube formation in order to verify whether sublethal PACT induced alterations in *C. albicans* virulence. Yeasts were incubated with MB followed by irradiation with parameters that causes no photoinactivation of cells (100 mW/cm^2 for 2 min). The ability of *C. albicans* to form germ tube significantly decreased after exposition to sublethal PACT ($p<0.001$). In control group, 32% of the cells were able to form germ tube, while in PACT group there were only 15% of germ tube formation (Figure 2).

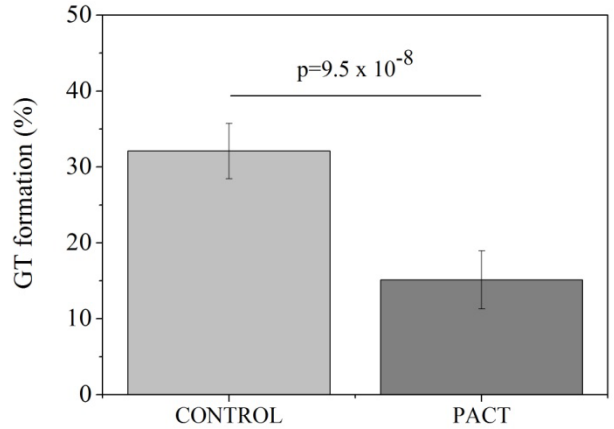

Figure 2. Effect of photodynamic antimicrobial chemotherapy on germ tube formation. Cells from PACT group were treated with sublethal MB-mediated PACT before incubation in medium to induce germ tube formation. Cells from control group were not treated either by MB or light. Column represents the mean percentage of GT formed in each group and error bar is standard deviation. There was a significant difference between groups ($p<0.05$)

C. albicans is a microorganism that has the ability to switch between distinct forms in response to external stimuli[18]. This ability has been considered a virulence attribute of this fungus, i.e. a trait of *C. albicans* that are needed to interact directly with the host and cause disease[19]. Among morphogenetic forms of *C. albicans*, germ tube is the initial elongating structure formed during the yeast-hyphal transition that is important to infect[20], as well as to evade or escape phagocytic cells[21]. In addition, germ tube is the dominant growth form of *C. albicans* in plasma[22]. The development of germ tube was reported to be inhibited as a response to oxidative stress caused by exposure of *C. albicans* to immune system cells [22]. The inhibition of germ tube formation observed in our study showed that the reactive oxygen species generated by sublethal MB-mediated PACT also produced injuries that altered this cell function.

Since sublethal PACT affected the ability of *C. albicans* to form germ tube, we tested whether the alterations caused by PACT could affect the pathogenicity of this microorganism. We used a mice model of systemic candidiasis induced by intravenous inoculation of *C. albicans*, which is very useful tool to study pathogenesis[23, 24]. The infection with *C. albicans* pre-treated with PACT was less aggressive than infection with untreated cells. Mice from PACT group started to die 3 days postinoculation, while all animals from control group died until this date (Figure 3). This data showed that the alterations caused by the oxidative stress of PACT diminished *C. albicans* pathogenitiy and slowed the infection progress. However, all mice from PACT group died within 10 days, showing that *C. albicans* cells injuried by sublethal PACT were able to develop the infection.

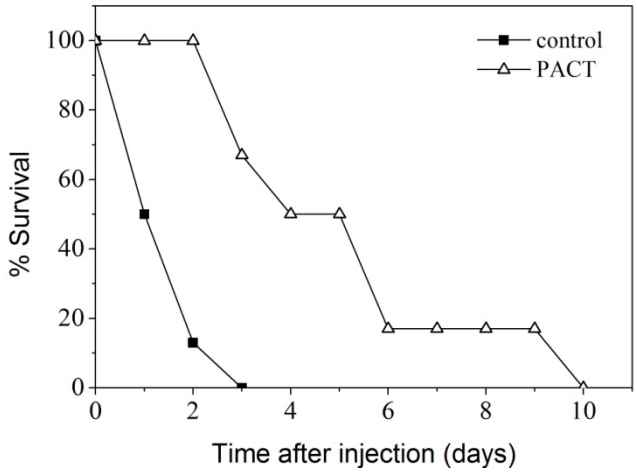

Figure 3. Survival curves for mice systemically infected with *C. albicans*. Mice infected with cells pre-treated with sublethal PACT survived for a longer period of time than mice infected with untreated cells

