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"Júlio de mesquita Filho"

Faculdade de Odontologia de Araraquara

Juliana Cabrini Carmello

Eficácia da Terapia fotodinâmica em modelo murino de candidíase oral e efeito genotóxico em células de *Candida albicans*.

Araraquara

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Juliana Cabrini Carmello

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Orientadora: Profª. Drª. Ana Cláudia Pavarina

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Dedico este trabalho.....

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Confie no Senhor de todo o teu coração e

não se apoie em seu próprio entendimento:

reconheça o Senhor em todos os seus

caminhos, e ele endireitará as suas veredas.

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Resumo

Carmello JC. Eficácia da Terapia fotodinâmica em modelo murino de candidíase oral e efeito genotóxico em células de *Candida albicans*. [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2015.

Resumo

O presente estudo avaliou a eficácia da terapia fotodinâmica (PDT) na inativação de *Candida albicans* em modelos murino de candidíase oral. Adicionalmente, este estudo avaliou se a PDT causa danos ao núcleo da célula fúngica. Dessa forma, foram conduzidos três estudos: 1. Avaliou a eficácia da cloro-alumínio-ftalocianina encapsulado em nanoemulsão catiônica (ClAlP NE) na inativação de *Candida albicans* presente em modelo murino de candidíase oral; 2. Avaliou a eficácia da PDT mediada pelo Photodithazine®(PDZ) como tratamento para candidíase oral induzida; 3. Avaliou o potencial da inativação fotodinâmica (PDT) mediado pelo fotossensibilizador (PS) Curcumina (CUR) associado ao LED azul em causar danos no DNA de *C albicans*. No estudo 1, 75 camundongos fêmeas com 6 semanas foram imunossuprimidos e inoculados com *C. albicans* para induzir candidíase oral. A PDT foi realizada sobre a língua pela aplicação dos fotossensibilizadores ClAlP NE ou ClAlP DMSO e luz LED (100 J/cm^2 – 660 nm). Em seguida, a avaliação microbiológica foi realizada por meio da recuperação de *C. albicans* da língua dos animais. Diluições seriadas foram realizadas, as colônias viáveis foram quantificado e o número de UFC mL determinado. Os animais foram sacrificados 24 horas após a PDT e as línguas foram removidas cirurgicamente para análise histológica e para análise de expressão de citocinas pró e anti-inflamatórias (IL-1, TNF-α e IL-6) por ensaio de RT-qPCR. Os dados foram analisados por One-Way ANOVA. A PDT mediada por ClAIP-NE reduziu 2,26 $\log_{10}$ da viabilidade celular de *C. albicans* em comparação ao grupo controle ($p < 0,05$). A PDT mediada por ClAlP DMSO não apresentou atividade antifúngica. A análise histológica revelou que a PDT não promoveu efeitos adversos sobre o tecido lingual dos animais. A terapia mediada por ambos os fotossensibilizadores induziu a expressão de TNF-α quando comparado com o controle, independentemente do tipo de veiculação utilizado($p < 0,05$). No estudo 2, 126 camundongos fêmeas com 6 semanas foram imunossuprimidos e inoculados com *C. albicans* para induzir candidíase oral. A PDT foi realizada sobre a língua dos animais por meio da aplicação de PDZ (100 mg/L) associado a luz LED (37,5 J/cm^2 – 660 nm). Para efeito de comparação, os animais também foram tratados com foram tratados com Nistatina (grupo - NIS). Os tratamentos foram realizados durante 5 dias consecutivos, sendo uma aplicação por dia. Além disso, foi realizada a avaliação macroscópica das lesões orais causadas por *C. albicans*. Após os tratamentos, as células de *C. albicans* foram recuperadas das línguas dos animais, as colônias viáveis foram quantificadas e o número de UFC/mL determinado. Os animais foram sacrificados 24 horas e 7 dias após os tratamentos e as línguas foram removidos cirurgicamente para análise histológica e análise de

expressão de citocinas pró e anti-inflamatórias (IL-1, TNF-α e IL-6) por RT-qPCR. Os dados foram analisados por Two-Way ANOVA ($p < 0,05$). A PDT mediada por PDZ foi tão eficaz quanto a NIS na inativação de *C. albicans*, reduzindo 3 e 3.1 $logs_{10}$ respectivamente, 24 h após o tratamento. Essa redução se manteve 7 dias após o tratamento para os animais submetidos à PDT, entretanto, houve um aumento significativo no número de UFC/ml para os animais tratados com NIS neste mesmo período. Os animais submetidos a PDT apresentaram remissão completa das lesões orais nos períodos avaliados, por outro lado, os animais do grupo - NIS apresentaram redução parcial das lesões orais NE mesmo período. A avaliação histológica revelou infiltrado inflamatório discreto nos grupos submetidos a PDT e NIS em ambos os períodos avaliados. Adicionalmente, foi observado que a PDT induziu a expressão do TNF-α quando comparado com o controle ($p < 0,05$), 24 h após o tratamento. Em resumo, o modelo murino de candidíase oral desenvolvido foi reprodutível a PDT mediada pelo PDZ foi eficaz no tratamento da candidíase oral induzida. No estudo 3, suspensões padronizadas de *C. albicans* foram tratadas com 2,5 μM de CUR e expostas a luz LED azul a 37,5 J/cm^2 (455 nm) (grupo P+L+). O efeito da aplicação isolada da CUR ou da luz também foi avaliado (P+L- e P-L+, respectivamente). O grupo controle negativo não foi tratado com CUR ou luz (P-L-). Para a comparação de danos ao DNA das células fúngicas, um grupo foi tratado com peróxido de hidrogénio (H_2O_2) a 10 mM. Após os tratamentos as células foram submetidas ao ensaio de cometa. Em seguida as lâminas foram coradas com GelRed ™ e 50 imagens de cada grupo foram visualizadas em microscópio de fluorescência a 400x (Leica Microsystems MS). As imagens foram analisadas pelo software de edição livre Comet Score TM e o comprimento da cauda dos cometas foi utilizado como parâmetro analítico (μm) e escolhido como unidade de danos no DNA. Adicionalmente avaliou-se a produção de espécies reativas de oxigênio (ROS) intracelular e a cinética de reparação do DNA fúngico. Os dados foram analisados por One-Way ANOVA e teste de Tukey post-hoc ($p<0,05$). A PDT mediada pela CUR assim como a luz LED azul aplicada isoladamente, promoveram danos significativos ao DNA de *C. albicans* quando comparadas com o grupo de controle negativo (P-L-) ($p < 0,05$). A produção intracelular de ROS também foi significativamente maior para o grupo tratado apenas com luz. A reparação do DNA foi significativamente mais lenta para as células submetidas com PDT em comparação às submetidas à luz azul isoladamente. O teste de cinética de reparação celular revelou que as caudas dos cometas formados apresentaram redução de 90% após 30 min da aplicação da luz azul isoladamente, enquanto que caudas dos cometas das células tratadas com PDT só diminuíram 45%. Estes resultados indicam que a PDT mediada por CUR e luz LED azul foi genotóxica ao DNA das células de *C. albicans*. Concluindo, a PDT mediada pela ClAlP-NE foi efetiva na redução de *C. albicans* presentes nas lesões orais de camundongos imunossuprimidos. A metodologia de indução de candidose oral em camundongos aqui instituída foi reprodutível da infecção e possibilitou o estabelecimento da PDT mediada pelo PDZ como tratamento para essa infecção. Adicionalmente, a PDT mediada pela CUR foi genotóxica às células de *C. albicans*.

Palavras-chave: *Candida albicans*, Candidíase bucal, Fotoquimioterapia, Genotoxicidade

Abstract

Carmello JC. Effectiveness of photodynamic therapy in a murine model of oral candidiasis and genotoxic effect of *Candida albicans* cells. [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2015.

Abstract

This study evaluated the efficacy of photodynamic therapy (PDT) in the inactivation of *C. albicans* in a murine models of oral candidosis. Additionally, this study evaluated the poetencial of PDT to promote DNA damage of fungal cell. For this purpose, three studies were conducted: 1. Evaluated the photodynamic effect of chloro-aluminum phthalocyanine encapsulated in cationic nanoemulsions (ClAlP NE) to inactivate *Candida albicans* in a murine model of oral candidiasis. 2. Evaluated the photoinactivation (PDT) of *Candida albicans* in a murine model of oral candidosis, mediated by Photodithazine® (PDZ). 3. Evaluated the potential of PDT mediated by Curcumin (CUR) associated with the blue LED in causing DNA damage of *C. albicans*. In the first study, 75 6-week-old female Swiss mice were immunosuppressed and inoculated with *C. albicans* to induce oral candidiasis. PDT was performed on the tongue by the application of the photosensitizers and LED light (100 J cm-² - 660 nm). Next, microbiological evaluation was carried out by recovering *C. albicans* from the tongue via colony forming units (CFU mL-1). Then, the animals were sacrificed and the tongues were surgically removed for histological analysis and analysis of inflammatory cytokines expression (IL-1, TNF-α and IL-6) by RT-qPCR. Data were analyzed by ANOVA test. ClAlP NE-mediated PDT was able to reduce *C. albicans* recovered from the tongue (2.26 log10) when compared with control ($p<0.05$). ClAlP DMSO-mediated PDT did not present antifungal activity. Histological analysis revealed that PDT did not promote adverse effects on the tongue tissue. PDT mediated by both photosensitizers induced the TNF-α expression when compared with control ($P<0.05$). ClAlP mediated by PDT induced the expression of inflammatory cytokines, TNF-α, irrespective of the drug system delivery used**.** In the second study, a new murine model of oral candidiasis was developed in order to allow the monitoring of the infection and the establishment of the treatment. Six-weeks-old female mice were immunosuppressed and inoculated with *C. albicans* to induce oral candidiasis. PDT was performed on the tongue by the application of PDZ (100 mg/L) and LED light (37.5 J/cm^2 – 660 nm), (P+L+). For comparison, animals were treated with nystatin (group - NIS). The treatments were carried out for 5 consecutive days with one application per day. Additionally it was performed the macroscopic evaluation of oral lesions caused by

C. albicans. After each treatment, the tongue was swabbed to recover *C. albicans* cells. Viable colonies were quantified and the number of CFU/ml determined. The animals were sacrificed 24 hours and 7 days after treatment and the tongues were surgically removed for histological analysis and analysis of inflammatory cytokines expression (IL-1, TNF-α and IL-6) by RT-qPCR. Data were analyzed by two-way ANOVA ($p<0.05$). PDZ-mediated PDT was as effective as NIS in the inactivation of *C. albicans*, reducing 3 and 3.1logs10 respectively, 24 h after treatment. These reduction were the same 7 days after treatment for the animals treated with PDT. However, there was a significant increase in the number of CFU/ml 7 days after treatment for animals treated with NIS. Animals underwent PDZ-mediated PDT showed complete remission of oral lesions, 24 h and 7 days after treatment. However, animals treated with NIS presented partial reduction of oral lesions. These results were the same 7 days after treatment. Histological evaluation revealed mild inflammatory infiltrate in the groups treated with PDT and NIS in both periods evaluated. PDT induced the TNF-α expression when compared with control ($p<0.05$), 24 h after treatment. In summary, the murine model of oral candidiasis developed was reproducible of this infection and PDZ- mediated PDT was effective in the treatment of mice with induced oral candidiasis. In the third study, it was investigated the potential of sub lethal photodynamic therapy (PDT) using light sensitive curcumin (CUR) in combination with blue (455 nm) light to promote reactive oxygen species (ROS) formation in the form of singlet oxygen and DNA damage of *C. albicans*. Surprisingly, CUR-mediated PDT but also light alone caused significantly longer comet tails, an indication of DNA damage of *C. albicans* when compared with the negative control. The intracellular ROS production was also significantly higher for the group treated only with light. However, PDT compared to blue light alone significantly slowed DNA repair. Comet tails decreased during 30 min visualized as a 90% reduction in length in the absence of light for cells treated with light alone, while comet tails of cells treated with PDT only diminished in size about 45%. These results indicate that complex mechanisms may result in PDT in a way that should be considered when choosing the photo-sensitive compound and other aspects of the treatment design. In conclusion, PDT mediated by ClAlP-NE was effective in reducing *C. albicans* recovered from the oral lesions of immunocompromised mice. The new murine model of oral candidiasis established here was reproducible of the infection and it enabled the establishment of PDZ-mediated PDT as a treatment for this disease. In addition, the CUR-mediated PDT was genotoxic on *C. albicans* cells.

Keywords: *Candida albicans*, Oral candidiasis, Photochemotherapy, Genotoxicity

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1 - Introdução

1 – Introdução

A *Candida albicans* é o fungo mais comumente encontrado nos seres humanos sendo responsável por provocar infecções superficiais tais como candidíase orofaríngea[27.]. A patogênese da candidíase pode estar associada com condições de risco relacionadas ao hospedeiro, tais como a hipossalivação, diabetes mellitus, o uso prolongado de antibióticos de amplo espectro e pacientes transplantados[64]. Muitos medicamentos estão disponíveis para o tratamento de infecções por *Candida* tais como agentes antifúngicos tópicos (polienos e os azois) e sistêmicos (anfotericina B, itraconazol). No entanto, a utilização destes medicamentos pode promover o desenvolvimento de hepatotoxicidade e resistência antifúngica[74]. Outro aspecto relacionado com recorrência das infecções e o desenvolvimento da resistência antifúngica é a capacidade que as espécies de *Candida* apresentam de formar biofilmes sobre superfícies bióticas ou abióticas[77]. Os biofilmes são comunidades estruturadas de micro-organismos, envoltos em matriz polimérica extracelular[75]. A organização do biofilme fornece vantagens à sobrevivência dos micro-organismos mesmo quando em ambientes hostis, além de aumentar a resistência aos antifúngicos convencionais[74-75]. Devido às dificuldades relacionadas ao uso de medicamentos convencionais e resistência antifúngica, a terapia fotodinâmica (PDT) tem sido sugerida como um método alternativo na inativação de *Candida* spp. e para o tratamento de infecções fúngicas superficiais[24, 35, 89].

O interesse pela PDT advém da simplicidade do mecanismo de ação resultante da interação entre um fármaco fotossensível (FS) e uma luz visível de comprimento de onda compatível ao espectro de absorção do fármaco com oxigênio, o que culmina na produção de espécies reativas de oxigênio (ROS) que são letais para os micro-organismos[13, 52]. Mais especificamente, o mecanismo de ação da PDT envolve a absorção de fótons da fonte de luz pelo FS, o que leva seus elétrons a um estado excitado. Na presença de oxigênio, o FS excitado pela luz pode reagir com moléculas vizinhas, por meio da transferência de elétrons ou hidrogênio (reação do tipo I) ou pela transferência de energia ao oxigênio (reação do tipo II), levando à produção de ROS[9]. Ambos os caminhos podem levar à morte celular. Tais espécies reativas possuem reatividade não específica com moléculas orgânicas, isto é, qualquer macromolécula celular pode ser um alvo em potencial para PDT[9].

De acordo com os aspectos citados, as ROS, tais como o radical superóxido (O_2), radical hidroxila (OH), peróxido de hidrogênio (H_2O_2) e oxigênio singlete, representam

uma ameaça significativa para integridade celular[41] sendo as responsáveis por causar o dano celular por meio de stress oxidativo. Tem sido sugerido que na presença de íons metálicos redox-ativos tais como Fe^{2+}, o oxigênio e H_2O_2 podem levar a reação química de Fenton, culminando na produção de radicais extremamente reativos aos constituintes celulares. O stress oxidativo pode provocar danos oxidativos em macromoléculas tais como DNA[8] o que contribui para a instabilidade genética e possível mutação[41].

Atualmente, os FSs de segunda geração têm sido avaliados[18, 56, 81], e entre esses compostos estão as ftalocianinas[54, 62]. As ftalocianinas apresentam alta banda de absorção no espectro eletromagnético que varia de 650 a 680 nm, o que permite maior penetração de luz nos tecidos[14]. As propriedades fotofísicas das ftalocianinas são dependentes de sua composição, mais especificamente do íon metálico central. Dentre as ftalocianinas estudadas, está a cloro-alumínio ftalocianina (ClAlPc), que produz grande quantidade de oxigênio singlete[72]. Um estudo anterior avaliou o potencial fotodinâmico da ClAlPc diluída em nanoemulsão catiônica e aniônica na inativação de culturas planctônicas e biofilmes de *C. albicans* em comparação com ClAlPc livre[70], e foi verificado que a inativação fúngica foi dependente do sistema de liberação, carga superficial e dose de luz. A nanoemulsão aniônica não apresentou atividade antifúngica, por outro lado, a nanoemulsão catiônica de ClAlPc reduziu significativamente a viabilidade e o metabolismo celular da *C. albicans*. Além disso, tanto a nanoemulsão catiônica de ClAlPc quanto a livre causaram danos significativos na membrana celular do fungo. Para os biofilmes, observou-se redução no metabolismo celular de 70% com a utilização do composto catiônico e a microscopia confocal de varredura demonstrou que a ClAlPc catiônica pode penetrar no biofilme[70]. Entretanto, são necessários estudos em modelo animal para definição de um protocolo de tratamento que possibilite a utilização da ClAlPc catiônica no tratamento da candidíase oral.

Outra classe de fotossensibilizadores de segunda geração que vem sendo amplamente estudado são as clorinas[3, 25, 67], FSs que apresentam forte banda de absorção na região vermelha do espectro fotomagnético. O Photodithazine® (PDZ) é uma clorina e6 que tem sido utilizada com sucesso na PDT contra o câncer, devido ao seu alto rendimento quântico de formação de oxigênio singlete[3]. Em estudos anteriores foi avaliada a eficácia da PDT mediada pelo PDZ na fotoinativação de cepas de *C. albicans, Candida glabrata* e *Candida tropicalis* isoladas de pacientes com estomatite protética. Os resultados demonstraram que a PDT promoveu redução significativa na viabilidade de suspensões celulares de *C. tropicalis* e *C. glabrata* enquanto cinco cepas de *C. albicans* foram

completamente inativadas após a PDT. A redução do metabolismo do biofilme para *C. albicans* foi de 62,1% enquanto para a *C. tropicalis* e *C. glabrata* foi equivalente a 76 e 76,9%, respectivamente[25]. Quando um biofilme misto formado por *C. albicans*, *C.glabrata*, e *Streptococcus mutans* foi submetido a PDT com o PDZ foi observado redução significativa na viabilidade das colonias das três espécies avaliadas, e redução significativa na atividade metabólica dos biofilmes submetidos a PDT[67]. Em estudo *in vivo* em um modelo murino de candidíase oral foi observado que a associação de PDZ (100 mg/L) a luz LED reduziu $4{,}36\log_{10}$ de *C. albicans* presente em lesões induzidas em lingua de camundongo[16]. Esses resultados demonstraram que a PDT foi eficaz na redução da *C. albicans,* entretanto, foi feita somente uma aplicação de PDT e não foi realizado o acompanhamento da evolução das lesões presentes na língua dos animais em todos os grupos avaliados.

Adicionalmente, a Curcumina (CUR), um composto de coloração amarela extraída do rizoma da planta Curcuma longa L (açafrão) tem sido sugerida como fotossensibilizador em potencial para ser utilizada em PDT[76]. Vários trabalhos sugerem que a CUR possui efeitos anti-inflamatórios, antioxidantes e imunomoduladores, que poderiam ser exacerbados com a sua iluminação em comprimento de onda adequado[12]. Recentemente, a PDT mediada pela CUR associada a luz LED mostrou-se efetiva na inativação de suspensões planctônicas e biofilmes de *C. albicans, C. glabrata,* e *C. dubliniensis*[1]. A CUR também mostrou seu efeito antifúngico, na inativação de suspensões celulares e biofilmes de isolados clínicos da *C. albicans, C. glabrata* e *C. tropicalis*, promovendo uma redução no metabolismo celular de 85, 85 e 73% respectivamente[23]. Esse composto também mostrou efetividade na inativação de *C. albicans* presentes em língua de camundongos com candidíase oral induzida, promovendo uma redução de aproximadamente 5 logs na viabilidade celular, sem causar danos ao tecido lingual dos animais[26].

Estudos têm demonstrado a efetividade da PDT mediada por diferentes FSs na inativação fúngica *in vitro*[6, 22]. Entretanto, os estudos que avaliaram sua interação com a resposta imune do hospedeiro são escassos[44]. A ativação da resposta imune do hospedeiro após PDT anti-câncer tem sido freqüentemente estudada[44]. Entretanto, essa ativação também parece estar envolvida nos efeitos terapêuticos da PDT para infecções fúngicas e bacterianas[44]. Estudos prévios realizados em modelos animais infectados, tem atribuído os benefícios terapêuticos da PDT à morte direta dos micro-organismos causada pelas ROS[44]. Porém, tem sido observado que as ROS podem interagir com diversas estruturas celulares do hospedeiro tais como proteínas, membranas lipídicas e ácidos nucléicos por diferentes mecanismos[66] e o estresse causado leva à ativação da cascata inflamatória, que promove a

liberação de diversas citocinas pró e anti-inflamatórias, capazes de mobilizar o sistema imune do hospedeiro para reverter esse quadro.

As citocinas são polipeptídios ou glicoproteínas extracelulares, hidrossolúveis, produzidas por diversos tipos de células no local da lesão e por células do sistema imunológico[79]. Essas substâncias se ligam a receptores específicos, ativando mensageiros intracelulares que regulam a transcrição gênica. Dessa forma, as citocinas influenciam a atividade, a diferenciação, a proliferação e a sobrevida da célula imunológica, assim como regulam a produção e a atividade de outras citocinas, que podem aumentar (pró-inflamatórias) ou atenuar (anti-inflamatórias) a resposta inflamatória[79, 91]. Durante as infecções, o TNF-α é um dos mediadores mais precoces e potentes na resposta inflamatória sendo responsável por provocar mudanças metabólicas e hemodinâmicas importantes e ativar outras citocinas [68,79]. A IL-1β é primariamente produzida por macrófagos e monócitos, assim como por células não imunológicas, tais como fibroblastos e células endoteliais ativadas durante lesão celular, infecção, invasão e inflamação, sendo responsável por desencadear inflamação sistêmica[91]. Ambas citocinas induzem a produção de IL-6, que é considerado o marcador mais relevante do grau de lesão tecidual, exercendo funções pró e anti-inflamatórias durante a infecção[42].

Assim, para considerar a PDT como um tratamento para candidíase oral, é necessário conhecer além do seu potencial antifúngico, o efeito citotóxico sobre as células do hospedeiro, bem como seu efeito sobre o DNA das células fúngicas, uma vez que todas as células são alvos de ROS produzidas pela PDT. Em face aos resultados promissores relatados na fotoinativação de *Candida* spp em estudos *in vitro* mediados pela ClAlP e nos trabalhos *in vitro* e *in vivo* mediados pelo PDZ, o presente estudo avaliou a eficácia da PDT, mediada pela ClAlP na inativação da *C. albicans* presente em candidíase experimental induzida em língua de camundongos (Capítulo 1) e avaliou também a eficácia fotodinâmica do PDZ em novo modelo animal de candidíase oral, simulando o tratamento para essa infecção (Capítulo 2). Adicionalmente, o presente estudo avaliou ainda o potencial da PDT mediada pela CUR associada a luz LED azul em promover dano no DNA de *C. albicans* devido ao estresse oxidativo produzido pela terapia (Capítulo 3).

2- Proposição

2 - Proposição

O presente estudo avaliou a eficácia da PDT na inativação de *C. albicans* em modelos murino de candidíase oral. Adicionalmente, este estudo avaliou se a PDT causa danos ao núcleo da célula fúngica. Dessa forma, 3 estudos foram conduzidos com os seguintes objetivos específicos:

1. Avaliar *in vivo* a efetividade da PDT mediada pela ClAlP na inativação *C. albicans* presente em candidíase experimental induzida em língua de camundongos.

2. Avaliar a efetividade da PDT mediada pelo PDZ como tratamento para candidíase oral por meio do desenvolvimento de novo modelo murino de candidíase oral. Realizar a avaliação macroscópica e microscópica das lesões orais, seguido da análise biomolecular da expressão gênica de citocinas pró e anti-inflamatórias.

3. Avaliar *in vitro* o potencial da PDT mediada pela Curcumina associada a luz LED azul em promover danos no DNA de *C. albicans*.

3 – Capítulos

3.1 Capítulo 1

In vivo photodynamic inactivation of *Candida albicans* and expression of inflammatory cytokines using chloro-aluminum phthalocyanine*

Short title: *In vivo* photoinactivation of *Candida albicans*

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Abstract

New drug delivery systems, such as nanoemulsions, have been developed in order to use hydrophobic drugs in antimicrobial photodynamic therapy (PDT). This study evaluated the photodynamic effect of chloro-aluminum phthalocyanine encapsulated in cationic nanoemulsions (ClAlP NE) to inactivate *Candida albicans* in a murine model of oral candidiasis compared with ClAlP diluted in DMSO (ClAlP DMSO). Seventy-five 6-week-old female Swiss mice were immunosuppressed and inoculated with *C. albicans* to induce oral candidiasis. PDT was performed on the tongue by the application of the photosensitizers and LED light (100 J cm^{-2}). Imediately after treatment, microbiological evaluation was carried out by recovering *C. albicans* from the tongue via colony forming units (CFU mL^{-1}). Then, the animals were sacrificed and the tongues were surgically removed for histological analysis and analysis of inflammatory cytokines expression (IL-1, TNF-α and IL-6) by RT-qPCR. Data were analyzed by ANOVA test followed by Tukey post-hoc ($p<0.05$). ClAlP NE-mediated PDT was able to reduce *C. albicans* recovered from the tongue (2.26 $\log_{10}$) when compared with control group (untreated animals) ($p<0.05$). ClAlP DMSO-mediated PDT did not present antifungal activity. Histological analysis revealed that PDT did not promote adverse effects on the tongue tissue. PDT mediated by ClAlP induced the TNF-α expression when compared with control independent on the formulation used ($p<0.05$). ClAlP mediated by PDT induced the expression of inflammatory cytokines, TNF-α, irrespective of the drug system delivery used. In summary, ClAlP-NE-mediated PDT was effective in reducing *C. albicans* recovered from the oral lesions of immunocompromised mice.

Introduction

Antimicrobial photodynamic therapy (PDT) has been widely suggested for the treatment of fungal infections due to increased resistance of these microorganisms to conventional antifungal treatments [1-3]. The interest in PDT also comes from the simplicity of the mechanism of action resulting from the interaction between a photosensitive drug (PS) and visible light wavelength compatible with absorption spectrum of drug with oxygen, culminating in the production of reactive oxygen species (ROS), which are lethal to microorganisms [4-5]. Photosensitive drugs used in antimicrobial PDT are predominantly from the families of porphyrins [6], phenothiazines [7], and 5-aminolevulinic acid (ALA) [8].

New compounds known as second generation photosensitizers, such as the phthalocyanines, have been evaluated [9-11]. These compounds have an absorption band in the electromagnetic spectrum that ranges from 650 to 680 nm, which allows greater light penetration into tissues [10] and their photophysical properties dependend on the composition, particularly central metal ion. Among the phthalocyanines, chloro-aluminum phthalocyanine has been suggested to have photophysical properties favorable for use in PDT as it produces high quantities of singlet oxygen [10]. The effectiveness of this photosensitizer associated with LED light has been proven in an *in vitro* study that assessed the photodynamic potential of chloro-aluminum phthalocyanine (ClAlP) diluted in cationic nanoemulsion to inactivate planktonic and biofilm cultures formed by *C. albicans* [9]. The photosensitizer was also effective for the inactivation of bacteria in patients with carious lesions [11]. Studies have shown that PDT mediated by ClAlP is effective for fungal inactivation. However, studies assessing the interaction between PDT and the host immune response are scarce [12]. The activation of the host immune response after photodynamic anti-cancer therapy has frequently been studied [12]. However, this activation also seems to be related to the therapeutic effects of PDT for fungal and bacterial infections [12]. Previous studies using infected animal models have attributed the therapeutic benefits of PDT to the direct death of microorganisms caused by ROS [12]. However, it has been observed that ROS can interact with several host cellular structures such as proteins, nucleic acids and membrane lipids by different mechanisms [13] and the oxidative stress leads to the activation of the inflammatory cascade that promotes the release of various pro- and anti-inflammatory cytokines, which mobilize the host's immune system to revert the situation.

Cytokines are soluble, extracellular polypeptides or glycoproteins produced by different types of cells at the lesion site and by cells of the immune system [14]. These substances bind to specific receptors, activating intracellular messengers that regulate gene transcription. Thus, the cytokines influence the activity, differentiation, proliferation and survival of immune cells, as well as regulating the production and activity of other cytokines, which may increase (pro-inflammatory) or attenuate (anti-inflammatory) inflammatory response [14-15]. During infection, TNF-α is one of the first and most potent mediators in the inflammatory response responsible for causing important metabolic and hemodynamic changes and distally activating other cytokines [15-16]. IL-1β is primarily produced by macrophages and monocytes, as well as non-immune cells such as fibroblasts and endothelial cells activated during cell injury, infection, invasion and inflammation, and it is responsible

for triggering systemic inflammation [15]. Both cytokines induce IL-6 production, which is considered the most relevant marker for the degree of tissue injury, acting as pro- and anti-inflammatory proteins during infection [17].

Thus, to consider PDT as a possible treatment for fungal infections, first it is important to know what cytotoxic effect it would cause on the host cells since all cells are targets of ROS produced by the therapy, in addition to its antifungal effectiveness [12]. Thus, the aim of the present *in vivo* study was to assess the efficacy of PDT mediated by chloro-aluminum phthalocyanine (ClAlP) in the inactivation of *C. albicans* present in induced experimental candidiasis in mice. It was also assessed the host response toward antimicrobial PDT by analyzing the gene expression of pro- and anti-inflammatory cytokines to evaluate inflammation after application of PDT.

Materials and Methods

Photosensitizer and light sources

ClAlP encapsulated in cationic nanoemulsion (NE) was kindly donated by the Center of Nanotechnology and Tissue Engineers, Photobiology and Photomedicine research Group of the University of São Paulo at Ribeirão Preto, Brazil. The NE with a cationic superficial load (ClAlP-NE) was obtained by spontaneous emulsification [13] and the final concentration of ClAlP was previously described [9]. The photophysical, chemical, and biological properties were evaluated and presented similar response [13]. The final concentration of ClAlP NE was 31.7 μM, in order to compare the photodynamic inactivation of *C. albicans* using the delivery system (ClAlP-NE, in cationic form), a stock solution of phthalocyanine (600 M) was purchased from Sigma–Aldrich Co. (St. Louis, MO) and was prepared in 10% DMSO (ClAlP-DMSO) and then diluted in PBS to obtain the same final concentration of 31.7 μM.

A handpiece with a red (630 nm) light-emitting diode device (LED, LXHL-PR09, Luxeon® III Emitter, Lumileds Lighting, San Jose, California, USA) with 5 mm of diameter was designed by the Physics Institute of São Carlos (University of São Paulo, São Carlos, SP, Brazil). The output power of light delivered at the end of the handpiece was 44.6 mW cm^{-2}.

Microorganisms and growth conditions

The standard strain of *C. albicans* ATCC 90028, (Rockville, MD USA) used in this study was stored in a microtube containing Tryptic Soy Broth culture medium (TSB, pH 7.2, Acumedia Manufacturers Inc., Baltimore, MD) and glycerol and frozen at -70ºC. Prior to the experiments, the microorganism was thawed and inoculated aerobically in a Sabouraud dextrose agar culture (SDA) and incubated at 37ºC for 48 hours for reactivation. To obtain the suspensions, the strain was grown in tubes containing 5 ml of RPMI 1640 at 37°C for 24 hours. The tube was then centrifuged at 2000 rpm for 10 min, washed twice, resuspended in sterile saline and the cell concentration was adjusted to 10^7 CFU mL^{-1}.

Induction of oral candidiasis and treatments

The study was performed in compliance with the relevant laws and institutional guidelines and was approved by the Committee on the Ethics of Animal Experiments (CEUA) of the Araraquara Dental School, Univ Estadual Paulista, UNESP (Permit Number: 1/2010). All surgeries were performed under ketamine anesthesia, and all efforts were made to minimize suffering. Seventy-five 6-week-old female Swiss mice specific pathogen free (SPF), with 20 g of weight approximately, were used for all animal experiments. The animals were allocated to experimental groups by randomization before each experiment. Thus, they were kept in cages housing 5 animals in a temperature controlled room (23 ± 2°C). Standard chow and tap water were given *ad libitum*.

To induce candidiasis in the animals, we used the method previously described by Takakura et al. (2003) [18], reproduced by our group [19-20]. During the experimental period, the animals were treated with tetracycline 0.83 mg mL^{-1} (Santa Paula Pharmacy, Araraquara, SP, Brazil) administered through the drinking water. The animals were immunosuppressed with two subcutaneous injections of prednisolone (Depo-Medrol, Pfizer Ltd., Guarulhos, SP, Brazil) at a dose of 100 mg kg^{-1} body weight 1 day before and 3 days after infection with *C. albicans*.

On the second day of the experiment, the animals were sedated with 0.1 mL of 2 mg ml^{-1} chlorpromazine hydrochloride (Santa Paula Pharmacy, Araraquara, SP, Brazil) by intramuscular injection. The tongue of the animals was gently taken out of the oral cavity and mini swabs (Cotton baby, Higie-Plus Cotton baby Ind. Com., Ltd, San Jose, SC, Brazil) embedded in standardized suspension of *C. albicans* were rubbed on the tongue of the anesthetized animals for 30 seconds to induce infection. The swabs contained 9 x 10^6 CFU mL^{-1} of *C. albicans*, approximately.

PDT was performed 48 hours after the second immunosuppression (on the 7th day of the experiment). The susceptibility of *C. albicans* strain to PDT was evaluated through exposure of the infected tongues of the mice to PS encapsulated in cationic nanoemulsion (P+L+ ClAlP-NE) and PS-free (P+L+ ClAlP-DMSO) at a concentration of 31.7 μM associated with a light dose of 100 J cm^{-2}. Additionally, the effect of the isolated application of PS (P+L- ClAlP and P+L- ClAlP-DMSO) and light dose (P-L+) was also evaluated in animals. The tongues of the animals in the positive control group (P-L-) received inoculation with *C. albicans*, but they were not photosensitized or received light. The animals in the negative control group (NC) did not receive any treatment or inoculation by the fungus. Furthermore, we evaluated two experimental groups consisting of healthy animals (no inoculation - NI) that were treated with PDT to assess the effects of therapy alone (P+L+ ClAlP-NE NI and P+L+ ClAlP-DMSO NI). The evaluation of possible reactions in the oral tissues of the animals was performed at the following time intervals: 24 hours after treatment (day 8) and 7 days after treatment (day 14). Each experimental group consisted of 5 animals (n = 5). (Fig. 1- Study desing).

After the treatments, mini swabs were rubbed on the tongue dorsum of the animals for 1 minute to collect *C. albicans*. These mini swabs were transferred to test tubes containing 1 ml of saline solution and stirred for 1 minute to detach the cells. Serial dilutions were made from the samples contained in the test tubes and plated in duplicate onto SDA plates containing 5 μg ml^{-1} of chloramphenicol. After 48 hours of incubation at 37°C, viable colonies were counted and the values of CFU ml^{-1} determined.

Macroscopic analysis of oral lesions

Standardized photographs were taken at the beginning of proposed treatments, 24 hours and 7 days after application to evaluate the progression of lesions on the tongue of the animals. Each photograph was rated by means of scores according to the classification proposed by Takakura et al. (Table 1) [18].

Table 1 - Clinical classification of oral lesions.

Classification of lesions	Clinical characteristics of tongue dorsum
Score 0	Healthy mucosa
Score 1	White patches on up to 20% of the tongue
Score 2	White patches on up to 21-90% of the tongue
Score 3	White patches on more than 91% of the tongue
Score 4	Thick white patches/pseudomembranes on more than 91% of the tongue

Histological analysis

The animals were sacrificed with a lethal dose of ketamine (0.2 mL) 24 hours or 7 days after the treatments proposed. After the sacrifice, the tongue of the animals was surgically removed and standardized longitudinal sections were made. Each half was submitted to histological or RT-qPCR tests. Immediately after removing the tongues, one of the halves of the tongue dorsum was submitted to routine laboratory procedure for fixing and inclusion of samples in paraffin. Then, sixteen histological sections of each 5-mm thick block were obtained, mounted on glass slides and stained with periodic acid-Schiff and hematoxylin (PAS-H) stain for histopathological examination and fungal detection under a light microscope (Carl Zeiss 62774, Oberkochen, West Germany). Tissue reaction due to *C. albicans* infection associated or not to PDT was examined by a pathologist blinded to all the groups of mice. A descriptive analysis of the histological characteristics of the tissue with and without local inflammatory response of different intensity was performed and the intensity of inflammation was expressed using a 5-point scoring system (Table 2).

Table 2. Classification of histopathological events (ISO 7405: 1997).

Score	Histological event
0	No inflammation
1	Discrete inflammation
2	Moderate inflammation
3	Severe inflammation
4	Abscess formation

Evaluation of gene expression of inflammatory cytokines

RNA extraction and cDNA synthesis

Part of the tissue was used to analyze the effect of PDT on gene expression of the inflammatory cytokines TNFα**,** IL-1β and IL-6. Total RNA was isolated using the TRIzol® method in accordance with manufacturer's protocol (lifetechnologies - Carlsbad, CA, USA). Briefly, after storing the tissues in Trizol, 0.1 ml of chloroform (Sigma-Aldrich, St. Louis, MO, USA) was added to each sample and they were incubated for 3 minutes at room temperature after homogenization. The samples were then centrifuged at 12,000 RCF (Eppendorf microcentrifuge, Model 5415R, Hamburg, Germany) for 15 minutes at 4°C. After centrifugation, the formation of three distinct phases were observed and the aqueous phase containing RNA was transferred to a new tube, followed by the addition of 0.5 mL of isopropanol (Sigma-Aldrich) to promote precipitation of RNA. The samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 RCF for 10 minutes at 4°C. After removal of the supernatant, 1 mL of 75% ethanol (Sigma-Aldrich) was added, followed by centrifugation at 7,500 RCF for 5 minutes at 4°C. The supernatant was again discarded and the RNA pellet was air dried by inversion for 10 minutes. The RNA was resuspended in 10 μL of ultrapure water (Invitrogen). The samples were then incubated at 55°C for 10 minutes (Thermomixer Comfort, Eppendorf, Hamburg, Germany). An aliquot of total RNA (1 μL) was diluted with ultra-pure water (1:49) to carry out the quantification of RNA in the BioPhotometer (Eppendorf BioPhotometer, Eppendorf model RS-232C, Hamburg, Germany).

For each total RNA sample, cDNA was synthesized using the high-capacity cDNA reverse transcriptase kit (Applied Biosystems) in accordance with the manufacturer's recommendations. The cDNA was stored at -20 ° C until use in RT-qPCR.

RT-qPCR

The effect of PDT on the expression of inflammatory cytokines (TNF-α, IL-1b and IL-6) was assessed by RT-qPCR. Therefore, it was selected specific primers for each target gene (TaqmanAssay, Applied Biosystems: βActin = Mm00607939_S1; TNF = Mm00443260_G1; IL-1β = Mm00434228_M1; IL-6 = Mm00446190_M1). Amplification analyses were performed in a Step-One Plus thermocycler and amplification cycles were analyzed and determined using the Step-One Plus software (Applied Biosystems, Foster City, CA, USA). The concentration of mRNA of the target genes in each sample was subsequently normalized to the amount of mRNA from the selected endogenous control (βActin) and expressed in percentage for comparison with the control group.

Statistical analysis

Data for viable colony counts were transformed into a logarithm to the base 10 and submitted to analysis of variance (ANOVA), followed by post-hoc Tukey's test at a 5% significance level. Additionally, the Spearman correlation test was used to correlate the number of colony forming units (CFU mL^{-1}) with the scores assigned to these lesion on the tongue of the animals. The data obtained from the RT-qPCR were analyzed by ANOVA followed by post-hoc Tukey's test for TNFα and IL-1β. For cytokine IL-6, the data were analyzed by the Kruskal-Wallis test and post hoc Dunn's test. The significance level adopted was 5%.

Results

C. albicans detection after treatments

The results showed a statistically significant difference in the CFU mL^{-1} values for the group that was submitted to PDT with PS in cationic nanoemulsion (P+L+ ClAlP-NE), achieving a reduction of 2.26 $\log_{10}$ ($p \leq 0.0001$). The other groups presented statistically similar CFU mL^{-1} values in comparison with P-L- [P-L+ (p=0.694); P+L- ClAlP NE (p= 0.505); P+L- ClAlP-DMSO (p=0.526); P+L+ ClAlP-DMSO (p= 0.801)] (Fig. 2).

The animals evaluated after 7 days of the completion of treatments showed no fungal growth on the culture plate, indicating the absence of infection on the tongue tissue.

Macroscopic and histological analyses of *Candida* lesions

All infected animals with *C. albicans* showed white patches/pseudomembranes on the tongue dorsum (score 2) (Fig. 3A). It was found a significant positive correlation between the scores assigned to oral lesions of animals with the CFU mL^{-1} number ($r_s = 0.70$; $p \leq 0.0001$). Twenty-four hours after treatment, the animals in the P+L+ ClAlP-NE group showed score 1 (Fig. 3B) (white patches on up to 21% of the tongue) and the other groups of animals that were infected by *C. albicans* maintained score 2. In addition, score 0 (healthy mucosa) was attributed to the tongues of the animals in the groups not infected by *C. albicans*, irrespective of treatment received (CN, P+L+ ClAlP-NE NI, P+L+ ClAlP-DMSO NI). After 7 days all infected animals presented score 0 (Fig. 3C).

According to Fig. 4A, the animals in the group P+L+ ClAlP-NE showed mild inflammatory infiltrate in the tissues 24 hours after treatment, with few hyphae/pseudohyphae in the thin keratin layer. The groups P+L-, P-L+, P-L-, and P+L+ ClAlP-DMSO showed moderate inflammatory infiltrate with presence of numerous hyphae/ pseudohyphae in the thin keratin layer without invasion of the epithelial tissue (Fig. 4B). On the other hand, the groups that received treatment with PDT and were not inoculated with *C. albicans* (P+L+ ClAlP-NE NI and P+L+ ClAlP-DMSO NI) showed normal histological appearance with a thick keratin layer, similar to the one found in the negative control group (NC) (Fig. 4C). The tissues showed normal neoformation of filiform papillae and absence of *C. albicans* on the animals sacrificed 7 days after treatment (Fig. 4C).

Gene expression data for TNFα, IL-1β and IL-6

The gene expression analysis of cytokines TNF-α, IL 1-β and IL 6 showed that there was an increase of 20% and 32% in comparison with the positive control group (P-L-) in the TNF-α expression for animals in the P+L+ ClAlP-NE ($p=0.008$) and P+L+ ClAlP-DMSO ($p=0.027$) groups, respectively. The expression of this cytokine was higher in the uninfected animals treated with PDT mediated by PS diluted in DMSO (P+L+ ClAlP-DMSO NI) ($p=0.044$). The same behavior was observed for the groups treated only with light (P-L+) ($p=0.033$) or PS formulated in DMSO (P+L- ClAlP-DMSO) ($p=0.048$) in comparison with

the P-L- group (Fig. 5). The IL-1 and IL-6 cytokines were present in all groups studied, but there was no statistical difference when compared with the P-L- group ($p \geq 0.05$) (Fig. 5).

Discussion

In the present study we observed that PDT mediated by ClAlP cationic NE presented antifungal effect on *C. albicans* as it promoted a reduction of $2.26 \log_{10}$. Furthermore, when the same substance was diluted in DMSO it showed no fungicidal effect. As PS with ClAlP used in the present study is insoluble in water, the cationic nanoemulsion was formulated (PBS, pH 7.4). It has been reported that the delivery vehicle of drugs in nanoemulsion increases availability and stability of PS, reducing the possibility of aggregation of the molecules [21]. The treatment of fungal infections using PS diluted in nanoemulsion has advantages due to its small particle size that allows a uniform deposition on the tissues and greater tissue penetration. In addition, the nanoemulsion can comprise particles ranging from 10 to 600 nm, allowing the transportation of oil-soluble compounds that can be dissolved in the oil phase of an emulsion or amphiphilic drugs that can be adsorbed at the oil-water interface [13, 22]. Moreover, the addition of a positive charge (cationic) to the nanoemulsion improves adhesion of PS o the cell membrane of the fungus, thereby increasing the amount of PS on the cell wall and effectiveness of PDT [23]. This justifies the greater effectiveness of PDT mediated by ClAlP encapsulated in the cationic nanoemulsion observed in the present study.

It is noteworthy that the group treated with PDT mediated by ClAlP in DMSO showed no reduction in cell viability of *C. albicans* present in the oral lesions of animals. These results are not in agreement with those obtained in *in vitro* studies in which the same compound was effective against bacteria and fungi on PDT [9, 24]. While DMSO is considered a compound that allows the penetration of hydrophilic and lipophilic drugs into the biological tissues [25], unlike the cationic nanoemulsion, it is no charge, so it cannot have promoted the adhesion of PS to the fungal cells. The adhesion and aggregation of *Candida* to biotic or abiotic surfaces depends on the reactions between the fungal cell wall and surface with the presence of covalent bonds between these structures [26-28]. As DMSO is an organic solvent and not a delivery system of PS, it shows no characteristic that favors the adhesion of PS to the cell membrane of *C. albicans*. Another disadvantage is that because DMSO is an organic solvent, it does not protect the PS, allowing it to undergo faster photodegradation when compared with PS encapsulated in nanoemulsions [29]. Thus, when PS, formulated in

DMSO, is in an excited state it easily degrades, which reduces its oxidative power and decreases the effectiveness of PDT [30].

Macroscopic analysis of the oral lesions revealed the presence of white patches or pseudomembranes on approximately 21-90% of the tongue dorsa (score 2) in all animals infected with *C. albicans*. Twenty-four hours after treatment, it was possible to assign score 1 to the P+L+ ClAlP NE group and score 2 remained for the other groups infected with *Candida*. These findings corroborate those obtained by Costa et al., [31] who investigated the effectiveness of PDT mediated by erythrosine in mice with induced oral candidiasis. Score 1 was assigned to group undergoing PDT 24 hours after treatment and score 2 to the other groups. Additionally, in the present study the relationship between oral lesions detected on the tongue dorsum of the animals and the results obtained in the cell viability assay (CFU ml^{-1}) was verified by the correlation coefficient. This analysis showed a high coefficient value, confirming that the high number of white patches and/or pseudomembrane on the tongue reflected the degree of *C. albicans* colonization in the tongue of animals. In the study conducted by Takakura et al. [18], the authors evaluated the therapeutic efficacy of fluconazole and amphotericin B in the treatment of oral candidiasis induced in mice and also relate microbiological data with the degree of oral lesions, reporting a high correlation coefficient between these two factors ($r_s = 0.70$, $n = 5$), as shown in the present study.

The histological analysis of the tongues removed 24 hours after treatment showed the presence of yeast and hyphae in the keratin layer and discrete inflammatory infiltrate on the tongue dorsum of all animals infected with *C. albicans*, including those undergoing PDT. The histological changes found in this study are characteristic of oral candidiasis and it has also been reported by other authors [19, 31-33]. Furthermore, the histological images from the tongues of the uninfected animals with *C. albicans* (CN, P+L+ ClAlP NE NI and P+L+ ClAlP DMSO NI) showed no inflammatory response in the tongue tissue. These results are in agreement with Fontana et al. [34], who evaluated the effect of different PS associated with LED light (630 or 660 nm) on the tongue tissue of histologically healthy mice and found that the tissues maintained normal histological characteristics. In addition, histological assessments carried out in animals sacrificed after 7 days, demonstrated the remission of oral lesions with neoformation of filiform papillae and absence of *C. albicans* in the tissues.

Moreover, the gene expression of inflammatory cytokines analyses demonstrated an increase in tumor necrosis factor-α (TNF-α) expression in the group with oral candidiasis that underwent PDT with PS encapsulated in cationic nanoemulsion (P+L+ ClAlP NE) when

compared with the control group (P-L-). These results are in agreement with the literature studies that show that the efficacy of PDT seems to be strongly related to the degree and nature of inflammation induced by therapy [35] and that PDT may induce a short term inflammation characterized by an increase in the cytokine TNF-α expression [36 -38]. Thus, it is possible to suggest that antimicrobial PDT induces an increase of TNF-α by increasing the inflammatory reaction. The inflammatory response is a host response that restores the integrity of the tissue by removing dead or damaged cells to accelerate tissue healing, maintaining homeostasis [39]. These findings can be considered positive since, in accordance with the macroscopic analysis, the animals that underwent PDT showed rapid resolution of inflammation of oral lesions. Furthermore, the first stage of *C. albicans* infection induces local production of TNF-α, which can mediate the sequence of inflammatory reactions [40].

Furthermore, PDT mediated by ClAlP DMSO with and without fungal inoculation (P+L+ ClAlP DMSO, P+L+ ClAlP DMSO NI, respectively) and the group treated only with PS without application of light (P+L- ClAlP DMSO) showed increased of TNF-α expression. This can be explained due to the presence of 10% DMSO. It has been reported that this compound alone is capable of causing damage to the cell membrane, dissociation of the extracellular collagen matrix [41-42] and cell death by apoptosis of the host cells [43], starting an inflammatory cascade that makes its citotoxicity evident. Similarly, TNF-α expression also increased in the group that received only light (P-L+). This production can be associated to oxidative stress generated by the light, leading to a low intensity inflammatory response.

In addition, the expression of cytokines IL-1β and IL-6 was similar in all groups evaluated in comparison with positive control (P-L-). These findings can be explained because the peak expression of these cytokines occurs at different time intervals after treatment. It has been reported that the peak of IL-1β and IL-6 release can occur within 6 to 8 hours after procedures that promote inflammatory reactions [43]. Since the animals in the present study were sacrificed 24 hours after the treatment, it is possible that the expression reached a plateau in 8 hours and decreased after this period. Another aspect that should be emphasized is that only one application of PDT was performed during this research and this may explain the similarity of the IL-1β and IL-6 cytokine expression among all groups evaluated.

Considering the results of this research, the delivery system using cationic nanoemulsion seems to be an effective vehicle for the distribution of phthalocyanine and PDT mediated by PSencapsulated was effective to inactivate *C. albicans* on the tongue dorsum of mice with induced oral candidiasis, as well as promoting the remission of candidiasis lesions.