After hematogenous inoculation of *C. albicans*, the development of disseminated candidiasis dependends on survival of microorganisms in bloodstream, growth, escape from bloodstream and infection of organs. This fungus uses different attributes to escape from the bloodstream and to penetrate endothelial layers and their surrounding tissues[22]. Hypha formation and hyphal-associated factors can help the fungus to escape from macrophages after phagocytosis[25, 26], to inhibit killing by neutrophils[22], to adhere to endothelial cells[27], to induce phagocytosis by endothelial cells in order to escape from the bloodstream[27], and to invade tissue[28]. When *C. albicans* is exposed to blood, the transition from yeast to hyphal cells rapidly initiates[29], helping this microorganism to survive in a such hostile enviroment. We observed that sublethal PACT reduced the ability of *C. albicans* to form germ tube, which affected the capability of this microorganism to escape from bloodstream and also favored host defenses, making the fungal cells more succeptible to be killed by immune cells. These events can explain the less aggressive infection caused by *C. albicans* pre-exposed to PACT. Furthermore, *C. albicans* damaged by ROS could also present a decrease in cellular growth rates[30] and inhibition of adhesion[31], which might also affected the infection.

Although PACT showed to induce cellular alterations that reduced *C. albicans* pathogenitiy, all mice from PACT group died whithin 10 days. This observation demonstrated that the damage induced by PACT in *C. albicans* cells did not

impede the establishment and progression of the infection in our model. After inoculation, the fungal cells pre-treated with PACT that survived the challenge of mice immune system probably recovered from injuries caused by ROS, becaming able to cause disease. One point of our methodology should be noted; mice were infected with an acutely lethal inocula of *C. albicans* that killed all animals from control group within 3 days. This aggressive infection was used to avoid massive killing of fungal cells by immune system, which allowed us to verify the ability of *C. albicans* to recovery from damage caused by PACT in a challenging environment as bloodstream.

Even though ROS is one of the major causes of DNA damage and mutations, fungal cells have evolved several repair mechanisms to counteract oxidative DNA damage [26]. Furthermore, studies fail to demonstrate that microorganisms exposed to PACT might express characteristics of genotoxicity promoted by PACT [32-34].

In summary, our data showed that oxidative damage caused by PACT in *C. albicans* inhibited an important virulence determinant, the ability to form germ tube, and reduced *in vivo* pathogenicity of this microorganism. The results of survival after hematogenously candidiasis suggest that the alterations caused by oxidative stress generated by sublethal PACT might be transitory.

ACKNOWLEDGMENTS

The authors thanks to Supporting Foundation for the Research of São Paulo State/Brazil (FAPESP) and Brazilian National Commission on Research (CNPq) for financial support.