Although the histological analysis has shown that PDT did not cause adverse effects to the tongue tissue, the biomolecular analysis revealed that this treatment caused the expression of pro- and anti-inflammatory cytokines. The level of expressed cytokines was not sufficient to cause significant histological changes. These results may be due to the single application of PDT. Therefore we suggest that the degree of inflammation induced by PDT and subsequent contribution to antimicrobial effectiveness depends on several parameters such as the chemical structure of the delivery system used and the amount of applications of PDT. These findings encourage further studies to evaluate the effectiveness of successive applications of PDT mediated by chloro-aluminum phthalocyanine entrapped in cationic nanoemulsion in animal model of oral candidiasis and more detailed identification of the molecular mechanisms involved in the effectiveness of this approach. In the future, these parameters will assist randomized clinical trials to be conducted safely and effectively.

Acknowledgements

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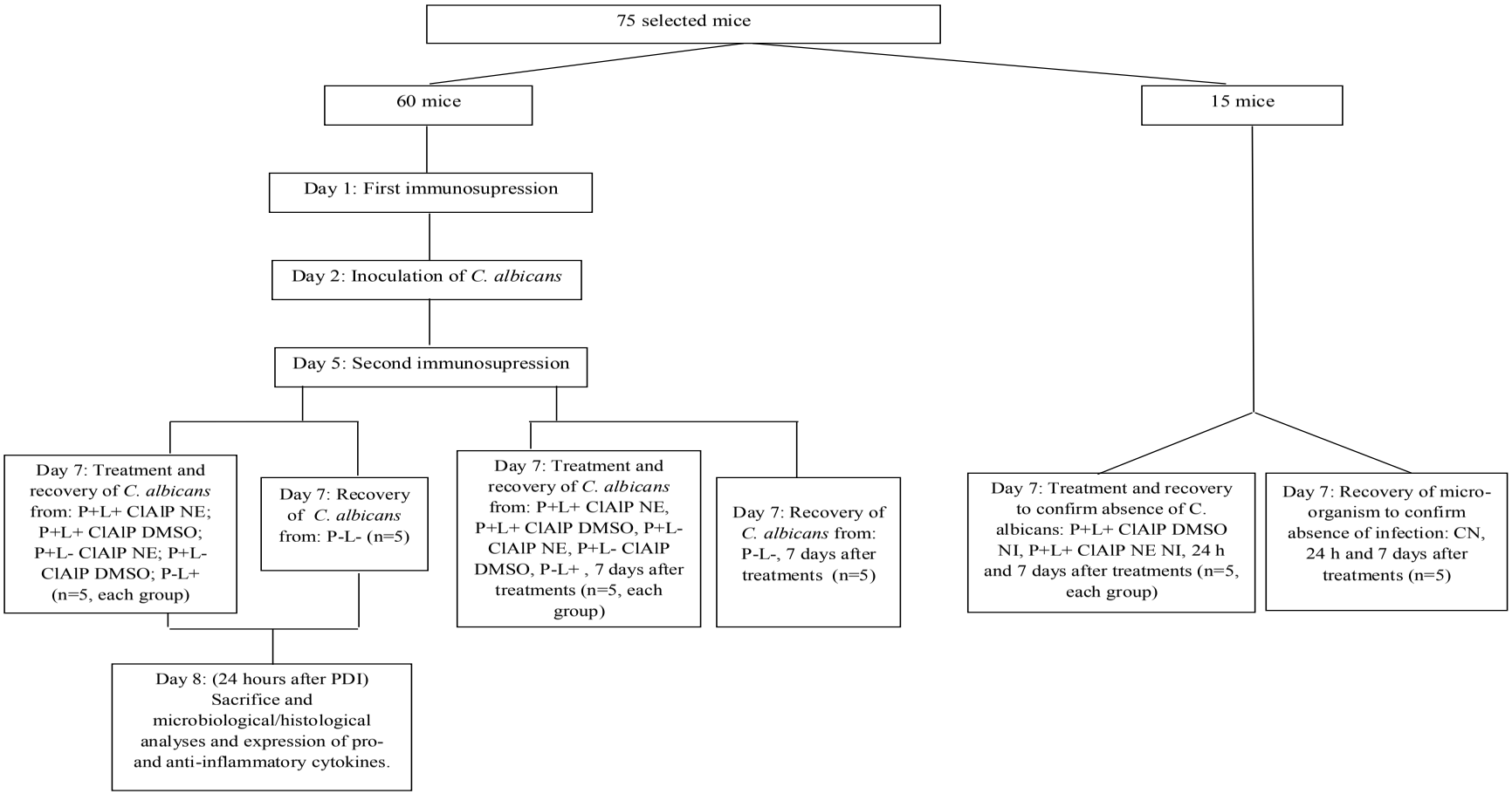

Fig. 1- Study desing: Tetracycline hydrochloride was given in drinking water during days 1 to 7; The P+L+ groups corresponded to the animals treated with both of PS and LED light (P+L+ ClAlP-NE and P+L+ ClAlP-DMSO); The P-L+ group corresponded to evaluation of LED light alone; The P+ L- groups corresponded to evaluation of the PS alone (P+L- ClAlP and P+L- ClAlP-DMSO); The P-L- group corresponded to infected animals that did not receive any treatment; Two experimental groups consisting of healthy animals that were treated with PDT (P+L+ ClAlP-NE NI and P+L+ ClAlP-DMSO NI); the NC group corresponded to the healthy and untreated animals.

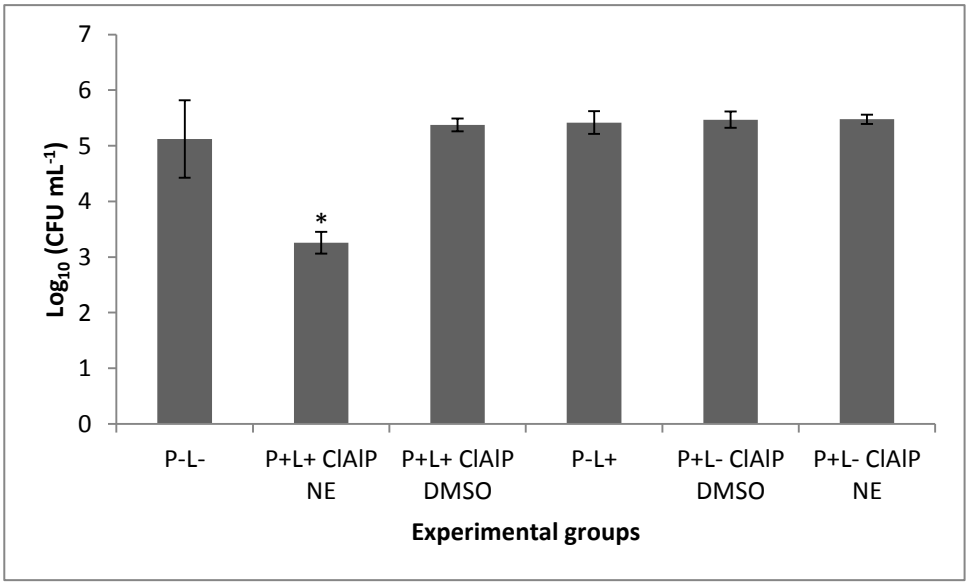

Fig 2: Mean Log_{10} (CFU mL^{-1}) of the groups evaluated 24 hours after treatments. * The analyses of variance (ANOVA) followed by Tukey post-hoc demonstrated statistically significance difference between P+L+ ClALP NE and P-L- group ($p<0.05$).

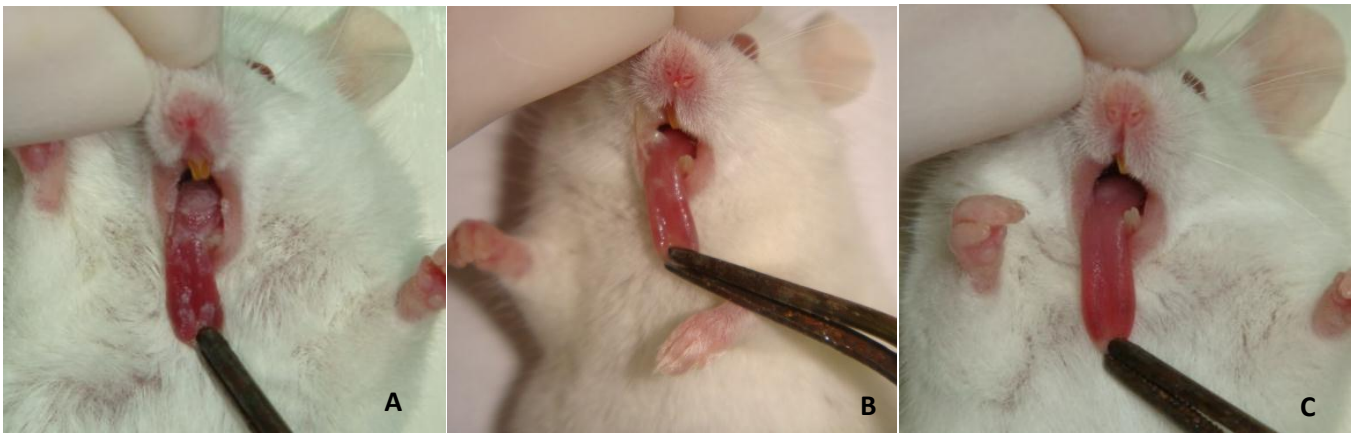

Fig. 3 - A: Representative image of the presence of white patches/pseudomembranes on the tongue dorsum of all animals after inoculation with *C. albicans*. B: Representative images of animals treated with PDT mediated by ClAlP NE, 24 hours after treatment. C: Representative image of the absence of white patches/pseudomembranes on the tongue dorsum of all animals 7 days after PDT and of the animals in the groups that were not infected by *Candida*.

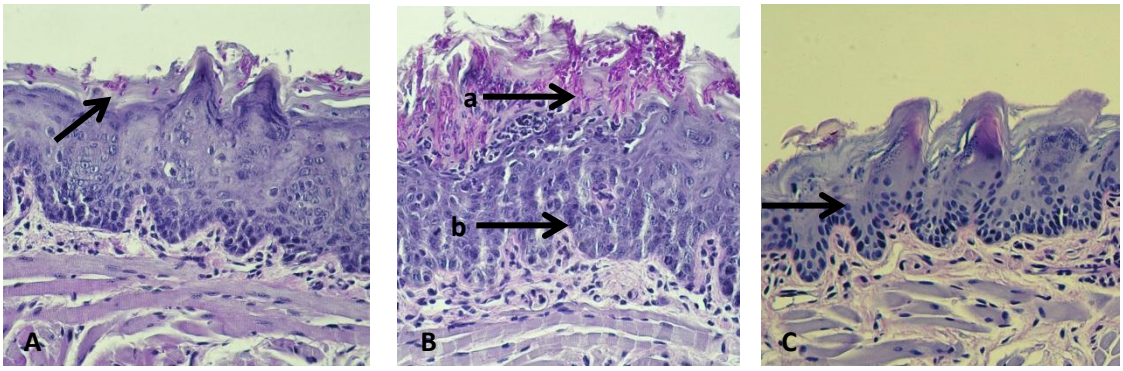

Fig. 4: Histological characteristics observed on tongues from *C. albicans* infected and not infected animals, subjected or not to PDT. A: Presence of some hyphae/pseudohyphae and blastospores (arrow) on the keratin layer presenting discrete inflammatory reaction (P+L+ ClAlP NE group). B: Presence of numerous hyphae/pseudohyphae on the keratin layer (arrow a) of groups P+L-, P-L+, P-L- and P+L+ClAlP DMSO presenting moderate inflammatory reaction (arrow b). C: Normal histological characteristics such as thick keratin layer, intact epithelium (arrow), connective tissue with numerous fibroblasts amid collagen and capillaries and absence of microorganisms were observed in the groups not infected by *C. albicans* and in all groups evaluated 7 days after treatment.

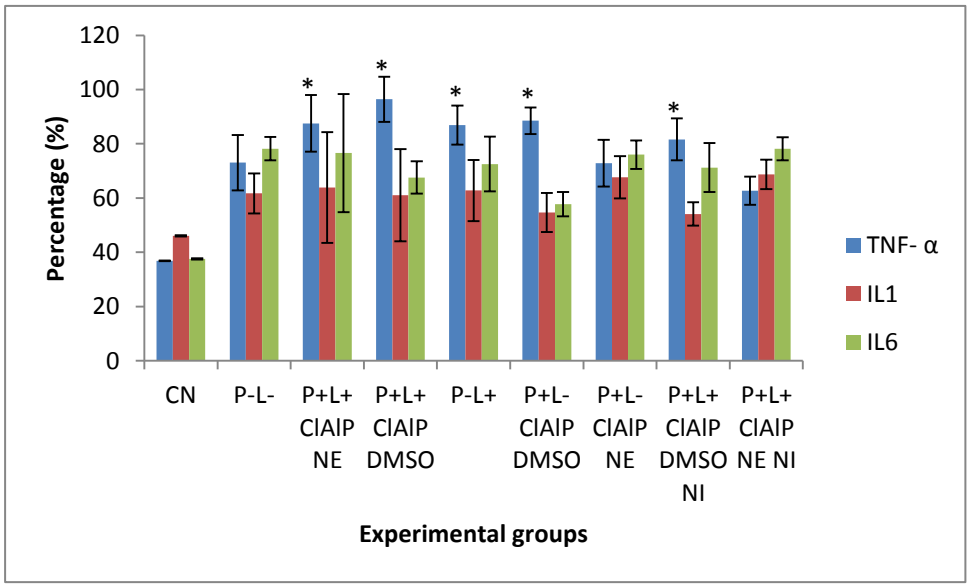

Fig. 5 - Mean and standard deviation in percentage (%) of gene expression of each cytokine evaluated of all experimental groups in comparison with the control group (P-L-). * The analyses of variance (ANOVA) followed by Tukey post-hoc demonstrated statistically significance difference between P+L+ ClALP NE, P+L+ ClAlP DMSO, P-L+, P+L- DMSO and P+L+ ClAlP DMSO NI in comparison with the control group (P-L-) for TNF-α ($p<0.05$).

3.2 Capítulo 2

Treatment of oral candidiasis using Photodithazine®- mediated photodynamic therapy *in vivo*.

Short title: Treatment of oral candidiasis using Photodithazine.

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*Artigo submetido e formatado de acordo com as normas do periódico **PLOS PATHOGEN**.

Abstract

This study evaluated the photoinactivation (PDT) of *Candida albicans* in a murine model of oral candidiasis using Photodithazine® (PDZ). A new murine model of oral candidiasis was developed to allow the monitoring of the infection and the establishment of the treatment. Six-week-old female mice were immunosuppressed and inoculated with *C. albicans* to induce oral candidiasis. PDZ-mediated PDT and nystatin treatment were carried out for 5 consecutive days with one application per day. The macroscopic evaluation of oral lesions was performed. After each treatment, the tongue was swabbed to recover *C. albicans* cells. Viable colonies were quantified and the number of CFU/ml determined. The animals were sacrificed 24 hours and 7 days after treatment and the tongues were surgically removed for histological analysis and analysis of inflammatory cytokines expression (IL-1, TNF-α and IL-6) by RT-qPCR. Data were analyzed by two-way ANOVA. PDZ-mediated PDT was as effective as Nystatin (NYS group) in the inactivation of *C. albicans,* reducing 3 and 3.2 $logs_{10}$ respectively, 24 h after treatment. Seven days after treatments, the animals treated with PDT maintained the level of *C. albicans* reduction, but the animals treated with Nys showed a significant increase of CFU/mL ($p<0.05$). Animals that underwent PDZ-mediated PDT showed complete remission of oral lesions, 24 h and 7 days after treatments. However, animals treated with NYS presented partial remission of oral lesions. These results were the same 7 days after treatment. Histological evaluation revealed mild inflammatory infiltrate in the groups treated with PDT and NYS in both periods assessed. PDT induced the TNF-α expression when compared with positive control (P-L-) ($p<0.05$), 24 h after treatment. In summary, the murine model of oral candidiasis was able to mimic the infection and PDZ-mediated PDT was effective to treat mice with induced oral candidiasis.

Author Summary

With the widespread use of light-emitting diodes (LED) in medical applications, for the treatment of human disease, concerns have been raised on the safety of this technology in photodynamic therapy (PDT). Thus, we investigated the photodynamic effect of Photodithazine against *Candida albicans* in a new murine model of oral candidiasis. Studies have shown that PDT mediated by Photodithazine is effective for fungal inactivation; however, it is important to point out that studies assessing the interaction between PDT and the host immune response are scarce. We also assessed the host response toward antimicrobial

PDT by analyzing the gene expression of pro- and anti-inflammatory cytokines to evaluate inflammation after PDT application. We found that PDT was effective for inactivation of *C. albicans* without causing any harmful effects on host tissues. Our results may contribute to the development of more effective protocols for *in vivo* photoinactivation of *Candida* spp, which is promising for future clinical trials.

Introduction

Candida species are part of the normal oral microbiota [1] being *Candida albicans* the most common species associated with clinical manifestations, which range from superficial mucocutaneous lesions, such as oropharyngeal candidiasis (OPC), to disseminated forms of the infection [2]. OPC is the result of adhesion and penetration of fungal species into the oral tissues [3] and it has a high incidence in patients that use immunosuppressive drugs, broad-spectrum antibiotics, anti-diabetic mediations, anticancer therapies, and in patients with the acquired immunodeficiency syndrome (AIDS) [4].

The widespread use of topical (azole) or systemic antifungal agents (polyenes) for the treatment of OPC has resulted in the development of resistant *Candida* species [5]. Additionally, the organization of microorganisms in biofilms is a protective shell, enabling the survival of these pathogens even in unfavorable conditions and providing high resistance to antifungal agents [6]. Considering the increased incidence of resistant pathogens to conventional antifungal treatments and drug toxicity, studies have searched for strategies to control fungal species.

Photodynamic therapy (PDT) is a common anticancer treatment that has been indicated for the inactivation of microorganisms [7-9]. The mechanism of action of PDT requires the use of a photosensitizing agent (PS), the application of light corresponding to the absorption band of PS, and the presence of oxygen [8]. The interaction of the appropriate wavelength of light with PS in the presence of oxygen results in reactive oxygen species (ROS) capable of inducing death of microorganisms by oxidative damage [10].

The photosensitive drugs used in the antimicrobial PDT are predominantly from the families of porphyrins [11], such as 5-aminolevulinic acid (ALA) [12] and phenothiazines [13]. The use of distinct PSs have shown effects on viability of *C. albicans,* for example, methylene blue (450 and 500 mg/L) associated with laser light promoted 3 $\log_{10}$ of reduction in the *C. albicans* viability [14], while Photogem® (500 and 1000 mg/L) associated with LED was able to reduce 1.59 $\log_{10}$ in the viability of *C. albicans* [15]. In these studies the reduction

in the number of microorganisms was only achieved when PDT was applied together with high concentrations of PS.

Photodithazine® (PDZ), a second-generation photosensitizer, is a glucosamine salt of chlorin (e_6) derivative and soluble in water [16]. This PS has short sensitization periods and high absorption band in the electromagnetic spectrum (650-680 nm), which allows greater depth into the biological membranes improving action of PDT [17], and producing higher levels of singlet oxygen [18]. *In vitro* studies have shown that PDZ associated with visible light was effective in the inactivation of cell suspensions of *C. albicans* and *Candida. guilliermondi* [19-20], and single [21] and mixed biofilms of *Candida* spp [22]. Moreover, an in vivo study showed that 100 mg/L of PDZ associated with LED light reduced $4.36\log_{10}$ of the cell viability of *C. albicans* present on the tongue of mice with induced oral candidiasis [23]. However, only one application of PDT was made and the follow-up of the clinical evolution of *Candida* lesion was not performed.

Thereby, given the prevalence of OPC and the clinical similarity of this infection between mice and human host [24], it is essential to carry out *in vivo* studies that present infections caused by fungal biofilms, so that conditions close to the clinical situations can be simulated and evaluated. In a murine model of oral candidiasis used in previous studies [15, 23, 25-26], the lesions on the dorsum of the tongue are maintained for up to 7 days and the effectiveness of the antifungal treatment is assessed 24 hours after treatment administration. For this reason, in the present investigation, it was developed an animal model of oral candidiasis in which induced infection was maintained over a longer period of time. Thus, it was possible to assess the effectiveness of successive applications of PDZ-mediated PDT, simulating a possible clinical treatment for this infection. In addition, to understand the effect of PDT on the host cells, it was evaluated the gene expression ratio of pro- and anti-inflammatory cytokines after treatments.

Materials and Method

Photosensitizer and light source

The photosensitizer used in this study was PDZ, a chlorin e_6 derivative (VETAGRAND, Co, Russia). Stock solutions of PDZ diluted in carboxiethilcellulose hydrogel (Pharmacy Santa Paula, Araraquara, SP, Brazil) at a concentration of 100 mg/L were prepared, storage at room temperature and kept in the dark until use.

The red LED light device (660nm) (LXHL-PR09, LUXEON® III Emitter, Lumileds Lighting, San Jose, California, USA) was designed by the Physics Institute of São Carlos, USP, (University of São Paulo, São Carlos, SP, Brazil). The light output power delivered at the end of the handpiece (5 mm diameter) was 44.6 mW cm^{-2}.

Animals and experimental oral candidiasis

The study was conducted according to the institutional laws and it was approved by the Ethics Committee on Animal Use, Araraquara Dental School, UNESP (Permit number 24/2012). A total of 126 6-week-old female mice were used. They were kept in individual cages and fed with food and water *ad libitum*.

The reference strain *C. albicans* ATCC 90028 (Rockville, MD) was used to induce oral candidiasis in mice. It was defrosted and reactivated in Sabureaud Dextrose Agar medium culture (SDA) at 37°C for 48 hours. Then the strain was cultured in tubes with 5 mL of RPMI 1640 at 37°C for 24 hours. The tubes were centrifuged at 2000 rpm for 10 minutes and the supernatant discarded. The cells were centrifuged at 2000 rpm for 10 minutes, rinsed with sterile saline and resuspended in 2 ml of sterile saline (10^7 CFU/ml).

To induce infection, the animals were immunosuppressed with subcutaneous injections of prednisolone on days 1, 5, 9 and 13 of the experiment at a dose of 100 mg/kg body weight according to the period evaluated (24 hours and 7 days after treatment). Tetracycline (0.83 mg/mL) was administered in the drinking water of animals throughout the trial period. On day 2, the animals were sedated with chlorpromazine hydrochloride 0.1 ml 2 mg/mL and mini-sterile swabs were soaked in *C. albicans* suspension and rubbed on the dorsum of the tongue for 30 seconds to produce oral candidiasis.

Treatments and microbiological evaluation

On day 7, the infection was present as verified by visual inspection (Fig. 2), the animals were randomly divided into groups and treatments were performed daily from day 7 to day 11 (Fig. 1 – flow chart). For this purpose, the animals were anesthetized with an intraperitoneal injection of ketamine at a concentration of 100 mg kg^{-1} body weight (National Pharmaceutical Chemistry Union S/A, Embu, SP, Brazil) and xylazine at 10 mg kg^{-1} body weight (Veterinary JA Ltda., Sponsor Paulista, SP, Brazil). The tongues of the animals were gently taken out of the oral cavity and PDZ diluted in hydrogel (100 mg/L) [23] was applied. The animals remained in the dark for 20 minutes (pre-incubation time). After this period, the

tongue of the mice was illuminated with LED light for 14 minutes, resulting in total fluency equivalent to 37.5 J/cm² (P+L+ group) [23]. The effect of the isolated application of PS (P+L-) and the LED light (P-L+) was also evaluated. The positive control group (P-L-) did not receive any PS or light. The negative control group (NC), did not receive *C. albicans* inoculation or any treatment. Additionally, there was another group in which animals were treatment with topical antifungal nystatin 100,000 IU oral suspension (NYS). The drug was applied on the dorsum of the tongues of the anesthetized animals, remaining in contact with the tissue for 34 minutes once a day for 5 days. Twelve animals were used for each experimental condition, except in the group NC (n = 3).

After 5 consecutive days of treatment, *C. albicans* cells were recovered from the tongue of mice. For this, the tongues were swabbed for 1 minute. The swabs were transferred to tubes containing 1 ml of saline solution and vortexed during 1 minute to detach *C. albicans* cells. Then, serial dilutions were made from the samples contained in the tubes, and plated in duplicate onto SDA culture medium with 5 μg/ml of chloramphenicol. After 48 hours of incubation at 37°C, the viable colonies were counted and the values of CFU/ml were determined.

Macroscopic analysis of candidiasis lesions

The lesions on the tongue of the animals were also assessed by standardized photographs taken before the beginning of the proposed treatments, 24 hours and 7 days after the application. All the photographs were taken with the same digital camera (Sony Cyber-Shot DSC-F717; Sony Corporation, Tokyo, Japan) by the same operator and under the same conditions (place, light, angle and position of mice) to facilitate reproducibility. Each photograph was classified in accordance with the scores proposed by Takakura et al. [25]: 0 = healthy mucosa; 1 = white patches on up to 20% of the tongue; 2 = white patches on 21 to 90% of the tongue; 3 = white patches on over 91% of the tongue; 4 = thick white patches or pseudomembrane on more than 91% of the tongue.

Sacrifice of animals

The animals were sacrificed 24 hours (Day 12) or 7 days (day 18) after application of the proposed treatments by intramuscular injection into the femur region, with a lethal dose of ketamine (0.2 mL). After the sacrifice, the tongue of each animal was surgically removed and standardized longitudinal sections were performed. The samples were then subjected to histological analyses and RT-qPCR.

Histological analysis

After fixation in 10% formalin, the samples were stained by periodic acid-Schiff reagent and hematoxylin and eosin followed by examination under a light microscope at 200 and 400X magnification. Histological changes were evaluated according to the intensity of inflammation, determined by the presence of inflammatory cells, alteration of the population of resident cells on the connective tissue, characteristics of the amorphous intercellular substance and intercellular fibrous substance, according to the following scores: 0: inflammation absent; 1: mild inflammation; 2: moderate inflammation; 3: severe inflammation; 4: abscess formation(ISO 7405:1997). The evaluation was performed by a single examiner blinded to each experimental group at the different time intervals assessed.

Assessment of gene expression of inflammatory cytokines by RT-qPCR

After storage of the samples in Trizol, 0.1 ml of chloroform (Sigma-Aldrich, St. Louis, MO, USA) was added to each sample and after homogenization the samples were incubated for 3 minutes at room temperature. The samples were then centrifuged at 12,000 rcf (Eppendorf microcentrifuge, Model 5415R, Hamburg, Germany) for 15 minutes at 4°C. After centrifugation, the aqueous phase containing RNA was transferred to a new tube, followed by addition of 0.5 mL of isopropanol (Sigma-Aldrich) to promote RNA precipitation. The samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 rcf for 10 minutes at 4°C. We added 1ml of 75% ethanol (Sigma-Aldrich) followed by centrifugation at 7,500 rcf for 5 minutes at 4°C. The supernatant was discarded before air drying the RNA pellet for 45 minutes. The RNA was resuspended in 10 μL of ultra-pure water (Invitrogen) and the samples were incubated for 10 minutes at 55°C (Thermomixer Comfort - Eppendorf, Hamburg, Germany). Then the total RNA obtained was purified using the QiagenMini-Elute kit in accordance with the manufacturer's instructions. The RNA quality and quantity was measured using OD 260 nm and the ratio OD260/280, respectively.

For each total RNA sample, the cDNA was synthesized using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems) according to manufacturer's recommendations. The cDNA was stored at -20°C until completion of the RT-qPCR.

After cDNA synthesis, the effect of PDT on the expression of pro- and anti-inflammatory cytokines (IL-1β, IL-6 and TNF-α), involved in the response of superficial infections, was evaluated. Specific primers were selected for each target gene (Taqman Assay

- Applied Biosystems): βActin = Mm00607939_S1; TNF = Mm00443260_G1; IL-1β = Mm00434228_M1; IL-6 = Mm00446190_M1.

It was used the Taqman assay (Applied Biosystems) for the RT-qPCR reaction (14µL), 1 µL of each specific oligonucleotide for each amplified gene and 1 pg of cDNA from the different samples. Amplification assays were performed in the Step One Plus thermal cycler and amplification cycles were analyzed and determined by the Step One Plus software (Applied Biosystems - Foster City, CA, USA). The process of thermal cycling consisted of 2 minutes at 50°C, 2 minutes at 95°C for reverse transcription, and 40 cycles of 15 seconds at 95°C, 30 seconds at 57°C and 60 seconds at 30°C. In all experiments negative controls were included to rule out possible contamination of RNA or genomic DNA. The RT-qPCR conditions were optimized for each target with optimal concentrations of oligonucleotides, absence of dimer formation of oligonucleotides and efficient amplification of target genes and β-actin control genes. Data related to cytokine expression of the groups receiving any treatment were compared with the negative control (healthy animals) and positive group (infected animals). All RT-qPCR experiments were performed according to the recommendations of the "MIQE Guidelines for qPCR" [27].

Statistical analysis

C. albicans counts (cfu/ml) were transformed into base-10 logarithms. As the data were normal and heterocedastic, the values were analyzed by two-way ANOVA with the treatment performed at different time intervals (24 h and 7 days after treatment) as main effects. There was interaction between the factors, treatment group and time interval, so one-way ANOVA with Welch correction followed by Games-Howell post-hoc test for multiple comparisons was performed. The Spearman correlation test ($p < 0.01$) was used to correlate the number of colony forming units (CFU/mL) with the scores assigned to the lesions on the tongues of animals infected with *C. albicans*.

Standard photographs were evaluated by two independent observers blinded to the treatment groups and experimental period. These observers were instructed to classify each photograph of the tongue as score 0 (healthy) or scores 1, 2, 3 or 4. The κ test was used to assess the agreement between the observers and the value was considered significant ($\kappa = 0.73$, $p \leq 0.0001$).

The data obtained from RT-qPCR for TNF-α cytokine and IL-1 β met the assumptions

of normality and homogeneity of variances. Thus, the two-way ANOVA followed by Tukey's test was used to compare the groups according to the different time intervals assessed ($p < 0.05$). For the cytokine IL-6, the data were normal and heterogeneous. Therefore, the data were submitted to two-way ANOVA test. As there was interaction between the factors, treatment group and time interval, data were submitted to one-way ANOVA test with Welch correction followed by Games-Howell post-hoc test for multiple comparisons. The significance level adopted was 5%.

Results

Model establishment

The murine model of oral candidiasis was successfully developed, which could be confirmed by the presence of white patches or pseudomembrane on the tongue of all immunosuppressed animals infected with *C. albicans* (score 3) in the control group throughout 16 days (Fig. 2). The severity of the lesions was assessed at day 7 until the last day of the experiment and it was recovered 10^5 CFU/ml of *C. albicans* from the oral cavity of animals (Fig. 3). Histopathological analysis showed presence of moderate inflammatory infiltrate with extensive colonization of hyphae/pseudohyphae on the entire keratin layer, invading the epithelial tissue of the tongues. Fig. 4 shows that a significant positive correlation was found between the scores assigned to oral lesions of the animals infected with the number of CFU /mL within the 5 to 16-day period pós- infection ($R = 0.92$; $p \leq 0.0001$).

The behavior of the infected mice was not altered after induction of oral candidiasis. The animals continued feeding and drinking water normally, there was no weight loss or any changes in the amount or volume of urine and feces. It is important to mention that the method developed in the present study did not cause the death of any animal.

Effectiveness of treatments performed

PDZ-mediated PDT and treatment with NYS promoted significant reduction in cell viability of approximately 3 and 3.2 $\log_{10}$, respectively, when compared to the positive control group (P-L-) ($p \leq 0.0001$ and $p \leq 0.0001$ respectively), 24 hours after the treatments. Seven days after treatment, the reduction in $\log_{10}$ remained statistically similar to the one observed in the 24-hour time interval in the group treated with PDT ($p \geq 0.601$). Regarding the group treated with NYS, there was a significant increase in the number of CFU/ml 7 days after treatment ($p \leq 0.001$), which suggests recurrence of infection for the animals treated with NYS.

Moreover, no statistical difference was found between the P-L-, P-L+ or P+L- groups throughout the experimental period ($p \geq 0.05$) (Fig. 5).

Twenty-four hours after completion of treatment (5 applications), all animals treated with PDT presented total remission of lesions (score 0) (Fig. 6A). For the group treated with NYS, the animals showed partial remission of lesions (score 1) (Fig. 6B). The animals treated with PDZ or light (P+L- and P-L+, respectively) presented white patches on the tongue (score 3). The macroscopic assessment of lesions 7 days after completion of treatment revealed that the scores attributed to lesions remained the same, irrespective of the groups.

The histopathological events observed on the tongue tissues of the animals 24 hours after treatments showed the presence of mild inflammatory infiltrates (score 1) for groups treated with PDT. The group that received NYS presented some hyphae/pseudohyphae on the layer keratin without fungal invasion into the epithelial tissue (Fig. 7A). The other groups (P-L+, P+L-) presented similar histopathological characteristics observed in the positive control group (P-L-) with moderate inflammatory infiltration and the presence of numerous hyphae/pseudohyphae on the entire keratin layer, and some hyphae/pseudohyphae invading the epithelial tissue of the tongues (Fig. 7B). The histological analysis 7 days after treatments proved to be similar to the one performed 24 hours after treatments.

Gene expression of TNF-α, IL-1-β and IL-6

A significant increase in TNF-α expression was found in the animals submitted to the PDT (P+L+) ($p \leq 0.014$) and NYS ($p \leq 0.007$) in comparison with the positive control group. No significant difference was found between the group treated with PDT and the one treated with NYS ($p = 0.605$). These results were observed 24 hours and 7 days after treatment. The group treated only with light (P-L+) also showed similar cytokine expression in comparison with the positive control group ($p = 0.530$), and the group treated with PDZ showed low cytokine expression similar to the negative control group ($p = 0.999$) at both time intervals (Fig. 8). There was no significant difference in the interaction among the experimental groups and period assessed ($p \geq 0.05$).

The animals submitted to PDT showed low expression of IL1-β cytokine in comparison with the positive control group ($p = 0.021$). The expression of this cytokine in the group of animals treated with NYS was similar to the control group ($p = 0.979$). The group treated only with light (P-L+) also showed cytokine expression similar to the control group ($p=0.996$). The P+L- group showed low cytokine expression similar to the negative control

group (p = 1.000). The above results were observed at both time intervals (Fig. 9). No significant difference was found in the interaction among the experimental groups and time interval assessed (p≥0.05).

As for the expression of IL-6 cytokine, the group treated with NYS showed similar expression as the control group 24 hours after treatment (p≥0.953). The other groups showed the expression similar to the negative control group [P+L+ (p = 0.967), P+L- (p = 0.146) and P-L+ (p = 0.272)]. Seven days after treatment, the cytokine expression in all animals evaluated was at baseline, similar among each other and the NC group (p≥ 0.05) (Fig. 10). A significant difference was found in the interaction among the experimental groups and time interval assessed (p≤0.05).

Discussion

The proposed model for the induction of oral candidiasis in immunocompromised mice used in the present investigation is reproducible since all animals infected with *C. albicans* showed white patches or pseudomembrane on the dorsum of the tongue within 5 to 16 days after inoculation. In order to allow the monitoring of infection and the establishment of the treatment, animals were immunosuppressed 4 times with prednisolone. It was collected 10^5 CFU/ml of *C. albicans* from the oral lesions of the animals from the P-L- group (positive control) throughout the experimental period. Histopathological analysis showed presence of moderate inflammatory infiltrate with numerous hyphae/pseudohyphae over the entire keratin layer, and some hyphae/pseudohyphae invading the epithelial tissue of the tongues. These findings are similar to those obtained by Takakura et al., [25] who also developed a method for inducing oral candidiasis in immunosuppressed mice and obtained 10^5-10^6 CFU/ml of *C. albicans* from the oral lesions of the animals. The histopathological analysis demonstrated the presence of severe inflammatory infiltrate with numerous hyphae, fungal invasion and destruction of epithelial layers. These findings can be explained because the strain used by Takakura et al., [25] was a clinicall isolated from patients with cutaneous candidiasis, whereas in the present study it was used a reference strain of *C. albicans* for the fungal inoculation. The relation between the scores attributed to the oral lesions and the CFU/mL values was assessed by the Spearman's correlation coefficient (R=0.92, n=12, 5-16 days after infection). This analyses showed a high coefficient value, confirming that the high number of white patches and/or pseudomembrane on the tongue reflected the degree of *C. albicans* colonization in the oral cavity of the animals infected. In the study conducted by Takakura et al., [25] the authors also correlated the microbiological data with the degree of oral lesions

and observed a high correlation coefficient between these two factors (R=0.75, n=26), as shown in this study. The results confirm that the induction of the oral candidiasis model is appropriate for reproducing the infection over an extended period of time, which allows the assessment of the effectiveness of treatments.

Other methodologies have also been proposed to induce oral candidiasis in animals [14, 28]. Teichert et al., [14] used a severe immunodeficiency protocol for inducing oral candidiasis and recovered approximately 2 x 10^2 CFU mL^{-1} of *C. albicans*. Totti et al., [28] used sialoadenectomized mice to induce oral candidiasis. The authors performed four inoculations with a suspension of *C. albicans*. Among the six animals used for each experimental group, only 2 presented fungal colonization up to 60 days after the infection and only 1 animal up to 75 days after infection. The histopathological analysis revealed the presence of pseudohyphae invading the epithelium, acanthosis and intraepithelial neutrophilic infiltration forming micro-abscesses for the animals that developed oral candidiasis [28]. Although this method facilitate longer colonization of *C. albicans*, it is not possible to establish a direct comparison with the present study as the number of CFU/ml from the oral lesions of the animals was not mentioned. Furthermore, in the present investigation, all mice showed typical lesions of oral candidíasis throughout the experimental period. In the study conducted by Totti et al., [28] the infection was not maintained throughout the desired period in most animals.

In the present study, the results showed that PDZ-mediated PDT was as effective as NYS to inactivate *C. albicans* present in the oral lesions of mice with experimental oral candidiasis. The association of 100 mg/L of PDZ with 37,5J/cm^2 of light promoted a reduction of 3 $logs_{10}$ in the cell viability, while NYS showed 3.2 log_{10}, when compared with the control group (P-L-). These results were observed 24 hours after the treatments and maintained up to 7 days after treatment in the group treated with PDT. As for the animals treated with antifungal NYS, an increase in the number of CFU/mL was observed 7 days after treatment and the log_{10} reduction was equivalent to 1.7 log_{10} in comparison with the control group, suggesting recurrence of infection. These findings are in agreement with the studies that reported frequent recurrence in patients with oral candidiasis weeks after treatment [29-31]. In the present investigation, the antifungal NYS was used as a comparison parameter, as it is considered the most commonly used topical antifungal for the treatment of oral candidiasis [32].

According to the literature, this is the first study that evaluated the effectiveness of successive applications of PDT for the treatment of oral candidiasis in mice. Thus, it is only

possible to make indirect comparisons with the studies reported in the literature as they only used a single application of PDT. Several photosensitizers have been evaluated for photoinactivation of *C. albicans* present in oral lesions in experimental oral candidiasis in mice, such as methylene blue [14], porphyrins [15], erythrosine [33] curcumin [26] and different results have been observed after single application. Carmello et al., [23] evaluated the effectiveness of PDZ (100 mg/L) associated with LED light (37.5J/cm^2) *in vivo* and obtained a 4.36 $\log_{10}$ reduction in the cell viability after a single application. These findings do not agree with those obtained in the present study because the five successive applications of PDT performed here, using the same PDZ concentration and light dose of the study conducted by Carmello et al., [23], promoted 3 $\log_{10}$ of reduction. Hypothetically, 5 applications of PDT should produce more reactive oxygen species (ROS) in cells, which would cause a greater reduction in cell viability when compared with a single application. However, it has been reported that fungal cells have the ability to adapt and protect themselves from oxidative stress and two mechanisms seem to be involved: the first one is related to a decrease in cell membrane permeability of ROS and the second is related to the increase in the regulation of antioxidants and other associated enzymes [34].

On the other hand, macroscopic analysis showed that 24 hours after completion of treatment, all animals submitted to PDT presented total remission of lesions (score 0). In the group treated with NYS, the animals showed partial remission of lesions (score 1) and the other groups showed extensive lesions on the entire dorsum of the tongue (score 3). These results were maintained up to 7 days after completion of treatment. In the present study, PDT was also more effective than NYS in the remission of lesion 24 hours and 7 days after treatment. One possible hypothesis to explain these findings is the biostimulator effect of red LED light. It has been reported that the red LED light is absorbed by components of the cellular mitochondrial respiratory chain, resulting in increased of ROS and adenosine triphosphate (ATP) or cyclic AMP, initiating a signaling cascade that promotes cell proliferation and cytoprotection [35-36]. More specifically, this type of light acts on the cells through the primary photoaceptor: cytochrome c oxidase, the terminal enzyme of the mitochondrial electron transport chain [37-38]. The absorption of light by the cytochrome c oxidase promotes increased amount of ATP and ROS, which leads to increased energy availability and signal transduction [39-40]. These biochemical changes have macroscopic effects, such as increased cell proliferation and consequently the rapid remission of infectious lesions. Thus, it is possible to suggest that the PDZ-mediated PDT associated with red LED light seems to have stimulated cell proliferation on the tongue tissue of animals, which would

explain the complete remission of the oral lesions in the animals treated with PDT, which did not occur in those treated with NYS. It is also important to mention that the pharmacodynamic and pharmacokinetic effects of NYS in a murine model of oral candidiasis are not completely elucidated in the literature.

Histological analysis of the tongues removed 24 hours after the treatment revealed the presence of mild inflammatory infiltrates (score 1) in the groups treated with PDT. The group that received NYS presented some hyphae/pseudohyphae on the keratin layer, without fungal invasion in the epithelial tissue. The other groups (P-L+, P+L-) were similar to the positive control group (P-L-), which showed moderate inflammatory infiltrate with the presence of numerous hyphae/pseudohyphae over the entire keratin layer, and some hyphae/pseudohyphae invading the epithelial tissue of the tongues. Histological changes found in this study are characteristic of oral candidiasis and have also been reported by other authors [13-15, 33]. The results of the study, we suggest that the inflammatory infiltrate found in the treated groups is associated with infection caused by *C. albicans*. Moreover, histological analysis conducted 7 days after the treatments showed to be similar to the one performed 24 hours after treatments.

It has been reported that the earliest stages of *Candida* infection induces the local production of TNF-α [41-43]. Here, the animals submitted to PDT presented TNF-α expression similar to the NYS group and 26% greater than the P-L- group. These results were observed 24 hours and 7 days after completion of treatment. These findings are in agreement with the studies that showed that the effectiveness of PDT may be related to TNF-α stimulation [44-47]. According to those authors, a slight increase in TNF-α expression can be considered beneficial since the fungal cells may have been killed or removed by cells present during the inflammatory reaction, promoting the rapid resolution of oral lesions.

In addition, we evaluated the expression of IL-1β and IL-6. The results showed that IL-1β expression in the group treated with PDT was lower than in the P-L- group and the group treated with NYS. As for IL-6, the group treated with PDT showed baseline values of this cytokine, which were similar to the negative control group. The antifungal NYS showed values similar to P-L- group 24 hours after treatments. It is important to point out that these cytokines have a peak release at different times, which may occur within 6 to 8 hours after procedures that stimulate inflammatory reactions [48]. As in this study, as the animals were sacrificed 24 hours and 7 days after treatment it is most likely that the expression of these cytokines would have reached a plateau in approximately 8 hours and then decreased, so the difference in expression could not be detected.

In summary, the oral candidiasis model established in this study was suitable to reproduce the infection. Five successive applications of PDZ-mediated PDT were as effective as the NYS to treat oral candidiasis in immunosuppressed mice. Although histological analysis has shown that the PDT did not cause adverse effects on the tongue tissue, the biomolecular analysis revealed that this treatment promoted TNF-α expression, which seems to be a beneficial factor for the remission of oral lesions. However, more detailed biomolecular techniques are required to evaluate the production of other pro- and anti-inflammatory cytokines at different time intervals to further explain the mechanism of action of PDT in the host. These results reinforce that PDT is a safe alternative for the treatment of superficial fungal infections, such as oropharyngeal candidiasis (OPC), since it has antimicrobial activity without harming the host. In the future, these parameters will assist the safe and effective conduction of randomized clinical trials.

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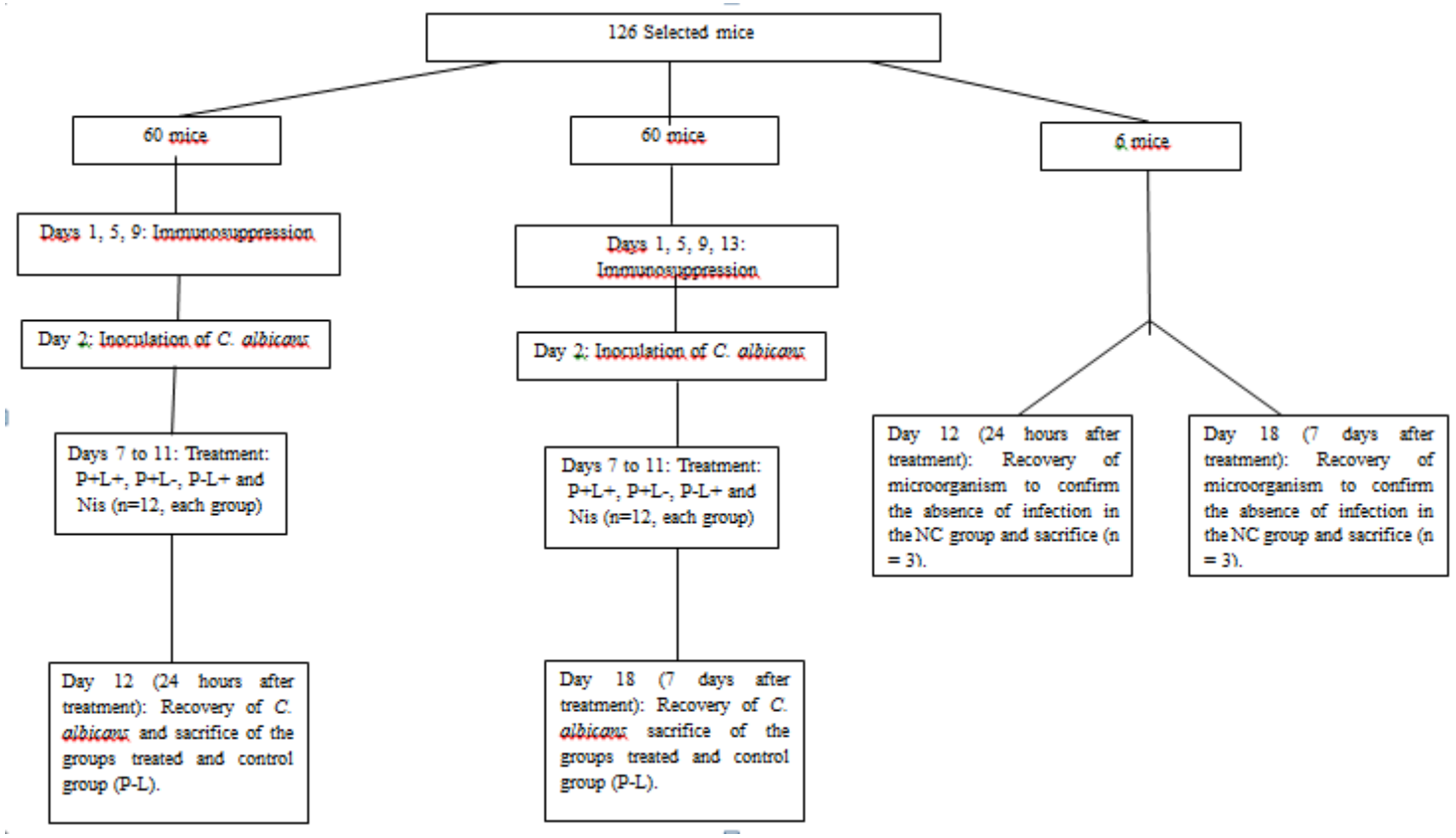

Fig. 1- Study desing: Tetracycline hydrochloride was given in drinking water during days 1 to 18; The P+L+ groups corresponded to the animals treated with PDZ and LED light (P+L+); The P-L+ group corresponded to evaluation of LED light alone; The P+L- groups corresponded to evaluation of the PS alone; The P-L- group corresponded to infected animals that did not receive any treatment; The NC group corresponded to the healthy and untreated animals.

Fig. 2 - White patches or pseudomembrane present on the dorsum of the tongue of the animals infected with *C. albicans* and not treated 5 to 16 days after infection.

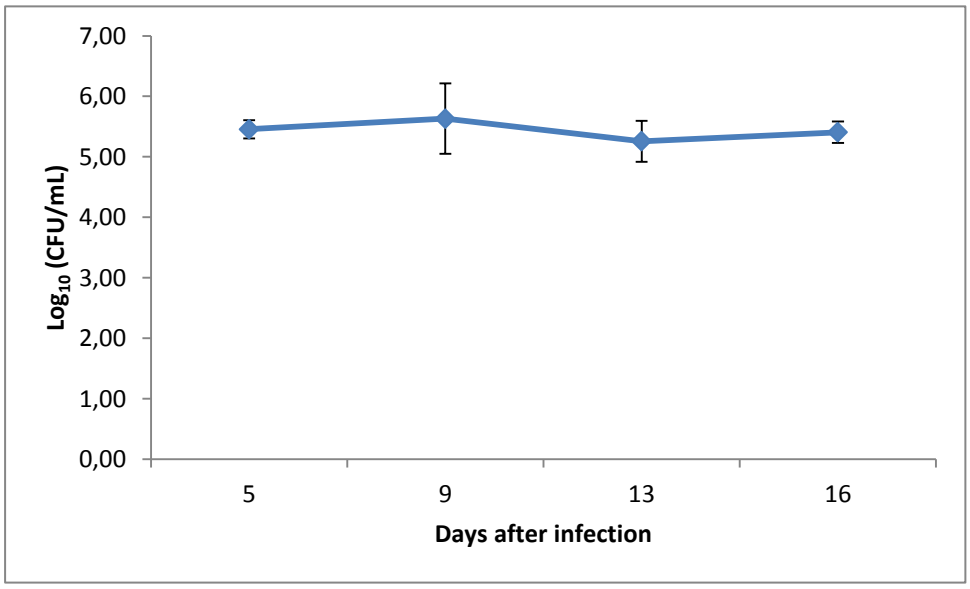

Fig. 3 - Kinetics of *C. albicans* colonization after 5 and 16 days of infection in the newly stablished murine model of oral candidiasis.