REFERENCES

[1] Cowen, L. E., Anderson, J. B. and Kohn, L. M., "Evolution of drug resistance in Candida albicans," Annu. Rev. Microbiol. 56, 139-165 (2002).
[2] Pfaller, M. A., Pappas, P. G. and Wingard, J. R., "Invasive Fungal Pathogens: Current Epidemiological Trends," Clin. Infect. Dis. 43(S1), S3–14 (2006).
[3] Gualco, L., Debbia, E. A., Bandettini, R., Pescetto, L., Cavallero, A., Ossi, M. C., Schito, A. M. and Marchese, A., "Antifungal resistance in Candida spp. isolated in Italy between 2002 and 2005 from children and adults," Int. J. Antimicrob. Agents. 29(2), 179-184 (2007).
[4] Pfaller, M. A., Diekema, D. J., Jones, R. N., Messer, S. A. and Hollis, R. J., "Trends in antifungal susceptibility of Candida spp. isolated from pediatric and adult patients with bloodstream infections: SENTRY Antimicrobial Surveillance Program, 1997 to 2000," J. Clin. Microbiol. 40(3), 852-856 (2002).
[5] Hamblin, M. R. and Hasan, T., "Photodynamic therapy: a new antimicrobial approach to infectious disease?," Photochem. Photobiol. Sci. 3(5), 436-450 (2004).
[6] Munin, E., Giroldo, L. M., Alves, L. P. and Costa, M. S., "Study of germ tube formation by Candida albicans after photodynamic antimicrobial chemotherapy (PACT)," J. Photochem. Photobiol. B 88(1), 16-20 (2007).
[7] Soares, B. M., da Silva, D. L., Sousa, G. R., Amorim, J. C., de Resende, M. A., Pinotti, M. and Cisalpino, P. S., "In vitro photodynamic inactivation of Candida spp. growth and adhesion to buccal epithelial cells," J Photochem Photobiol B 94(1), 65-70 (2009).
[8] Prates, R. A., da Silva, E. G., Yamada, A. M., Suzuki, L. C., Paula, C. R. and Ribeiro, M. S., "Light parameters influence cell viability in antifungal photodynamic therapy in a fluence and rate fluence-dependent manner," Laser Phys. 19(5), 1038-1044 (2009).
[9] Suzuki, L. C., Prates, R. A., Raele, M. P., Freitas, A. Z. and Ribeiro, M. S., "Real time optical coherence tomography monitoring of Candida albicans biofilm in vitro during photodynamic treatment," Proc. SPIE 7715, 7715241- 7715245 (2010).
[10] Demidova, T. N. and Hamblin, M. R., "Effect of cell-photosensitizer binding and cell density on microbial photoinactivation," Antimicrob. Agents Chemother. 49(6), 2329-2335 (2005).
[11] Tegos, G. P. and Hamblin, M. R., "Phenothiazinium antimicrobial photosensitizers are substrates of bacterial multidrug resistance pumps," Antimicrob. Agents Chemother. 50(1), 196-203 (2006).
[12] Lin, X. H., Wu, P., Chen, W., Zhang, Y. F. and Xia, X. H., "Electrochemical DNA biosensor for the detection of short DNA species of Chronic Myelogenous Leukemia by using methylene blue," Talanta 72(2), 468-471 (2007).