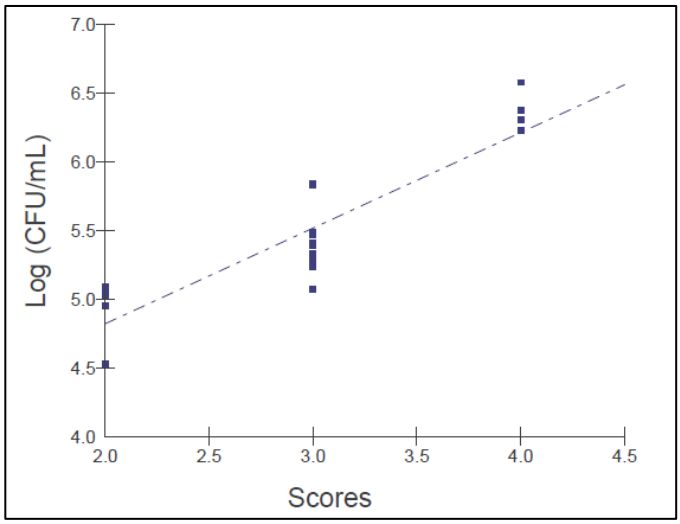

Fig. 4- Correlation between the scores assigned to oral lesions with the CFU number/mL within the 5 to 16-day period pós- infection in the newly murine model of oral candidiasis.

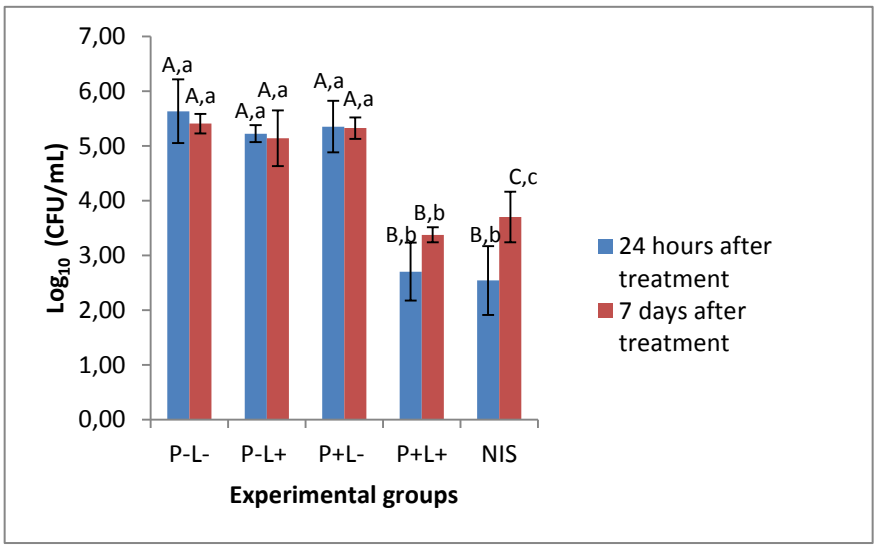

Fig. 5: Mean values of log^{10} (CFU/mL) obtained for the control and experimental groups. The superscript capital letters denote statistical differences between the time intervals assessed (24 hours and 7 days after treatment) and the superscript lowercase letters denote statistical difference between the experimental groups ($p < 0.05$).

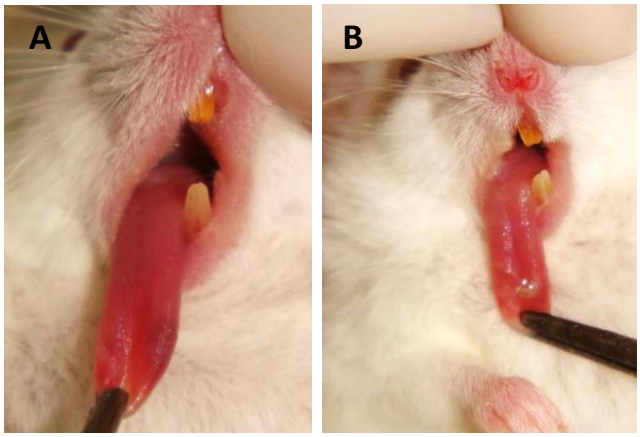

Fig. 6 - Representative image of absence of white patches or pseudomembrane on the dorsum of the tongue of the animals submitted to the PDT (24 hours and 7 days after treatment) (A); representative image of the lesions in animals treated with NYS (24 hours and 7 days after treatment) (B).

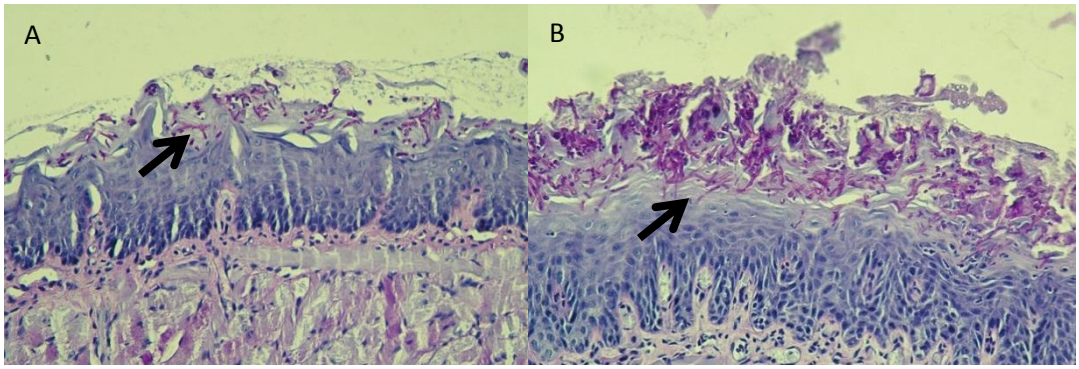

Fig. 7 - Representative images of histological sections in groups treated with PDT and NIS showing mild inflammatory infiltrate with a few hyphae/pseudohyphae on the keratin layer (arrows) 24 hours and 7 days after treatment) (A); Images related to the control group (P-L-) and treated with PS (P+L-) or only light (P-L+) 24 hours and 7 days after treatment (B).

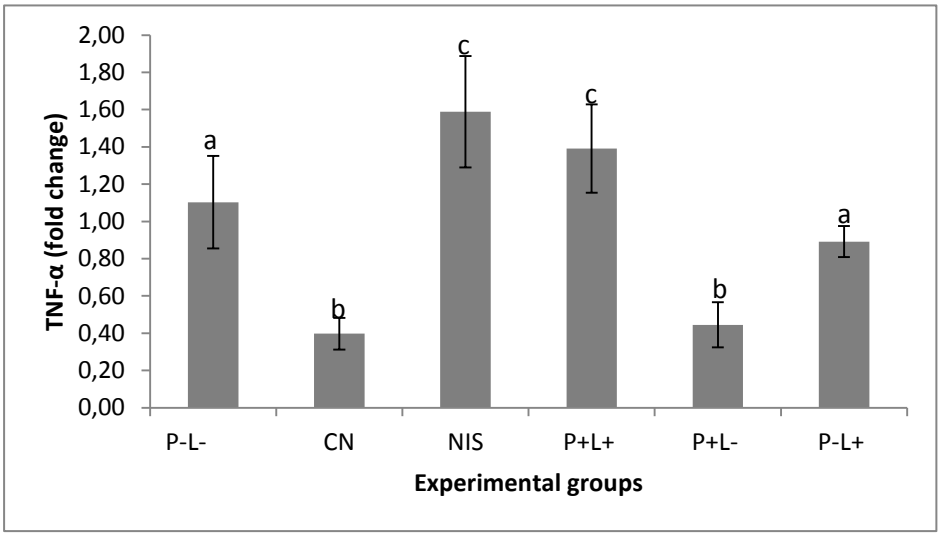

Fig. 8 - Mean values and standard deviation of the TNF-α gene expression in each experimental group during the time intervals assessed (24 hours and 7 days after treatment). Equal superscript lowercase letters denote statistical similarity among the groups ($p<0.05$).

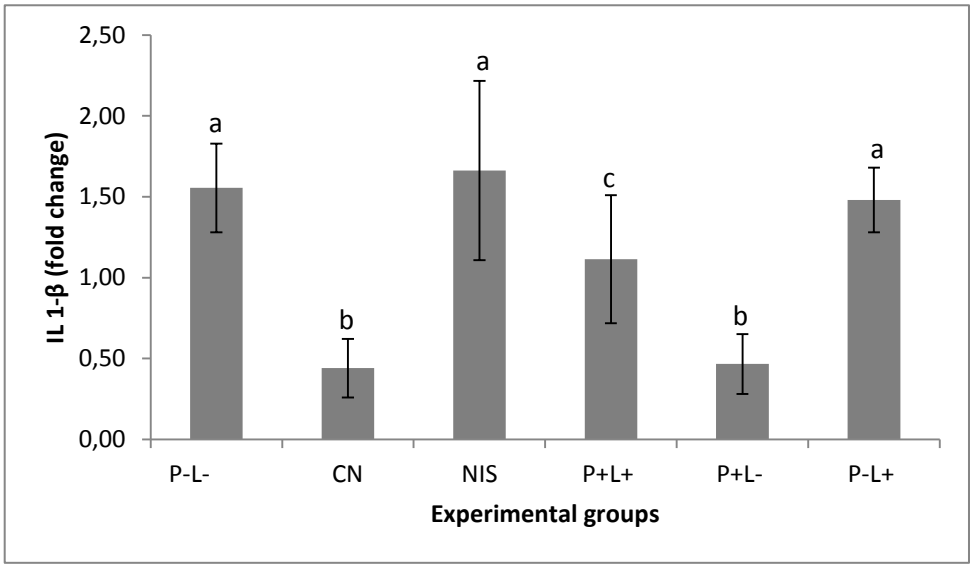

Fig. 9 – Mean values and standard deviation of the gene expression of IL-1 β assessed for each experimental group depending on time interval assessed (24 hours and 7 days after treatment). Equal superscript lowercase letters denote statistical similarity between the groups ($p <0.05$).

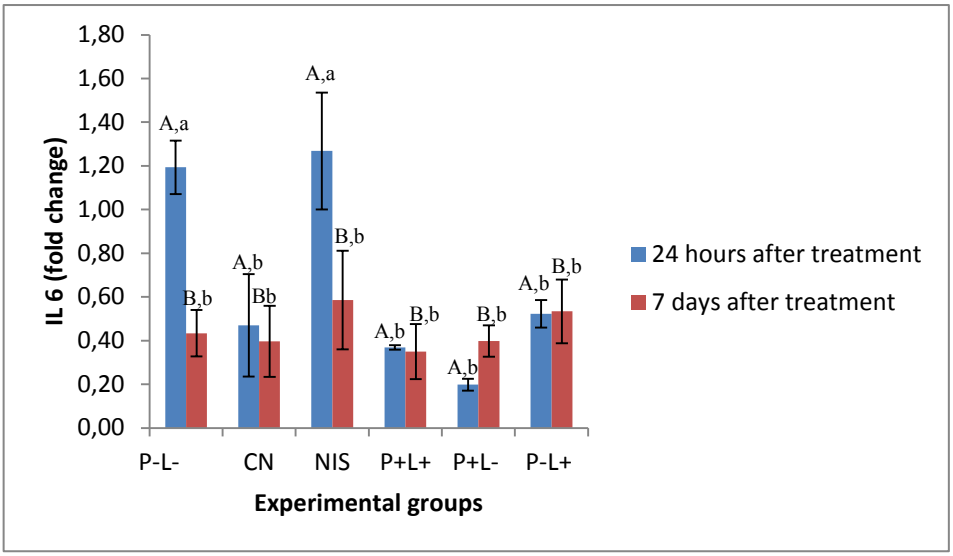

Fig. 10 - Mean values and standard deviation of IL-6 gene expression evaluated for each experimental group. Equal superscript capital letters denote similarity between the time intervals assessed (24 hours and 7 days after treatment) and equal superscript lower case letters denote statistical similarity between the experimental groups ($p<0.05$).

3.3 Capítulo 3

Genotoxic effect of photodynamic therapy mediated by curcumin on *Candida albicans

Runing Title: Curcumin-mediated PDT-induced DNA damage on *Candida albicans*

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ABSTRACT

Photodynamic therapy (PDT) is a promising method for localized and specific inactivation of fungi and bacteria. A nontoxic light-sensitive compound is taken up by cells, which are then exposed selectively to light, which activates toxicity of the compound. We investigated the potential of sub lethal photodynamic therapy (PDT) using light sensitive curcumin (CUR) in combination with blue (455 nm) light to promote reactive oxygen species (ROS) formation in the form of singlet oxygen and DNA damage of *Candida albicans*. Surprisingly, CUR-mediated PDT but also light alone caused significantly longer comet tails, an indication of DNA damage of *C. albicans* when compared with the negative control. The intracellular ROS production was also significantly higher for the group treated only with light. However, PDT compared to blue light alone significantly slowed DNA repair. Comet tails decreased during 30 min visualized as a 90% reduction in length in the absence of light for cells treated with light alone, while comet tails of cells treated with PDT only diminished in size about 45%. These results indicate that complex mechanisms may result in PDT in a way that should be considered when choosing the photo-sensitive compound and other aspects of the treatment design.

INTRODUCTION

The species of *Candida* genus are considered as commensal microorganisms that are part of the oral microflora of healthy individuals. Under certain circumstances, these microorganisms can act as opportunistic pathogens, being responsible for the development of infections. Oropharyngeal candidiasis (OPC) has been reported as the most common opportunistic infection caused by an accentuated growth and penetration of fungal species in the oral tissues (Canon *et al*., 1995; Pfaller & Diekema, 2007). Some predisposing factors such as poorly fit dentures, AIDS, and broad-spectrum antibiotic use over extended periods of time can enhance host susceptibility to develop this infection (Samaranayake, 1990; Scully *et al*., 1994; Shapiro *et al*., 2011). Disseminated candidiasis in immunocompromised patients can cause high mortality rates (Kromery & Barnes 2002; Pfaller & Diekema, 2007). Treatments used against infections caused by *Candida* spp. are routinely based on the use of medications that may be topical or systemic (Samaranayake, 1990; Pappas *et al*., 2009). However, the use of standard antifungal therapy may be limited due to its toxicity, low efficacy, or microorganism resistance after medication exposure over extended periods of

time (Perezous *et al*., 2005). Thus, studies have been conducted in an endeavor to find alternative strategies to inactivate *Candida* species. Within this context, photodynamic therapy (PDT) has been suggested for inactivation of some viruses (Wainwright, 2004), bacteria (Gois *et al*., 2010) and fungi (Dovigo *et al*., 2011a, c).

The use of PDT therapy for treating non-oncological diseases, including microbial inactivation and infection treatments, is still a recent field of scientific investigations (Konopka & Golinski, 2007; Donnelly *et al*., 2008). The photodynamic process requires the use of a photosensitizing agent (PS), light application that corresponds to the absorbance spectrum of the PS and the presence of oxygen (Wainwright, 2004; Jori *et al*., 2006). Photosensitization results in reactive species capable of inducing cell inactivation (Bonnett & Martínez, 2001). It has been suggested that the mechanism involves absorption of photons by the PS, which lead electrons to an excited state. In the presence of oxygen, light-excited PS can react with neighboring molecules through the transfer of electrons or hydrogen (type I reaction) or by energy transfer to oxygen (type II reaction), culminating to the production of reactive oxygen species (ROS) (Bonnett & Martínez, 2001). These ROS have non-specific reactivity with organic molecules. This means that any cellular macromolecule may be a potential target for PDT (Henderson & Dougherty. 1992; Bonnett & Martínez, 2001). Therefore, PDT seems to have a significant advantage over conventional antifungal treatments, since the plurality of targets decreases the likelihood of development of resistance of the exposed microorganisms in this procedure.

The inactivation of *Candida* spp. has been discussed in several studies (Sonis *et al*., 2000; Lambrechts *et al*., 2005; Konopka & Golinski, 2007; Junqueira *et al*., 2009), however it has been reported that it is more difficult to eliminate this type of fungus than gram-positive bacteria (Lambrechts *et al*., 2005). According to Demidova & Hamblin (Demidova & Hamblin, 2005), the presence of the nuclear membrane, the greater fungal cell size, and the reduced number of targets for singlet oxygen per unit volume of cell require a higher concentration of photosensitive drug and higher light doses to inactivate the fungus. Furthermore, the similarity of the fungal cell with mammalian cells complicates the selective accumulation of the photosensitive drug in the fungus (Donnelly *et al*., 2008).

Different types of PSs have been proposed in *in vitro* studies, including porphyrins (Bliss *et al*., 2004; Dovigo *et al*., 2011b), phenothiazines (Paardekopper *et al*., 1992; Zeina *et al*., 2001), chlorins (Strackhovskaya *et al*., 2002; Copley *et al*., 2008) and phthalocyanines

(Mantareva *et al*., 2011). Additionally, another PS that has been studied in anti-cancer therapy is curcumin (CUR), a yellow-colored compound extracted from the rhizome of *Curcuma longa L* (saffron) (Sharma *et al*., 2005). Several studies suggest that CUR has anti-inflammatory, antioxidant, and immunomodulatory effects, which could be exacerbated by light at a suitable wavelength (Bruzell *et al*., 2005). Recently, PDT mediated by CUR associated with light emitting diode (LED) was effective in inactivating planktonic suspensions and biofilms of *C. albicans, C. glabrata*, and *C. dubliniensis* (Andrade *et al*., 2013). CUR has also shown an antifungal effect on the inactivation of biofilms and cell suspension cultures of clinical isolates of *C. albicans, C. glabrata* and *C. tropicalis*, promoting a reduction in cellular metabolism by 85, 85, and 73%, respectively (Dovigo *et al*., 2011a). The compound was effective for the inactivation of *C. albicans* present in the tongue of mice with induced oral candidiasis, promoting an approximate 5-$\log_{10}$ reduction in cell viability, without causing any damage to the tongue tissues of the animal (Dovigo *et al*., 2013).

It is important to point out that there are no studies in the literature reporting that PDT, PS or light alone can be genotoxic to fungal cells. Damage can be the result of various processes and agents of endogenous or exogenous origin that may induce mutagenic processes (Henle *et al*., 1996). Examples of damage are double-strand breaks or oxidized bases that can lead to single strand breaks with subsequent DNA relaxation, increasing its electrophoretic mobility. The extent of DNA damage can be assessed by single-cell gel electrophoresis, also known as comet assay, where the chromosomal DNA migration distance correlates with the extent of damage of the DNA in low melting agarose-embedded cells (Azevedo *et al*., 2011). The comet assay has been used in a number of different applications, such as testing for genotoxicity, ecological monitoring, and human biomonitoring (Collins, 2004). To our knowledge among the studies that evaluated this type of test, only one was performed in yeasts of the genus *Candida* (Farrell *et al*., 2011). The explanation for this fact is that the amount of DNA per cell in these microorganisms is considerably lower than in other eukaryotic cells, which could make the visualization of the comet difficult (Rank *et al*., 2009).

According to the above-mentioned aspects, the mechanism of action of PDT is based on the production of ROS, which are responsible for causing cell damage through oxidative stress. ROS production, such as superoxide radical ($^{\cdot}O_2^-$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), and singlet oxygen constitute a significant threat to cellular integrity (Henle

et al., 1996). It has been suggested that the presence of redox-active metal ions such as Fe^{2+}, oxygen, and H_2O_2 can lead to Fenton chemical reaction, culminating in the production of highly reactive radicals to the cellular components. Oxidative stress can cause oxidative damage to macromolecules such as the DNA (Boiteux & Guillet, 2004), which contributes to genetic instability and possible mutations (Henle *et al*., 1996). Thus, determining DNA damage by the comet assay would provide information on the genotoxic potential of PDT and/or of its components (PS and light) due to oxidative stress caused on cells. Currently, CUR has shown promising results in the inactivation of *Candida* spp. Thus, the aim of this *in vitro* study is to assess the potential of PDT mediated by CUR in causing DNA damage on *C. albicans* cells.

MATERIAL AND METHOD

Analysis of DNA damage of *C. albicans* cells

Photosensitizing agent and light source

For the experiments we used natural CUR (Sigma-Aldrich, St. Louis, MO). A stock CUR solution (600 mM) was prepared in 10% dimethyl sulfoxide and then diluted in saline to obtain the concentrations to be tested. The blue LED device was developed by the Institute of Physics of São Carlos (University of São Paulo, São Carlos, SP, Brazil) and consisted of a handpiece (LXHL-PR09, Luxeon1 III emitter, Lumileds Lighting, San Jose, CA) that provided uniform emission of 440-460 nm with light energy equivalent to 89.2 mW/cm^2.

Microorganisms and growth conditions

All assays were performed on the type strain *C. albicans* ATCC 18804, a clinical isolate in Uruguay purchased from ATCC (American Type Culture Collection; www.atcc.org/). Fungal cells used in the study were cultured in YPD medium (1% w/v yeast extract, 2% w/v peptone and, 2% w/v dextrose) at 37ºC, 200 rpm and frozen at -70°C. Prior to the experiments, cultures of yeast were grown aerobically at 37°C for 48 h on solid YPDA medium (YPD with 2% w/v agar).

Planktonic cultures of *Candida albicans* and PDT

To conduct the study, a pre-culture of *C. albicans* was prepared in a tube containing 5 ml of 0.67% (w/v) YNB medium (Yeast Nitrogen Base; Difco) supplemented with 2% (w/v) glucose, which was incubated in an orbital shaker at 37°C at 200 rpm for 16 h. After incubation, standardized suspensions of *C. albicans* were obtained by diluting the culture and further incubation for two generations time until mid-exponential growth phase (0.4 optical density at 600 nm, which corresponds to 10^6 cells/ml). The cells were then centrifuged at 18,000 x*g*, washed twice for 2 min and suspended in lyticase buffer (1.0 M sorbitol, 25 mM KH_2PO_4, 50 mM β-mercaptoethanol, and 500 U/ml lyticase (Sigma-Aldrich, Ireland, pH 6.5). The suspension was incubated at 30°C for 30 min. After this incubation, the cells were centrifuged (18,000 x*g*) and washed twice with S buffer (1.0 M sorbitol and 25 mM KH_2PO_4, pH 6.5).

To perform PDT, 100 µL aliquots of the suspension were placed in 96-well microplates and the experimental groups were determined. The samples of the group treated with PDT were exposed to 2.5 µM CUR for 20 min in the dark (pre-irradiation time) followed by illumination for 7 min, giving a total light irradiance of 37.5 J/cm^2 (group P+L+). The effect of the application of isolated PSs (group P+L-) was performed by photosensitization of the samples in 2.5 µM CUR, which remained at rest in the dark for 20 min and then left on the laboratory bench for 7 min (time equivalent to the light dose applied to samples P+L+). To assess the effect of light alone (P-L+), the microplate was left at rest in the dark for 20 min and subsequently illuminated by the device at the light dose proposed (37.5 J/cm^2). Additionally, we assessed the negative control group (P-L-) that was composed of cells that received no treatment. The H_2O_2 group consisted of samples treated with 10 mM H_2O_2, which was considered as positive control since this substance promotes the formation of comet tails in *C. albicans* cells. Thus, five experimental conditions were performed on three different occasions.

Comet assay

After the established treatments, each sample was removed from the microplate wells and placed in microtubes, which were centrifuged again at 18,000 x*g* for 2 min. Then, the supernatants were discarded and the pellets were resuspended in 100 µL of 1.5% (w/v in S buffer) low-melting agarose (LMA) at 30ºC, placed on glass slides, covered with coverslips and incubated in at 4°C for 10 min for gelation of agarose. After this period, the

coverslipswere removed and the slides were first immersed in lysis buffer (30 mM NaOH, 1 M NaCl, 50 mM ethylenediamine tetraacetic acid (EDTA), 10 mM tris-HCl, 0.05% (w/v) lauroylsarcosine, pH 10) for 20 min, followed by electrophoresis buffer (30 mM NaOH, 10 mM EDTA, 10 mM Tris-HCl, pH 10) for 20 min. Electrophoresis was performed at 0.7 V/cm for 10 min, the slides were submerged in neutralization buffer (10 mM Tris-HCL, pH 7.4) followed by fixation in 96% methanol for 10 min at room temperature and then they were air-dried. The samples were stained with GelRed™ (Biotium Uniscience) and then analyzed by fluorescence microscopy at 400X magnification, equipped with excitation filter of 475-490 nm. At least 50 representative images of each of the glass slide were analyzed with the help of the free edition of the CometScore™ software version 1.5 (free access at http://autocomet.com/products_cometscore.php). The analytical parameter tail length (in μm) was chosen as the unit of DNA damage, corresponding to the head diameter of the comet subtracted from the total length. Thus, the occurrence of DNA damage was identified by the formation of stained comets, unlike intact DNA that presented circular morphology (Azevedo *et al*., 2011).

Analysis of intracellular ROS production

The test was conducted to assess the amount of ROS produced by the groups tested using the fluorescent probe 2',7'-dichlorofluorescein diacetate (H_2DCF-DA; Life Technologies, San Francisco, CA, USA). For this purpose, a 5 mL cell suspension of *C. albicans* was centrifuged once at 18,000 x*g* for 5 min and resuspended in ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). Then, 500 μL of untreated cells were removed for evaluation of autofluorescence and kept in the dark at 4ºC (AF group). H_2DCF-DA (50 μM) was added to the remaining cell suspension and incubated at 37°C for 1 h in the dark. The cells were washed twice with the same amount of PBS and 500 μL aliquots were distributed to the different experimental conditions treatment with PDT (P+L+), only light (P-L+), only CUR (P+L-) or 10 mM H_2O_2. The control consisted of H_2DCF-DA-loaded cells without any treatment (P-L-). After treatments, the cells were washed twice with PBS and quantification of ROS was analyzed by fluorimetric test using Fluoroskan (Thermo Scientific Fluoroskan Ascent- Thermo®) at 495-527 nm. Immediately after the reading, *C. albicans* cells were placed on round glass slides and fixed in 4% paraformaldehyde for 30 min at 37°C, and then analyzed by fluorescence microscopy.

Analysis of DNA repair in *C. albicans* cells

To assess the ability of DNA damage repair after the treatments proposed, analysis of DNA damage was performed using the comet assay, as described above. For this, *C. albicans* cells were centrifuged at 18,000 x*g* for 2 min and resuspended in 100 µL S buffer. The samples were incubated at 37°C for different time intervals of up to 30 min to allow DNA repair. Then they were centrifuged at 18,000 x*g* at 4°C for 2 min and embedded in LMA to perform the comet assay.

Statistical analysis

The results are expressed as mean and standard deviation of at least three replicates obtained in independent experiments. In each replica of the comet assay a corresponding mean value of at least 50 comets was used to calculate the mean value of the 3 replicates. Statistical analysis was performed using analysis of variance (ANOVA) at the 1% significance level and the post-hoc Tukey test (p <0.05) to compare groups, including the fluorescence analysis test.

RESULTS

Analysis of DNA damage to *C. albicans* cells

To assess genotoxicity of PDT, cells were treated with 2.5 µM CUR and blue LED (37.5 J/cm^2; group P+L+) and the DNA damage was analyzed by the comet assay. Controls were included in the analysis: no treatment (P-L-), CUR only (P+L-), blue LED only (P-L+) and 10 mM H_2O_2 (group H_2O_2). Upon microscopic inspection of the comets after the treatments, as expected, group P-L- did not display considerable comet tails (Figure 1A) unlike group P+L+, which displayed longer comet tails (Figure 1B), indicating that PDT is genotoxic. The length of the comet tails was used as DNA damage parameter, longer tails being proportional to more abundant DNA damage. As depicted in Figure 1C, a statistically significant difference was observed between the group that received photodynamic therapy (P+L+) ($p \leq 0.0001$), the group that received only blue LED light (P-L+) ($p \leq 0.0001$), and the group treated with hydrogen peroxide (H_2O_2) ($p \leq 0.0001$). These groups displayed statistically significant differences from the negative control group (P-L). Interestingly, blue LED only was able to promote DNA damage (see P-L+ and P-L- in Figure 1C), which was significantly

increased when cells were previously treated with CUR when irradiated in PDT (see P+L+ in Figure 1C). In addition, the group treated with CUR (P+L-) was similar to the P-L- group, suggesting that this compound is not genotoxic ($p>0.993$).

Analysis of intracellular ROS production

Photosensitization is usually mediated by the production of ROS when the PS is excited by light. To investigate if the genotoxicity of PDT is provoked by ROS upon irradiation, cells were loaded with the redox sensitive fluorochrome H_2DCF-DA and then treated as in the previous experiment. The apolar nature of the fluorochrome allows free permeation through biological membranes, however, once inside cells, esterases deacetylate H_2DCF-DA yielding the polar compound dichlorofluorescein (H_2DCF), which becomes trapped inside the cells. Upon oxidation H_2DCF is converted into the fluorescence-emitting oxidized form. Therefore, fluorescence of H_2DCF-DA-loaded cells is proportional to the amount of ROS produced during the treatments. Figure 2 shows that the fluorescence emitted by cells treated only with light (P-L+) was significantly higher than that of the groups receiving PDT (P+L+) ($p\leq0.032$) or H_2O_2 ($p\leq0.028$), which were similar among them, suggesting that ROS production was more significant in group P-L+ ($p\leq0.0001$). These groups displayed a statistically significant increase of fluorescence when compared with the negative control (P-L-), while the group treated only with CUR (P+L-) showed a slight but non-significant increase in fluorescence when compared to group P-L- ($p>0.437$). Figure 3 shows the difference in the fluorescence of cells that have undergone oxidative stress of the groups P-L- and P-L+, which corroborated the intracellular origin of the fluorescence. In addition, further testing was conducted with the group treated with CUR (without H_2DCF-DA) to assess whether CUR alone would emit the same fluorescence spectrum as H_2DCF-DA. The results showed absence of fluorescence of the cells analyzed (data not shown).

ROS production induced by blue LED (P-L+; Figure 2) correlates with the genotoxicity observed in the previous experiment (see P-L+ in Figure 1C) and suggests that this radiation promotes DNA damage through the induction of production of ROS inside the cells. Nevertheless, considering that blue LED is able to induce the highest amount of ROS, DNA damage would be expected to be more extensive in this group than in the PDT group (P+L+; see Figures 1C and 2). One reason for this could be an inhibition of DNA damage repair by CUR that would exacerbate blue LED genotoxicity.

Analysis of DNA damage repair in *C. albicans* cells

To investigate if CUR is inhibiting DNA damage repair during PDT, cells were exposed to treatments as in the previous experiments and then they were incubated for different periods of time to allow the activity of the DNA damage repair systems, before DNA damage analysis by the comet assay. Figure 4 shows the DNA damage repair activity through the analysis of DNA damage along time for each group of cells. Except for untreated cells (P-L-) and cells treated only with CUR (P+L-), where the initial DNA damage was negligible, the size of comet tails decreased over time, suggesting that DNA damage is actively repaired during the experiment.

Repair was significantly more accentuated in cells in the P-L+ group in which the tail length decreased approximately 90% after 30 min when compared with time zero, which reached similar levels of DNA damage as in P-L-, the negative control group (Figures 4, 5A and 5B). The cells submitted to PDT (P+L+) showed a reduction in tail length of only approximately 45% after 30 min compared with time zero (Figures 4, 5C and 5D). These results strongly suggest that CUR inhibits DNA damage repair during PDT, which contributes to an enhanced genotoxic effect of blue LED. However, P+L+ cells still display more DNA damage than cells of group P-L+, indicating that other factor(s) might be contributing to enhance the genotoxic effect in PDT.

DISCUSSION

Photodynamic therapy (PDT) is considered a minimally invasive method for the treatment of infectious diseases such as OPC, which is mediated by the production of ROS that are responsible for the inactivation of target cells (Lyon *et al*., 2011). Some investigations have demonstrated that ROS are the main causative agent of DNA damage on fungi cells, and the level of this alteration can be easily detected by the comet assay (Henle *et al*., 1996; Azevedo *et al*., 2011). The comet assay has widespread applications allowing an estimation of the degree of genotoxicity of potentially mutagenic and/or carcinogenic agents in studies of DNA damage and repair (Speit & Hartmann, 1999; Speit *et al*., 1999). The present study was conducted to assess the DNA damage caused by the oxidative stress generated by CUR-mediated PDT associated with blue LED light on *C. albicans* and the inhibition of DNA repair provoked by CUR.

Oxidative stress is caused by ROS, such as, superoxide and hydroxyl radical, and more specifically singlet oxygen that pose a significant threat to cell integrity by causing damage to the DNA, lipids, proteins, and other cellular macromolecules (Slupphau *et al*., 2003). In the present study, it was found that PDT using CUR (2.5 μM which correspond to 0,9 mg/L) associated with blue LED at 37.5 J/cm^2 (group P+L+) induced DNA damage. These modifications were more significant when compared with the group treated with H_2O_2 (Figure 1C). These results may be justified by the fact that yeast cells are aerobic and produce ROS during their natural metabolism in the mitochondrial respiratory chain and in peroxisomal metabolism that generates H_2O_2 in reactions catalyzed by oxidases (Moradas-Ferreira *et al*., 1996). Endogenous ROS are dispersed from the mitochondrial electron transport chain and peroxisomes, diffusing freely through the cell membranes, attacking other components inside the cell (Salmon *et al*., 2004). Oxidative damage produced by endogenous ROS can induce DNA base modifications, single- and double-strand breaks, in addition to the formation of purine/pyrimidine lesions, many of which are liable to be toxic and/or mutagenic (Girard & Boiteux, 1997). Endogenous ROS production added to those produced by light associated with CUR may have been responsible for accentuating the genetic damage of the *C. albicans* cells.

After the application of blue LED light (group P-L+), DNA cell damage was also observed regardless of the use of a PS. Studies have reported that the wavelength used in the present study (455 nm) causes oxidative stress that may be associated with the photoexcitation of endogenous intracellular porphyrins in a way that the absorption of these photons would promote energy transfer to oxygen, culminating in the production of ROS (Hamblin *et al*., 2005; Maclean *et al*., 2008). It has been reported that microorganisms can detect blue light, which may induce physiological responses caused by the light receptors (Daia *et al*., 2012). Thus, photosensitized excitation of blue light chromophores, including cytochromes and flavins, within the mitochondria and/or peroxisomes could induce the production of endogenous ROS (Daia *et al*., 2012). The presence of comets in *C. albicans* cells was also observed after UV irradiation (352 nm) (Farrell *et al*., 2011). Furthermore, it has been reported that blue LED light has an antimicrobial effect on bacteria such as *Staphylococcus aureus* (Guffey & Wilborn, 2006), including methicillin-resistant strains (Enwemeka *et al*., 2008) and *Pseudomonas aeruginosa* (Guffey & Wilborn, 2006). According to studies that have evaluated the visible band spectrum, the wavelength range from 402 to 420 nm has been reported to be the most effective against microorganisms, while the

spectrum from 455 to 470 nm showed antimicrobial potential only in some bacterial species (Daia *et al*., 2012). Studies have shown that the wavelength (455 nm) and light dose of 37.5 J/cm^2 used in the present study has no antifungal effect (Dovigo *et al*., 2011a; Dovigo *et al*., 2013). However, this investigation demonstrated that this wavelength was able to promote DNA damage on *C. albicans*. It can be justified by the fact that *C. albicans* cells have the ability of efficiently repair the DNA damage under these conditions so that they can survive.

Fluorescence assessment with H_2DCF-DA revealed that PDT, light, and H_2O_2 provoked oxidative stress. The fluorescence intensity of the group treated only with light (P-L+) was more accentuated, suggesting that ROS production was higher in this group (Figure 2). These results may have occurred because the blue light stimulated intracellular ROS production, as discussed above. Interestingly, lower fluorescence was observed when cells were submitted to PDT. It has been reported that a high concentration of CUR alters the expression of genes that regulate the metabolism, and mitochondrial integrity of *C. albicans* (Kumar *et al*., 2014). Possibly, CUR may have acted in the mitochondria, impairing cellular respiration, resulting in lower endogenous ROS production. Although, a low concentration of CUR was used in the present study, we suggest that light might have enhanced the effect of CUR in the cell. Thus, it is possible to mention that low concentration of CUR associated with blue LED light altered the cellular metabolism, which can result in lower quantity of ROS produced in the mitochondria during the cell respiration. On the other hand the antioxidant activity of CUR (Barzegar & Moosavi-Movahedi, 2011) might have also contributed to lower fluorescence in PDT. Scavenging of light-induced ROS by CUR in PDT would contribute to attenuate the oxidative stress.

Furthermore, we evaluated the kinetics of DNA repair in fungal cells with the purpose of verifying if the damaged DNA would be repaired over time. The results showed that CUR when combined with blue LED light (PDT; P+L+) was genotoxic and hindered DNA repair of *C. albicans* cells, which was characterized by the reduction of the comet tails size in only approximately 45% after 30 min, unlike the group of cells exposed to blue LED without CUR (P-L+) where the reduction was 90% (Figures 4 and 5). The influence of CUR on DNA repair pathways in yeast cells is still not completely understood, according to the literature (Azad *et al*., 2013). Topoisomerases are the main enzymes responsible to directly alter topological changes in DNA and thereby playing an important role in the regulation of the replication, transcription, recombination and chromosome condensation (Corbett & Berger, 2004). The activities of this enzyme are crucial in cells with DNA damage (Champoux, 2001). To our

knowledge, only one study reported the influence of CUR on the enzymatic activities of topoisomerases I and II (top I and II) of *S. cerevisiae* and *C. albicans* (Roth *et al*., 1998). According to the authors a low concentration of CUR (0,001mg/L) was able to promote the inhibition of the activity of *C. albicans* top I and II (Roth *et al*., 1998). Thus, it is possible to suggest that CUR also may have interacted with enzymes such as topoisomerase in the present study, hindering DNA repair, which would explain the slower DNA repair in the P+L+ group in comparison with P-L+.

In summary, the comet assay can be considered an important tool for assessing the integrity of the genome of *C. albicans* cells in the study of the toxicity of natural compounds and therapies targeted against fungal DNA. Results from the present investigation suggest that PDT provoked extensive DNA damage that was not repaired efficiently due to the inhibition caused by CUR. This may be the main mechanism of the antifungal activity of CUR-mediated PDT. It is possible that virulence factors of this microorganism might also be affected upon PDT, which would exacerbate the therapeutic effect. Therefore it is important to investigate the effects of PDT also in *in vivo* studies by monitoring DNA damage and virulence factors. This will help to understand the mechanism of action of PDT on *C. albicans* cells that will allow implementing efficiently this therapeutic approach in the clinical practice.

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Figures

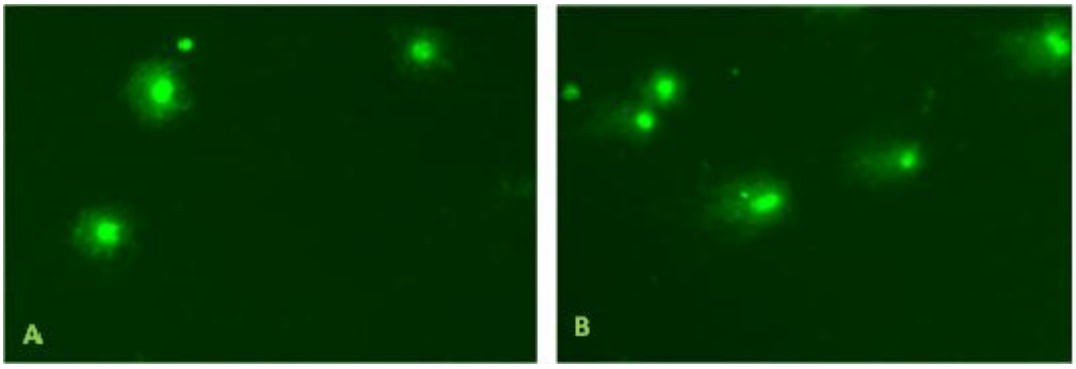

Figure 1 (A) – Imaging by fluorescence microscopy, representative of the group P-L-, showing the absence of comets; and (B) group P+L+ showing the presence of comets in the *C. albicans* cells.

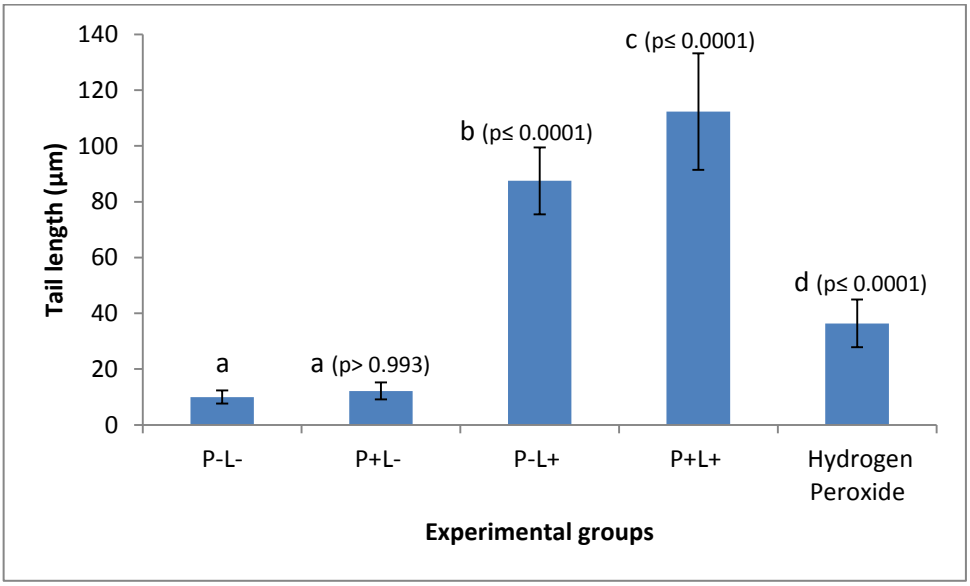

Figure 1 C- Mean and standard deviation of the length of the tails formed by the exposure of the *C. albicans* cells to PDT (group P+L+), CUR (P+L), light (P-L+), or 10 mM hydrogen peroxide (H_2O_2). Negative control (P-L-) refers to untreated cells. The same superscript letters on the bars denote statistical similarity among groups (post-hoc Tukey test - p <0.05).

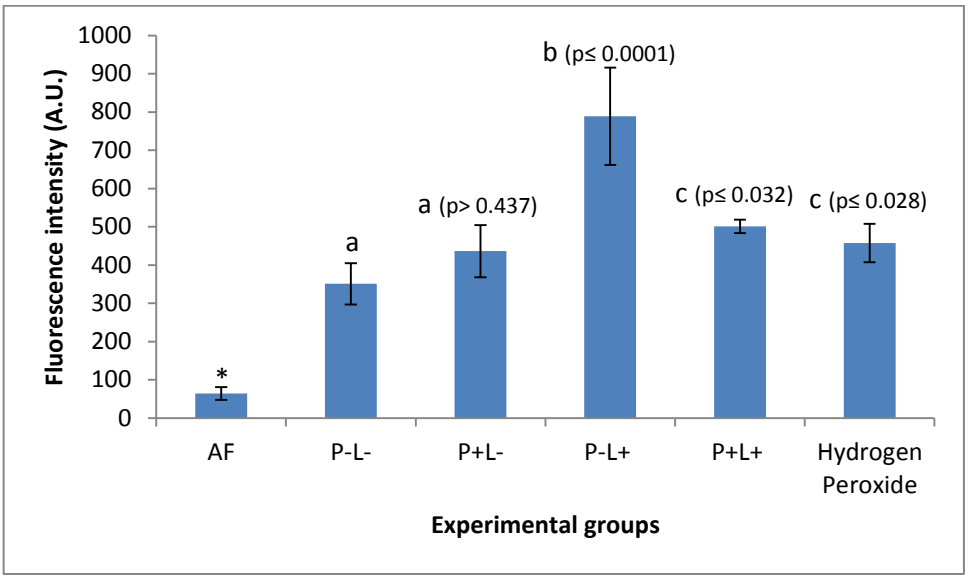

Figure 2 - Mean value and standard deviation of ROS production, measured as fluorescence intensity upon oxidation of H2DCF-DA by fluorimetry, of *C. albicans* submitted to PDT (P+L+), CUR (P+L-), light alone (P-L+), or 10 mM hydrogen peroxide (H2O2) in comparison with the control group (P-L-) ($p<0.05$). *Group with untreated cells and without fluorochrome. A.U. - arbitrary units.

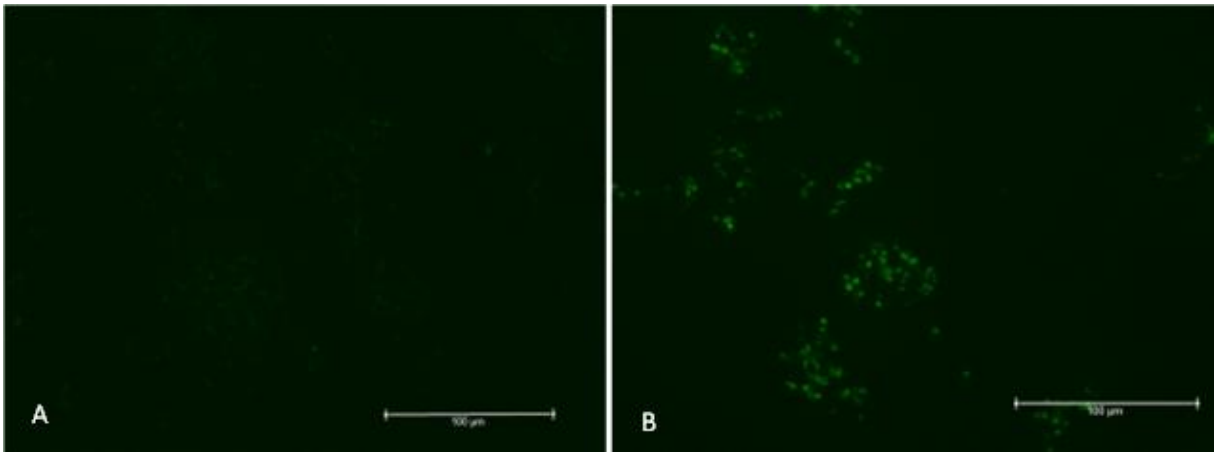

Figure 3 - Representative images of fluorescence intensity of H2DCF-DA-loaded *C. albicans* cells of groups P-L-, untreated (A), and P-L+, treated only with blue LED (B). Magnification: 20x.

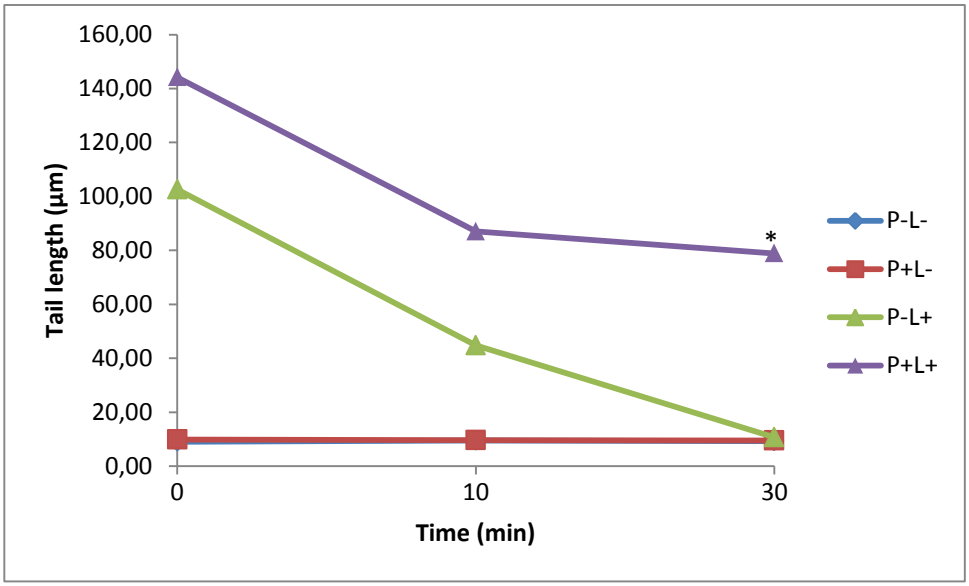

Figure 4 - DNA damage, represented as mean comet tail lengths, along time after treatments in groups P+L+ (PDT), P+L- (treated with CUR), P-L+ (treated with blue LED), and P-L- (untreated).* Statistically different from P-L+ ($p<0.05$).

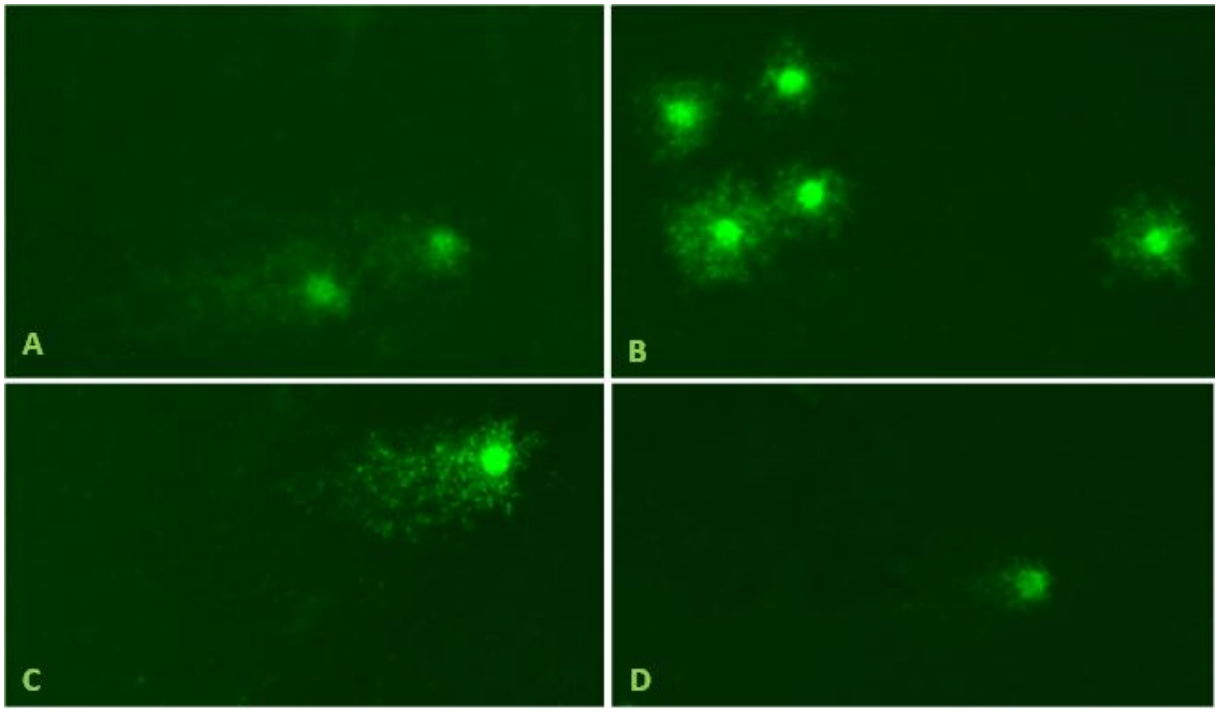

Figure 5 - Representative images of DNA damage, depicted as yeast comets, in the DNA damage repair experiments with group P-L+ (irradiation with blue LED only; A and B) and with group P+L+ (PDT; C and D). DNA damage was assessed immediately after light application (A and C) and after 30 min incubation (B and D).