[13] Jett, B. D., Hatter, K. L., Huycke, M. M. and Gilmore, M. S., "Simplified agar plate method for quantifying viable bacteria," Biotechniques 23(4), 648-650 (1997).
[14] Mackenzie, D. W., "Serum tube identification of Candida albicans," J. Clin. Pathol. 15(6), 563-565 (1962).
[15] Navarathna, D. H., Hornby, J. M., Krishnan, N., Parkhurst, A., Duhamel, G. E. and Nickerson, K. W., "Effect of farnesol on a mouse model of systemic candidiasis, determined by use of a DPP3 knockout mutant of Candida albicans," Infect. Immun. 75(4), 1609-1618 (2007).
[16] Maidan, M. M., De Rop, L., Relloso, M., Diez-Orejas, R., Thevelein, J. M. and Van Dijck, P., "Combined inactivation of the Candida albicans GPR1 and TPS2 genes results in avirulence in a mouse model for systemic infection," Infect. Immun. 76(4), 1686-1694 (2008).
[17] de Souza, S. C., Junqueira, J. C., Balducci, I., Koga-Ito, C. Y., Munin, E. and Jorge, A. O., "Photosensitization of different Candida species by low power laser light," J. Photochem. Photobiol. B 83(1), 34-38 (2006).
[18] Whiteway, M. and Bachewich, C., "Morphogenesis in Candida albicans," Annu. Rev. Microbiol. 61, 529-553 (2007).
[19] Calderone, R. A. and Fonzi, W. A., "Virulence factors of Candida albicans," Trends Microbiol. 9(7), 327-335 (2001).
[20] Brown, A. J. and Gow, N. A., "Regulatory networks controlling Candida albicans morphogenesis," Trends Microbiol. 7(8), 333-338 (1999).
[21] Ghosh, S., Navarathna, D. H., Roberts, D. D., Cooper, J. T., Atkin, A. L., Petro, T. M. and Nickerson, K. W., "Arginine-induced germ tube formation in Candida albicans is essential for escape from murine macrophage line RAW 264.7," Infect. Immun. 77(4), 1596-1605 (2009).
[22] Fradin, C., De Groot, P., MacCallum, D., Schaller, M., Klis, F., Odds, F. C. and Hube, B., "Granulocytes govern the transcriptional response, morphology and proliferation of Candida albicans in human blood," Mol. Microbiol. 56(2), 397-415 (2005).
[23] Spellberg, B., Ibrahim, A. S., Edwards, J. E., Jr. and Filler, S. G., "Mice with disseminated candidiasis die of progressive sepsis," J. Infect. Dis. 192(2), 336-343 (2005).
[24] de Repentigny, L., "Animal models in the analysis of Candida host-pathogen interactions," Curr. Opin. Microbiol. 7(4), 324-329 (2004).
[25] Borg-von Zepelin, M., Beggah, S., Boggian, K., Sanglard, D. and Monod, M., "The expression of the secreted aspartyl proteinases Sap4 to Sap6 from Candida albicans in murine macrophages," Mol. Microbiol. 28(3), 543-554 (1998).
[26] Lorenz, M. C., Bender, J. A. and Fink, G. R., "Transcriptional response of Candida albicans upon internalization by macrophages," Eukaryot. Cell 3(5), 1076-1087 (2004).
[27] Filler, S. G. and Sheppard, D. C., "Fungal invasion of normally non-phagocytic host cells," PLoS Pathog. 2(12), 1099-1105 (2006).
[28] Felk, A., Kretschmar, M., Albrecht, A., Schaller, M., Beinhauer, S., Nichterlein, T., Sanglard, D., Korting, H. C., Schafer, W. and Hube, B., "Candida albicans hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs," Infect. Immun. 70(7), 3689-3700 (2002).
[29] Ernst, J. F., "Transcription factors in Candida albicans - environmental control of morphogenesis," Microbiology 146 (8), 1763-1774 (2000).
[30] Alby, K. and Bennett, R. J., "Stress-induced phenotypic switching in Candida albicans," Mol. Biol. Cell 20(14), 3178-3191 (2009).
[31] Soares, B. M., da Silva, D. L., Sousa, G. R., Amorim, J. C., de Resende, M. A., Pinotti, M. and Cisalpino, P. S., "In vitro photodynamic inactivation of Candida spp. growth and adhesion to buccal epithelial cells," J. Photochem. Photobiol. B 94(1), 65-70 (2009).
[32] Cassidy, C. M., Donnelly, R. F. and Tunney, M. M., "Effect of sub-lethal challenge with Photodynamic Antimicrobial Chemotherapy (PACT) on the antibiotic susceptibility of clinical bacterial isolates," J. Photochem. Photobiol. B 99(1), 62-66 (2010).
[33] Lauro, F. M., Pretto, P., Covolo, L., Jori, G. and Bertoloni, G., "Photoinactivation of bacterial strains involved in periodontal diseases sensitized by porphycene-polylysine conjugates," Photochem. Photobiol. Sci. 1(7), 468-470 (2002).
[34] Griffiths, M. A., Wren, B. W. and Wilson, M., "Killing of methicillin-resistant Staphylococcus aureus in vitro using aluminium disulphonated phthalocyanine, a light-activated antimicrobial agent," J. Antimicrob. Chemother. 40(6), 873-876 (1997).