4- Discussão

4 - Discussão

O estudo 1 avaliou a eficácia da PDT mediada pela ClAlP, associada a fonte de luz LED na fotoinativação de *C. albicans* presente em lesões orais de camundongos imunossuprimidos. Foi observado que a PDT mediada por ClAlP-NE catiônica apresentou efeito antifúngico sobre a *C. albicans* reduzindo 2,26log_{10} da viabilidade celular. Por outro lado a mesma substância quando diluída em DMSO, não reduziu o número de micro-organismo. O FS ClAlP utilizado no presente estudo por ser insolúvel em água, foi formulado em nanoemulsão catiônica (PBS, pH 7.4). Tem sido relatado que a veiculação de fármacos em nanoemulsão aumenta a disponibilidade e estabilidade do FS, reduzindo a possibilidade de agregação das moléculas[92]. O tratamento de infecções fúngicas utilizando este PS diluído em nanoemulsão apresenta vantagens devido ao pequeno tamanho de suas partículas os quais permitem uma deposição uniforme sobre os tecidos aumentando a penetração. Além disso, a nanoemulsão pode englobar partículas que variam de 10 a 600 nm, permitindo o transporte de substâncias que podem ser dissolvidos na fase óleo de uma emulsão ou de fármacos anfifílicos que podem ser adsorvidos na interface óleo-água[10, 63, 66]. Além disso, a adição da carga positiva à nanoemulsão (catiônicas) melhora a adesão do FS à membrana celular do fungo, aumentando assim a quantidade de FS na célula e a efetividade da PDT[56]. Os aspectos citados justificam a efetividade da PDT mediada pela ClAlP veiculada em nanoemulsão catiônica observada no presente estudo.

É importante ressaltar que nesta investigação o grupo tratado com PDT mediada por ClAlP veiculada em DMSO não reduziu a viabilidade celular de *C. albicans* presentes na lesões orais dos animais. Esses resultados não estão de acordo com os obtidos em estudos in vitro, uma vez que esse mesmo composto foi efetivo na inviabilização de bactérias e fungos[69-70]. Embora o DMSO seja considerado um composto que proporciona a penetração de medicamentos hidrofílicos e lipofílicos em tecidos biológicos[57], ao contrário da nanoemulsão catiônica, ele não apresenta carga, o que dificulta a adesão do FS às células fúngicas. Tem sido relatado que a adesão e agregação de espécies de *Candida* a superfícies bióticas ou abióticas dependem de reações entre a parede celular fúngica com a superfície em questão, havendo a presença de ligações covalentes entre essas estruturas[48, 51]. Assim, a presença de um composto que se adere à membrana celular do fungo, poderia dificultar a adesão do mesmo ao substrato[85]. Como o DMSO é um solvente orgânico, não apresenta características que facilite a adesão do FS à membrana celular de *C. albicans,* o que pode ter limitado a infiltração do FS no biofilme oral formado no dorso da língua dos animais. Outra

desvantagem do DMSO é que por ser solvente orgânico, ele não protege o FS, permitindo que o mesmo sofra fotodegradação mais rapidamente se comparado a FS veiculados em nanoemulsões[39]. Dessa forma o FS, quando em estado excitado degrada-se facilmente, ficando com seu poder oxidativo reduzido, diminuindo também a efetividade da PDT[87].

A análise macroscópica das lesões orais revelou a presença de placas brancas ou pseudomembranosas em torno de 21 a 90% sobre o dorso da língua (Escore 2) em todos os animais infectados por *C. albicans*. Vinte e quatro horas após a aplicação dos tratamentos foi possível atribuir escore 1 ao grupo P+L+ ClAlP NE, e os demais grupos infectados por *Candida* mantiveram Escore 2. Estes achados corroboram aos obtidos por Costa et al.[20], que investigaram a efetividade da PDT mediada por eritrosina em camundongos com candidose oral induzida. Foi atribuído escore 1 ao grupo submetido à PDT 24h após o tratamento e escore 2 aos demais grupos. Adicionalmente, na presente investigação a relação entre as lesões orais presentes no dorso lingual dos animais e os resultados obtidos no teste de viabilidade celular (CFU/mL) foi verificada por meio do coeficiente de correlação ($r_s = 0.70$, $n = 5$). Esta análise demonstrou um coeficiente de valor alto, correlacionando o número elevado de placas brancas e/ou pseudomembranosas na língua com o grau de colonização de *Candida* na cavidade bucal dos animais. No trabalho conduzido por Takakura et al.[83], os autores avaliaram a eficácia terapêutica do Fluconazol e Anfotericina B no tratamento de candidose oral induzida em camundongos. Neste estudo os autores também relacionam os dados microbiológicos com o grau das lesões orais e relataram um alto coeficiente de correlação entre estes dois fatores, assim como demonstrado no presente estudo.

A análise histológica das línguas removidas 24 horas após os tratamentos demonstrou a presença de leveduras e hifas na camada de ceratina e discreto infiltrado inflamatório no dorso da língua de todos os animais infectados com *C. albicans,* incluindo os que foram submetidos à PDT. As alterações histológicas encontradas no presente estudo são características de candidose oral, e também foram relatadas por outros autores[20, 46, 59, 84]. Além disso, as análises histológicas realizadas nos animais não infectados com *C. albicans* (CN, P+L+ ClAlP NE NI e P+L+ ClAlP DMSO NI) demonstraram ausência de resposta inflamatória nos tecidos linguais. Esses resultados estão de acordo com Fontana et al.[32] que avaliaram o efeito da aplicação de diferentes FSs associados a luz LED (630 ou 660 nm) no tecido lingual de ratos saudáveis e observaram histologicamente que os tecidos permaneceram com características histológicas de normalidade. Adicionalmente, nas avaliações histológicas realizadas nos animais submetidos aos tratamentos e sacrificados 7 dias após, foi possível

observar a remissão das lesão orais com neoformação das papilas filiformes e ausência de *C. albicans* nos tecidos.

No presente estudo também foi avaliado a produção da citocina fator de necrose tumoral-α (TNF-α), após os tratamentos. De acordo com os resultados do presente estudo, houve aumento da expressão de TNF-α para os animais com candidose oral submetidos a PDT veiculada em nanoemulsão catiônica (P+L+ ClAlP NE) em comparação ao grupo controle positivo (P-L-). Estes resultados estão de acordo com a literatura, em que trabalhos demonstram que a eficácia da PDT parece estar fortemente relacionada com o grau e a natureza da inflamação induzida pela própria terapia[11] e que a PDT pode induzir uma inflamação aguda significativa caracterizada por um aumento da expressão da citocina TNF-α[29, 36-37]. Assim, é possível sugerir que a PDT antimicrobiana induziu ao aumento de TNF-α, intensificando a reação inflamatória tecidual. A reação inflamatória é uma resposta do hospedeiro que se desenvolve para restaurar a integridade do tecido por meio da remoção das células mortas ou danificadas, para acelerar a cicatrização do tecido mantendo a homeostase do mesmo[31]. Esses achados podem ser considerados positivos, uma vez que de acordo com a análise macroscópica, os animais submetidos à PDT apresentaram rápida resolução do quadro inflamatório das lesões orais. Adicionalmente, tem sido relatado que os primeiros estágios da infecção por *Candida* spp induz a produção local de TNF-α, que pode mediar a seqüência de reações inflamatórias[15].

É importante ressaltar que a PDT mediada por ClAlP DMSO com e sem inoculação fúngica (P+L+ ClAlP DMSO, P+L+ClAlP DMSO SI, respectivamente), assim como o grupo tratado apenas com o Fs sem aplicação de luz (P+L- ClAlP DMSO) também demonstraram aumento na expressão de TNF-α. Isso pode ser justificado pela presença do DMSO. Tem sido relatado que esse composto por si só é capaz de causar lesão na membrana celular, dissociação da matriz de colágeno extracelular[90, 93] e também a morte celular do hospedeiro por apoptose[58], iniciando a cascata inflamatória e evidenciando sua toxicidade. Da mesma maneira, o grupo que recebeu apenas luz (P-L+) também apresentou aumento na expressão de TNF-α. Essa produção pode estar associada ao estresse oxidativo gerado pela luz, causando reação inflamatória.

Adicionalmente, avaliou-se a produção das citocinas interleucina 1β (IL-1β) e interleucina-6 (IL-6). A expressão dessas citocinas foi semelhante entre todos os grupos avaliados em comparação ao controle positivo. Esses achados podem ser explicados pelo fato de que essas citocinas inflamatórias apresentam seu pico de expressão em diferentes momentos após o tratamento. Tem sido relatado que o pico de liberação de IL-1β e IL-6 pode

ocorrer no período de 6 a 8 horas após procedimentos que estimulem reações inflamatórias[45]. Como no presente estudo, os animais foram sacrificados 24 horas após os tratamentos, é possível que a expressão tenha chegado a um platô em até 8 horas, e tenha diminuído após esse período. O que pode justificar a similaridade da expressão das citocinas IL-1β e IL-6 entre todos os grupos avaliados.

Assim, os resultados apresentados no estudo 1 sugerem que o sistema de veiculação em nanoemulsão catiônica parece ser um meio eficaz para a distribuição da ftalocianina. A PDT mediada por este FS foi efetiva na inviabilização de *C. albicans* presente no dorso lingual de camundongos com candidose oral induzida, bem como na remissão das lesões. Embora a análise histológica tenha demonstrado que a PDT não causou efeitos adversos ao tecido lingual, a análise biomolecular revelou que este tratamento provocou a expressão de citocinas pró e anti-inflamatórias, entretanto o nível de citocinas expressas não foi suficiente para causar alterações histológicas significativas. Esses resultados podem ser decorrentes da uma única aplicação de PDT realizada. Contudo, é possível sugerir que o grau de inflamação induzida pela PDT e sua subsequente contribuição na eficácia antimicrobiana depende de vários parâmetros tais como estrutura química do sistema de liberação empregado e do FS, e o número de aplicações de PDT realizada. Estes achados encorajam a realização de estudos que avaliem os efeitos da PDT mediada por novos FSs como um tratamento por meio da realização de sucessivas aplicações em modelo animal com candidose oral induzida. Dessa forma, o estudo 2 foi conduzido com o objetivo de desenvolver um novo modelo murino de candidose oral para avaliar o efeito fotodinâmico do FS PDZ simulando tratamento para essa infecção.

O modelo proposto para indução de candidose oral em camundongos imunossuprimidos empregado no estudo 2 é reprodutível, uma vez que todos os animais infectados por *C. albicans* apresentaram placas brancas ou pseudomembranosas sobre o dorso da língua no período de 5 a 16 dias pós-inoculação. Para possibilitar o acompanhamento da infecção e o estabelecimento do tratamento, 4 imunossupressões com predinisolona foram realizadas com o objetivo de manter os animais por mais tempo com a infecção de forma que 10^5 UFC/mL de *C. albicans* foram coletados das lesões orais dos animais do grupo P-L- (controle positivo) durante todo período experimental. A análise histopatológica demonstrou presença de infiltrado inflamatório moderado com inúmeras hifas/pseudohifas sobre toda a camada de ceratina, e algumas hifas/pseudohifas invadindo o tecido epitelial das línguas. Esses achados são semelhantes aos obtidos por Takakura et al.[83], que também desenvolveram uma metodologia para indução de candidose oral em camundongos imunossuprimidos e

obtiveram valores de 10^5-10^6 UFC/mL de *C. albicans* das lesões orais dos animais. Na análise histopatológica foi observado a presença de infiltrado inflamatório severo, com numerosas hifas, invasão fúngica e destruição das camadas epiteliais. Esses achados podem ser explicados pelo fato de que Takakura et al.[83], utilizaram cepa proveniente de isolado clínico de pacientes com candidose cutânea, ao passo que no presente estudo foi utilizado cepa padrão de *C. albicans*. Foi avaliada a relação entre os escores atribuídos às lesões orais dos animais e os valores de UFC/mL por meio do Coeficiente de Correlação de Spearman (R=0.92, n=12, de 5 a 16 dias pós-infecção). Esta análise demonstrou um coeficiente de valor alto, confirmando que o número elevado de placas brancas e/ou pseudomembranosas na língua refletiu o grau de colonização de *C. albicans* na cavidade bucal dos animais infectados. No trabalho conduzido por Takakura et al.[83], os autores também relacionaram os dados microbiológicos com o grau das lesões orais e observaram alto coeficiente de correlação (R=0.75, n=26), assim como demonstrado no presente estudo. Os resultados obtidos confirmam que o modelo de indução de candidose oral aqui estabelecido é adequado para reprodução da infecção por um período de tempo mais prolongado o que permite a avaliação da eficácia de tratamentos sucessivos.

Outras metodologias também foram propostas para induzir candidose oral em animais[84, 86]. Teichert et al.[84] utilizaram um protocolo de imunodeficiência severa para induzir candidose oral e recuperou cerca de 2 x 10^2 UFC mL^{-1} de *C. albicans*. Outro método para induzir essa infecção foi usado por Totti et al.[86]. Neste estudo os camundongos foram sialoadenectomizados, e realizou-se quatro inoculações com suspensão de *C. albicans.* Dentre os 6 animais utilizados de cada grupo experimental, apenas 2 apresentaram colonização fúngica até 60 dias pós-infecção, e apenas 1 animal até 75 dias pós-infecção. Nos animais que desenvolveram candidose oral, a análise histopatológica revelou presença de pseudohifas invadindo o epitélio, acantose e infiltrado neutrofílico intra-epitelial formando micro-abscessos[86]. Embora esse método favoreça a colonização de *C. albicans* por mais tempo, não é possível estabelecer uma comparação direta com o presente estudo uma vez que o número de UFC/mL das lesões orais dos animais não foi mencionado. Adicionalmente, no presente estudo, todos os animais avaliados apresentavam lesões típicas de candidose oral por todo período experimental. Já no estudo conduzido por Totti et al.[86], na maioria dos animais, a infecção não foi mantida por todo período pretendido.

No presente estudo os resultados demonstraram que o tratamento por meio da utilização PDT foi tão eficaz quanto a NIS, na inativação de *C. albicans* presente nas lesões orais dos camundongos com candidose oral induzida. A associação de 100 mg/L de PDZ com

37,5 J/cm^2 de dose de luz promoveu 3 logs$_{10}$ de redução da viabilidade celular fúngica enquanto que a NIS reduziu 3,2 log$_{10}$, quando comparados com os animais não tratados (P-L). Esses resultados foram observados 24 horas após os tratamentos e se mantiveram até 7 dias para o grupo tratado com PDT. Já para os animais tratados com antifúngico NIS, foi observado aumento no número de UFC/mL 7 dias após o último tratamento, e a redução em log$_{10}$ de UFC/mL passou a ser de apenas 1,7log$_{10}$ em relação ao grupo controle, sugerindo possível recidiva da infecção. Esses achados corroboram com os estudos que relataram frequentes recidivas em pacientes portadores de candidose oral semanas após o tratamento[5, 7, 60].

De acordo com a literatura pertinente, este é o primeiro estudo que avaliou os efeitos de aplicações sucessivas da PDT no tratamento de candidose oral em modelo murino. Dessa forma, somente é possível a realização de comparações indiretas com os estudos relatados na literatura uma vez que os mesmos foram conduzidos realizando somente uma aplicação de PDT. Diferentes fotossensibilizadores têm sido avaliados para a fotoinativação de *C. albicans* presentes em lesões orais de camundongos com candidose oral induzida, tais como azul de metileno[84], porfirina[59], eritrosina[20], curcumina[26] e após uma única aplicação tem sido observado resultados diversos. Carmello et al.,[16] avaliaram a eficácia in vivo do PDZ (100mg/L) associado a luz LED (37,5J/cm^2) e obtiveram redução de 4,36 log$_{10}$ na viabilidade celular fúngica após uma única aplicação. Esses achados diferem dos resultados obtidos no presente estudo, pois embora tenham sido realizadas cinco aplicações sucessivas de PDT na mesma concentração e dose de luz do estudo acima citado, foi observado redução de aproximadamente 3 log$_{10}$. Hipoteticamente, as 5 aplicações de PDT deveriam produzir maior quantidade de espécies reativas de oxigênio (ROS) sobre as células, o que causaria maior redução na viabilidade celular se comparado a uma aplicação. Entretanto, tem sido relatado que células fúngicas tem a capacidade de se adaptar e se proteger do estresse oxidativo e dois mecanismos parecem estar envolvidos; o primeiro estaria relacionado a diminuição da permeabilidade da membrana celular às ROS e o segundo diz respeito ao aumento da regulação de antioxidantes e outras enzimas associadas[88].

Por outro lado, a análise macroscópica revelou que 24 horas após o término dos tratamentos, todos os animais submetidos a PDT apresentaram total remissão das lesões (escore 0). Para o grupo tratado com NIS, os animais apresentaram remissão parcial das lesões (escore 1) e os demais grupos apresentaram extensas lesões por todo dorso da língua (escore 3). Esses resultados se mantiveram até 7 dias após o término dos tratamentos. No presente estudo, a PDT também foi mais efetiva do que a NIS na remissão das lesões orais 24

horas e 7 dias após os tratamentos. Uma possível hipótese para explicar esses achados pode ser atribuída ao efeito bioestimulador da luz LED vermelha. Tem sido relatado que a luz LED vermelha é absorvida por componentes da cadeia respiratória mitocondrial celular, o que resulta no aumento das espécies reativas de oxigênio (ROS) e trifosfato de adenosina (ATP) /ou AMP cíclico, iniciando uma cascata de sinalização que promove a proliferação celular e citoproteção[33, 80]. Mais especificamente, esse tipo de luz age sobre as células por meio do fotoaceptor primário: citocromo C oxidase, enzima terminal do transporte de elétrons da cadeia mitocondrial[49-50]. A absorção da luz pelo citocromo c oxidase promove o aumento da quantidade de ATP e ROS, o que conduz ao aumento da disponibilidade de energia e de transdução de sinal[43, 82]. Estas mudanças bioquímicas levam a efeitos macroscópicos, tais como aumento da proliferação celular e consequentemente a remissão acelerada de lesões infecciosas. Dessa forma, é possível sugerir que a PDT mediada pelo PDZ associado a luz LED vermelha estimulou a proliferação celular do tecido lingual dos animais o que explicaria a total remissão das lesões orais dos animais tratados com PDT, o que não ocorreu para os tratados com NIS. É importante mencionar também, que muito pouco se sabe sobre os efeitos farmacodinâmicos e farmacocinéticos da NIS em modelo murino de candidose oral.

A análise histológica das línguas removidas 24 horas após os tratamentos revelou a presença de infiltrado inflamatório discreto (escore 1) para os grupos tratados com PDT e para o grupo que recebeu NIS, com a presença de algumas hifas/pseudohifas sobre a camada de ceratina, sem invasão fúngica no tecido epitelial. Os demais grupos (P-L+, P+L-) foram semelhantes ao grupo controle positivo (P-L-), que apresentaram infiltrado inflamatório moderado com presença de inúmeras hifas/pseudohifas sobre toda a camada de ceratina, e algumas hifas/pseudohifas invadindo o tecido epitelial das línguas. As alterações histológicas encontradas no presente estudo são características de candidose oral, e também foram relatadas por outros autores[20, 46, 59, 84]. Com base nos resultados do presente estudo pode-se sugerir que o infiltrado inflamatório encontrado nos grupos tratados está associado à infecção por *C. albicans*. Adicionalmente, a análise histológica realizada 7 dias após os tratamentos revelou-se semelhante à realizada 24 horas após os mesmos.

Tem sido relatado que os primeiros estágios da infecção por espe´cies de *Candida* induz a produção local de TNF-α[15, 47, 65]. De acordo com os resultados do presente estudo, os animais submetidos a PDT apresentaram expressão de TNF-α semelhante ao grupo NIS, e 26% maior do que o grupo P-L-. Esses resultados foram observados 24 horas e 7 dias após término dos tratamentos. Esses achados corroboram com os obtidos por Brackett e Gollnick[11], que demonstram que a eficácia da PDT parece estar relacionada à produção de TNF-α[11, 36-37].

Segundo esses autores, o discreto aumento na expressão de TNF-α pode ser considerado benéfico[31] uma vez que as células fúngicas podem ter sido mortas ou removidas pelas células presentes na reação inflamatória, auxiliando na rápida resolução das lesões orais. Dessa forma, é possível sugerir que o aumento na produção de TNF-α, observados no presente estudo, foi causado pela infecção por *C. albicans* associado a PDT.

A expressão das citocinas interleucina 1β (IL-1β) e interleucina-6 (IL-6) também foram avaliadas. Os resultados obtidos revelaram que a expressão de IL-1β para o grupo tratado com PDT foi menor em relação ao grupo P-L- e ao grupo tratado com NIS. Com relação à IL-6, o grupo tratado pela PDT exibiu valores basais na expressão dessa citocina, os quais foram semelhantes ao grupo controle negativo. Já o antifúngico NIS mostrou valores semelhantes ao grupo P-L- 24 horas após os tratamentos. É importante ressaltar que essas citocinas apresentam um pico de liberação em diferentes momentos, os quais podem ocorrer no período de 6 a 8 horas após procedimentos que estimulem reações inflamatórias[45]. Como no presente estudo, os animais foram sacrificados 24 horas e 7 dias após os tratamentos, muito provavelmente a expressão dessas citocinas deve ter atingido um platô em até 8 horas e diminuído após esse período de forma que suas produções não pôde ser detectada.

Em face aos resultados apresentados no estudo 2, o modelo de candidose oral estabelecido no presente estudo foi adequado para reproduzir a infecção. A realização de 5 aplicações sucessivas de PDT mediada pelo PDZ foi tão efetiva quanto a NIS no tratamento de candidose oral de camundongos imunossuprimidos. Embora a análise histológica tenha demonstrado que a PDT não causou efeitos adversos ao tecido lingual, a análise biomolecular revelou que este tratamento provocou a expressão de TNF-α, o que parece ser um fator benéfico para a remissão das lesões orais. Entretanto, técnicas biomoleculares mais detalhadas que avaliem a produção de outras citocinas pró e anti-inflamatórias em diferentes intervalos de tempo são necessárias para esclarecer o mecanismo de ação da PDT no hospedeiro.

Adicionalmente, o estudo 3 avaliou se o estresse oxidativo causado pela PDT mediada por Curcumina (CUR) associada a luz LED azul promove danos ao DNA da *C albicans* por meio do ensaio cometa. O estresse oxidativo é causado pelas espécies reativas de oxigênio (ROS), tais como o radical superóxido e hidroxila, e mais especificamente o oxigênio singleto que representam uma ameaça significativa no que diz respeito à integridade celular uma vez que podem causar danos ao DNA, lipídios, proteínas e outras macromoléculas celulares[78]. Na presente investigação, observou-se que a realização da PDT mediada pela CUR (2.5 μM que corresponde a 0,9 mg/L) associado a 37.5 J/cm^2 (P+L+) promoveu danos no DNA das células. Essas alterações foram mais acentuadas quando comparados ao grupo tratado com H_2O_2.

Estes resultados podem ser justificados pelo fato das leveduras serem células aeróbias, as quais apresentam formação de espécies reativas de oxigênio (ROS) durante seu metabolismo natural na cadeia respiratória mitocondrial e pela geração de reações de H_2O_2 catalisadas pelas oxidases[61]. As ROS endógenas são produzidas pela dispersão das mesmas da cadeia de transporte de elétrons mitocondrial, se difundindo livremente através das membranas celulares, agredindo outros componentes no interior da célula[73]. O dano oxidativo produzido por ROS endógenas pode provocar modificações de bases de DNA, quebras de fitas simples e dupla, além da formação de lesões purínica/pirimidínicas, muitas das quais são tóxicas e/ou mutagênicas[34]. Essas ROS endógenas somadas às produzidas pela luz associadas a CUR podem ter sido responsáveis por acentuar o dano genético às células de *C. albicans*.

Após aplicação da luz LED azul (P-L+) também foi observado dano no DNA das células independentemente da utilização do agente fotossensibilizador. Estudos têm mostrado que o comprimento de onda utilizado (455 nm) no presente estudo causa estresse oxidativo que pode estar associado à foto-excitação das porfirinas intracelulares endógenas de forma que a absorção desses fótons promoveria a transferência de energia ao oxigênio culminando na produção de ROS[40, 55]. Tem sido relatado que a luz azul pode ser detectada pelos micro-organismos podendo induzir respostas fisiológicas provocadas pelos receptores de luz[21]. Assim, a foto-excitação de cromóforos sensibilizados pela luz azul, incluindo flavinas e citocromos, dentro da mitocôndria e/ou peroxissomos, poderia induzir a formação de ROS endógenos[21]. A presença de cometas em células de *C. albicans* também foi observada após a irradiação com UV (352 nm)[30]. Além disso, tem sido relatado que a luz LED azul apresenta efeito antimicrobiano na inativação de bactérias, tais como *Staphylococcus aureus*[38], incluindo as cepas resistentes à meticilina[28] e *Pseudomonas aeruginosa*[38]. De acordo com estudos que avaliaram o espectro de banda visível, o intervalo de comprimento de onda de 402 a 420 nm, tem sido relatado como sendo o mais eficaz para inativação de micro-organismos, enquanto que o espectro de 455 a 470 nm mostrou potencial antimicrobiano apenas em algumas espécies bacterianas[21]. Estudos têm demonstrado que o comprimento de onda de 455 nm utilizado no presente estudo, quando avaliado isoladamente não apresenta efeito antifúngico[26]. No entanto, esta investigação demonstrou que esse comprimento de onda foi capaz de promover danos no DNA de *C. albicans*. Esses achados podem ser justificados pelo fato de que as células de *C. albicans* têm a habilidade de reparar danos no DNA com eficiência, o que permitiu a sobrevivência desse micro-organismo sob as condições empregadas neste estudo.

Avaliação da fluorescência com H2DCF-DA revelou que a PDT, luz e H_2O_2 provocaram estresse oxidativo. A intensidade de fluorescência do grupo tratado apenas com luz (P-L+) foi mais acentuada, o que sugere que a produção de ROS foi maior neste grupo. Estes resultados podem ter ocorrido porque a luz azul estimulou a produção intracelular de ROS, como discutido anteriormente. É importante ressaltar que menor fluorescência foi observada quando as células foram submetidas a PDT. Tem sido reportado que alta concentração de CUR altera a expressão de genes que regulam o metabolismo e a integridade mitocondrial de *C. albicans*[53]. Possivelmente, a CUR pode ter agido na mitocôndria, prejudicando a respiração celular, resultando em menor produção de ROS endógenos. Embora, baixa concentração de CUR tenha sido utilizada no presente estudo, nós sugerimos que a luz pode ter aumentado o efeito da CUR sobre a célula. Assim, é possível mencionar que a baixa concentração de CUR associada a luz LED azul alterou o metabolismo celular, o que pode resultar em menor quantidade de ROS produzido nas mitocôndrias durante a respiração celular. Por outro lado, a atividade antioxidante da CUR[4] também pode ter contribuído para diminuir a fluorescência no grupo tratado com PDT, contribuindo assim para atenuar o estresse oxidativo.

Adicionalmente, avaliou-se a cinética de reparação do DNA das células fúngicas com a finalidade de verificar se o DNA danificado seria reparado ao longo do tempo. Os resultados mostraram que CUR quando associada à luz LED azul (PDT; P+L+) foi genotóxica e impediu a reparação do DNA das células de *C. albicans*, a qual foi caracterizada pela redução do comprimento das caudas dos cometas em aproximadamente 45% após 30 minutos. Por outro lado, as células expostas a luz LED azul isoladamente (P-L+), apresentaram redução no comprimento das caudas de 90%. De acordo com a literatura, a influência da CUR na reparação do DNA de células fúngicas ainda não está completamente elucidada[2]. As topoisomerases são as principais enzimas responsáveis por causar diretamente mudanças topológicas no DNA e desempenham papel importante na regulação da replicação, transcrição, recombinação e condensação cromossômica[19]. As atividades desta enzima são cruciais para as células com danos no DNA[17]. De acordo com nosso conhecimento, apenas um estudo relatou a influência de CUR sobre a atividades enzimática das topoisomerases I e II (topo I e II) de *Sacharomycces. cerevisiae* e *C. albicans*[71]. Segundo os autores, baixa concentração de CUR (0,001mg / L) foi capaz de promover a inibição da atividade da topo I e II em células de *C. albicans*[71]. Com base nesses achados é possível sugerir que a CUR também pode ter interagido com as enzimas topoisomerase no presente estudo, impedindo a

reparação do DNA, o que explicaria a reparação do DNA mais lento nas células do grupo P+L+ em comparação com as células do grupo P-L+.

Em face aos resultados obtidos no estudo 3, o ensaio cometa pode ser considerado uma importante ferramenta para a avaliação da integridade do genoma de células de *C. albicans* em estudos que avaliem a toxicidade de compostos naturais e terapias dirigidas sobre DNA fúngico. Na presente investigação a PDT provocou grandes danos no DNA que não foi reparado de forma eficiente devido à inibição causada pela CUR. Este pode ser o principal mecanismo da atividade antifúngica da PDT mediada pela CUR. É importante ressaltar que os fatores de virulência deste micro-organismo também podem ter sido afetados pela PDT, o que ampliaria seu efeito terapêutico.

Assim, a realização dos 3 estudos mostrou o potencial da PDT na inativação de *C. albicans* com a utilização de diferentes fotossensibilizadores. Os resultados aqui apresentados reforçam ainda que a PDT é uma alternativa segura no tratamento de infecções fúngicas superficiais, tais como a candidose orofaríngea, uma vez que exibe atividade antimicrobiana sem causar danos ao hospedeiro, provocando apenas danos no DNA do micro-organismo. No futuro, estes parâmetros auxiliarão na realização segura e eficaz de pesquisas clínicas randomizadas.

5 - Conclusão

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Com base nas condições experimentais avaliadas e considerando as limitações do presente estudo, foi possível concluir que:

1. A associação de 31.7μM de cloro-alumínio ftalocianina veiculada em nanoemulsão catiônica com 100J/cm^2 de dose de luz promoveu a redução de 2,26log$_{10}$ da viabilidade celular de *C. albicans* presentes nas lesões linguais de camundongos imunossuprimidos.
2. Não foram observadas alterações histológicas no tecido lingual dos animais após 1 aplicação de PDT mediada pela cloro-alumínio ftalocianina em nanoemulsão catiônica.
3. O novo modelo murino de candidose oral desenvolvido foi reprodutível da infecção uma vez que os animais infectados por *C. albicans* apresentaram lesões linguais até 16 dias pós-infecção, de forma que 10^5 UFC/mL de *C. albicans* foram coletados das lesões orais durante todo período experimental.
4. As aplicações sucessivas da PDT mediada pelo Photodithazine® promoveram redução de 3log$_{10}$ da viabilidade celular de *C. albicans* e foi tão eficaz quanto a Nistatina no tratamento dos camundongos com candidose oral.
5. A análise macroscópica demonstrou rápida remissão das lesões orais nos animais tratados com PDT mediada pelo Photodithazine® em relação aos tratados com Nistatina.
6. Não foram observadas alterações histológicas nos tecidos linguais dos animais após 5 aplicações de PDT, entretanto a análise biomolecular revelou que a PDT mediada pelo Photodithazine® provocou a expressão da citocina TNF-α.
7. A PDT mediada pela curcumina (2,5μM), assim como a aplicação da luz LED azul (455nm) isoladamente promoveram danos no DNA de *C. albicans*.
8. A curcumina retardou a reparação do DNA de *C. albicans* causado pela aplicação de luz LED azul.

6 - Referências

6 - Referências

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*De acordo com o manual da FOAr/UNESP, adaptadas das normas Vancouver
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7 - Apêndice

7 – Apêndice: Análise estatística realizada nos 3 Capítulos

A seguir estão descritos os resultados originais obtidos por meio das metodologias realizadas nos Capítulos 1, 2 e 3. Adicionalmente, as análises estatísticas realizadas estão apresentadas para posterior interpretação dos resultados. Os dados estão apresentados na mesma sequência em que se encontram no respectivo Capítulo.

7.1 – Capítulo 1

Avaliação das unidades formadoras de colônias (CFU/mL) após PDT

A avaliação da viabilidade de *C. albicans* das amostras foi realizada por meio de contagem de colônias e cálculo das ufc/mL. Após a transformação dos valores em logaritmo (base 10), procedeu-se a análise descritiva dos dados para a verificação da normalidade e homocedasticidade por meio da realização dos testes de Shapiro-Wilk e Levene ($\alpha = 0,05$). O resultado de ambos os testes indicou valores de probabilidade maiores a 0,05, indicando que o conjunto dos dados atendia aos pressupostos necessários para utilização de testes paramétricos. Por essa razão, o teste estatístico empregado para comparação das médias dos grupos foi a ANOVA, ao nível de significância de 5% (Tabela A1). O valor de probabilidade encontrado indicou haver diferença estatisticamente significativa em pelo menos uma das médias dos grupos. Por isso, foi necessária a utilização de um teste de comparações múltiplas das médias (post hoc). O teste selecionado foi o teste de Tukey, ao nível de significância de 5%, e os resultados estão apresentados na Tabela A2.

Tabela A1- Sumário da Análise de Variância empregada para comparação das médias de log (UFC/mL) de todos os grupos avaliados.

	Df	Sum. Sq	Mean Sq.	F value	Pr (>F)
Entre grupos	5	19,031	3,806	37,865	0,000
Resíduo	24	2,412	0,101		

Tabela A 2 - Resultado das comparações múltiplas entre as médias dos grupos, por meio do teste de Tukey, ao nível de significância de 5%

Condições experimentais	**Média de log**	**Tukey test***
P-L-	5,43	a
P+L- CLAFt NE	5,49	a
P+L- CLAFt DMSO	5,46	a
P-L+	5,45	a
P+L+ CLAFt NE	3,18	b
P+L+ CLAFt DMSO	5,38	a

*: letras iguais denotam médias consideradas estatisticamente semelhantes pelo teste de Tukey (p>0,05).

Avaliação da expressão gênica de IL-1B, IL-6 e TNF-A

Após a extração e purificação de RNA e síntese do cDNA, foi avaliado o efeito da PDT sobre a expressão das citocinas pró e anti-inflamatórias (TNF-α, IL-1β e IL-6), envolvidas nas respostas de infecções superficiais. Para cada gene alvo foram selecionados primers específicos e sonda Taqman (Taqman Assay – Applied Biosystems): βActin = Mm00607939_S1; TNFα = Mm00443260_G1; IL-1β = Mm00434228_M1; IL-6 = Mm00446190_M1. Os níveis relativos da expressão dos genes foram calculados utilizando a β-actina como gene normalizador.

Os dados foram expressos usando o método de Ciclo limite (*Cq* ou *quantification cycle*), ou seja, o número de ciclos no qual a curva logarítmica de PCR cruza uma linha de corte arbitrariamente calculada. O cálculo da média do ΔCq da duplicata das amostras (Cq do gene alvo menos o Cq do gene normalizador) foi submetido a regressão linear. O primeiro parâmetro utilizado foi o valor de limiar da curva de amplificação obtida pela amplificação do cDNA alvo (Cq). Foi realizada a normalização dos valores entre a razão do Cq obtido da amplificação específica do gene alvo pelo Cq do gene normalizador (ΔCq).

Para as citocinas TNFα e IL-1β, como os dados obtidos atenderam aos pressupostos de normalidade e homocedasticidade foi utilizado os testes de Shapiro-Wilk e Levene, respectivamente (α = 0,05). Em seguida os dados foram analisados por ANOVA (Tabelas A3 e A4) seguido do teste post-hoc de Tukey ($p<0.05$). Para a citocina IL-6, de acordo com os testes de Shapiro-Wilk e Levene, os dados não atenderam aos pressupostos de normalidade e homogeneidade ($p<0.05$). Dessa foram a análise estatística foi realizada pelo teste não paramétrico de Kruskal-Wallis e Dunn post-hoc (Tabela A5) para comparação entre os grupos ($p<0.05$).

Tabela A3- Sumário da Análise de Variância empregada para comparação das médias de todos os grupos avaliados para a citocina TNFα, 24 horas após os tratamentos.

	Df	Sum. Sq	Mean Sq.	F value	Pr (>F)
Entre grupos	7	3342,192	477,456	7,369	0,000
Resíduo	24	1555,038	64,793		

Tabela A4- Sumário da Análise de Variância empregada para comparação das médias de todos os grupos avaliados para a citocina IL-1β, 24 horas após os tratamentos.

	Df	Sum. Sq	Mean Sq.	F value	Pr (>F)
Entre grupos	7	812,447	116,064	0,761	0,625
Resíduo	24	3659,604	152,483		

Tabela A5- Resultados das comparações múltiplas dos grupos experimentais em relação ao grupo controle por meio de teste não paramétrico para a expressão da citocina IL-1β, 24 horas após os tratamentos.

Condições experimentais	**Valores de p**	**Comparações múltiplas***
P+L- CLAFt NE	1,000	a
P+L- CLAFt DMSO	1,000	a
P-L+	0,508	a
P+L+ CLAFt NE	0,938	a
P+L+ CLAFt DMSO	0,380	a
P+L+ CLAFt NE NI	1,000	a
P+L+ CLAFt DMSO NI	0,857	a

*Letras iguais denotam grupos considerados estatisticamente semelhantes pelo teste estatístico não paramétrico.

7.2 – Capítulo 2

Avaliação das unidades formadoras de colônias (CFU/mL) após PDT

Da mesma forma descrita no Capítulo 1, as contagens de colônias de *C. albicans* (ufc /mL) foram transformadas em logaritmos (base10). De acordo com a análise descritiva os dados apresentaram-se normais e heterocedásticos ($\alpha = 0,05$). Para realização da análise estatística os dados foram submetidos a Análise de Variância ANOVA Two-Way para verificação de interação entre grupo de tratamento com o período de tempo avaliado (24 h e 7 dias após o tratamento) (Tabela A6). Como os dados apresentaram-se heterogêneos e foi observada interação entre os fatores grupo e período de tempo, os dados foram ainda submetidos a Análise de Variância ANOVA One-Way com correção de Welch seguido do post-hoc de Games-Howell para comparações múltiplas entre os grupos (Tabela A7).

Tabela A6- Sumário da Análise de Variância empregada para comparação das médias de log (UFC/mL) de todos os grupos avaliados.

	Type III Sum. Sq	df	Mean Sq.	F value	Pr (>F)
Grupo	13,064	4	34,516	189,311	0,004
Período de tempo	1,569	1	1,569	8,604	0,000
Interação (grupo x tempo)	7,474	4	1,869	10,648	0,000
Erro	20,056	110	0,182		

Tabela A7- Resultado das comparações múltiplas entre as médias de todos os grupos em função do período avaliado (24 horas e 7 dias após os tratamentos).

Condições experimentais	**Média de log_{10} (24 horas)**	**Média de log_{10} (7 dias)**	**Games-Howell test***
P-L-	6,05	5,40	A a
P+L- PDZ	5,35	5,32	A a
P-L+	5,22	5,14	A a
P+L+ PDZ	3,06	3,37	A b
Nis	2,87	3,70	B b

*: Letras maiúsculas iguais denotam semelhança entre os períodos de tempo avaliados (24 horas e 7 dias após os tratamentos) para cada grupo e letras minúsculas iguais denotam semelhança estatística entre os grupos experimentais pelo teste de Games-Howell ($p>0,05$).

Avaliação da expressão gênica de IL-1B, IL-6 e TNF-A

Após a extração e purificação de RNA e síntese do cDNA, foi avaliado o efeito da PDT sobre a expressão das citocinas pró e anti-inflamatórias (TNF-α, IL-1β e IL-6), envolvidas nas respostas de infecções superficiais. Para cada gene alvo foram selecionados primers específicos e sonda Taqman (Taqman Assay – Applied Biosystems): βActin = Mm00607939_S1; TNFα = Mm00443260_G1; IL-1β = Mm00434228_M1; IL-6 = Mm00446190_M1. Os níveis relativos da expressão dos genes foram calculados utilizando a β-actina como gene normalizador.

Os dados foram expressos usando o método de Ciclo limite (Cq ou quantification cycle), ou seja, o número de ciclos no qual a curva logarítmica de PCR cruza uma linha de corte arbitrariamente calculada. O cálculo da média do ΔCq da duplicata das amostras (Cq do gene alvo menos o Cq do gene normalizador) foi submetido a regressão linear. O primeiro parâmetro utilizado foi o valor de limiar da curva de amplificação obtida pela amplificação do cDNA alvo (Cq). Foi realizada a normalização dos valores entre a razão do Cq obtido da amplificação específica do gene alvo pelo Cq do gene normalizador (ΔCq).

Os dados obtidos a partir do RT-qPCR para a citocina TNF-α e IL 1-β obedeceram aos pressupostos de normalidade e homogeneidade das variâncias. Dessa forma realizou-se ANOVA two-way seguido do teste de Tukey para comparação entre os grupos em função dos diferentes períodos de tempo avaliados ($p<0.05$) (Tabelas A8 e A9 respectivamente). Para a citocina IL 6, os dados apresentaram-se normais e heterogêneos. Assim os dados foram submetidos ao teste de análise de variância ANOVA Two-Way (Tabela A10). Como houve interação entre a expressão das citocinas em função do grupo experimental em relação ao período de tempo avaliado, os dados foram submetidos ainda a análise de variância ANOVA One-Way com correção de Welch seguida do teste post-hoc Games-Howell para comparações múltiplas ($p<0.05$) (Tabela A11).

Tabela A8- Sumário da Análise de Variância empregada para comparação das médias de todos os grupos avaliados para a citocina TNFα, 24horas e 7 dias após os tratamentos.

	Type III Sum. Sq	df	Mean Sq.	F value	Pr (>F)
Grupo	0,069	1	0,069	189,311	0,004
Período de tempo	7,130	5	1,426	8,604	0,000
Interação (grupo x tempo)	0,028	5	0,006	10,648	0,060
Erro	1,085	24	0,045		

Tabela A9- Sumário da Análise de Variância empregada para comparação das médias de todos os grupos avaliados para a citocina IL 1-β, 24horas e 7 dias após os tratamentos.

	Type III Sum. Sq	df	Mean Sq.	F value	Pr (>F)
Grupo	9,054	5	1,811	26,752	0,000
Período de tempo	1,357	1	1,357	20,050	0,000
Interação (grupo x tempo)	0,700	5	0,140	2,069	0,105
Erro	1,625	24	0,068		

Tabela A10- Sumário da Análise de Variância empregada para comparação das médias de todos os grupos avaliados para a citocina IL 6, 24horas e 7 dias após os tratamentos.

	Type III Sum. Sq	df	Mean Sq.	F value	Pr (>F)
Grupo	1787,167	5	357,433	6,552	0,001
Período de tempo	58,778	1	58,778	1,077	0,310
Interação (grupo x tempo)	724,722	5	144,944	2,657	0,048
Erro	1309,333	24	54,556		

7.3 – Capítulo 3

Análise de dano ao DNA de células de C. albicans

No Capiítulo 3 os resultados estão expressos em média e desvio padrão de, pelo menos, três réplicas obtidas em experimentos independentes. Em cada réplica obteve-se um valor correspondente à média de pelo menos 50 cometas que foi usado para o cálculo das médias das 3 réplicas. De acordo com a análise descritiva, os dados referentes ao comprimento das caudas dos cometas se apresentaram paramétricos e homogêneos. Dessa maneira, a análise estatística foi efetuada por meio da análise da variância ANOVA One-Way (Tabelas A11) e Tukey post-hoc para comparação entre grupos ($p < 0.05$) (Tabelas A12).

Tabela A11- Sumário da Análise de Variância empregada para comparação das médias de comprimento das caudas dos cometas para todos os grupos avaliados.

	Df	Sum. Sq	Mean Sq.	F value	Pr (>F)
Entre grupos	4	145996,460	36499,115	308,141	0,000
Resíduo	95	11252,700	118,449		

Tabela A12 - Resultado das comparações múltiplas entre as médias dos grupos, por meio do teste de Tukey, ao nível de significância de 5%

Condições experimentais	**Média de comprimento da cauda (μM)**	**Tukey test***
P-L-	10	a
P+L- CUR	12	a
H_2O_2	36	b
P-L+	87	c
P+L+ CUR	112	d

*: letras iguais denotam médias consideradas estatisticamente semelhantes pelo teste de Tukey (p>0,05).

Análise da produção de ROS intracelular

A análise da produção de ROS intracelular foi avaliada por meio da aplicação do fluorocromo H2DCFDA após os tratamentos instituídos sobre as suspensões planctônicas de *C. albicans*. Os dados obtidos estão expressos em unidades arbitrárias (AU), e indicam a intensidade de fluorescência dos grupos avaliados. De acordo com a análise descritiva, os dados atenderam aos pressupostos de normalidade e homogeneidade. Dessa forma foi realizada análise da variância ANOVA One-Way (Tabelas A13) e Tukey post-hoc para comparação entre grupos ($p < 0.05$) (Tabelas A14).

Tabela A13- Sumário da Análise de Variância empregada para comparação das médias de intensidade de fluorescência para todos os grupos avaliados.

	Df	Sum. Sq	Mean Sq.	F value	Pr (>F)
Entre grupos	5	2,482E7	4963940,373	41,431	0,000
Resíduo	24	2875476,000	119811,500		

Tabela A14 - Resultado das comparações múltiplas entre as médias dos grupos, por meio do teste de Tukey, ao nível de significância de 5%

Condições experimentais	**Média da intensidade de fluorescência (A.U.)**	**Tukey test***
P-L-	351	a
P+L- CUR	435	a
H_2O_2	460	b
P+L+ CUR	501	b
P-L+	789	c

*: letras iguais denotam médias consideradas estatisticamente semelhantes pelo teste de Tukey (p>0,05).

Análise de reparação do DNA de células de C. albicans

Esta análise avaliou a reparação de DNA observada nos grupos experimentais, a qual foi caracterizada pela diminuição do tamanho das caudas dos cometas ao longo do tempo.

De acordo com a análise descritiva os dados apresentaram-se normais e homogêneos ($\alpha = 0,05$). Para realização da análise estatística os dados foram submetidos a Análise de Variância ANOVA Two-Way para verificação de interação entre grupo de tratamento com o período de tempo avaliado (0, 10 e 30 minutos após tratamento) (Tabela A15) e Tukey post-hoc para comparação entre grupos ($p < 0.05$) (Tabelas A16).

Tabela A15- Sumário da Análise de Variância empregada para comparação das médias de comprimento das caudas dos cometas para todos os grupos avaliados.

	Type III Sum. Sq	df	Mean Sq.	F value	Pr (>F)
Grupo	595400	11	54130	143,3	0,0001
Tempo	8507	23	369,9	0,9793	0,4927
Erro	95550	253	377,7		

Tabela A16 - Resultado das comparações múltiplas entre as médias dos grupos, por meio do teste de Tukey, ao nível de significância de 5%

Condições experimentais	**Média da comprimento da cauda (μM) em função do tempo:**			**Tukey test***
	0	**10**	**30**	
P-L-	9,04	9,46	9,25	a
P+L- CUR	11,42	11,29	11,17	a
P+L+ CUR	107,50	87	78,9	b
P-L+	83,83	44,75	10,67	c

* Letras minúsculas iguais denotam semelhança estatística entre os grupos experimentais pelo teste de Tukey ($p>0,05$).

8 - Anexos

8.1 – Autorização do Comitê de Ética em Pesquisa (CEUA) – Capítulo 1.

UNIVERSIDADE ESTADUAL PAULISTA
Faculdade de Odontologia
Câmpus de Araraquara
Comissão de Ética no Uso de Animais - CEUA

C E R T I F I C A D O

Certificamos que o protocolo nº **1/2010** referente à pesquisa **"Avaliação do efeito da terapia fotodinâmica utilizando solução de Cloro Alumínio Ftalocianina veiculada em nanoemulsão catiônica e em solvente orgânico (DMSO) em camundongos com candidose induzida"** sob a responsabilidade da **Profa. Dra. Ana Cláudia Pavarina, Prof. Dr. Carlos Alberto de Souza Costa** e **Ana Paula Dias Ribeiro** está de acordo com os Princípios Éticos em Experimentação Animal adotado pela legislação brasileira atualmente em vigor, tendo sido aprovado pela Comissão de Ética no Uso de Animais (CEUA) da Faculdade de Odontologia de Araraquara-UNESP.

C E R T I F I C A T E

We certify that the protocolo **1/2010** referring to the research **"Effectiveness of photodynamic therapy using Aluminum Chlorine Phthalocyanine solution entrapped in cationic nanoemulsion and organic solvent (DMSO) in mice with induced candidiasis"** under responsibility **Profa. Dra. Ana Cláudia Pavarina, Prof. Dr. Carlos Alberto de Souza Costa** and **Ana Paula Dias Ribeiro** in agreement with the nowadays specific brazilian laws and was approved by the Araraquara Dental School-UNESP Ethical Committee for Animal Research (CEUA).

Araraquara, 9 de Dezembro de 2013.

Elisa Maria Aparecida Giro

Profa. Dra. ELISA MARIA APARECIDA GIRO
Coordenador Substituto da CEUA/FOAr/UNESP

8.2 – Autorização do Comitê de Ética em Pesquisa (CEUA) – Capítulo 2.

unesp
UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Araraquara
FACULDADE DE ODONTOLOGIA

Proc. CEUA nº 24/2012

Araraquara, 15 de junho de 2015.

Senhores Pesquisadores:

A Comissão de Ética no Uso de Animal – CEUA desta Faculdade, procedeu a análise do Relatório Parcial do projeto de pesquisa de sua responsabilidade intitulado ***"ESTUDO DO EFEITO DA TERAPIA FOTODINÂMICA ANTIMICROBIANA NO TRATAMENTO DE CANDIDOSE ORAL INDUZIDA EM LÍNGUA DE CAMUNDONGO. AVALIAÇÃO IN VIVO DE UM HIDROGEL DE PHOTODITHAZINE E NANOEMULSÃO CATIÔNICA DE CLORO ALUMÍNIO FTALOCIANINA"*** (Proc. CEUA nº 24/2012), e considerou-o APROVADO, bem como sua solicitação de alteração da metodologia e prorrogação do prazo da pesquisa.

Lembramos que o Relatório Final deste projeto deverá ser entregue em **NOVEMBRO/2015**.

Atenciosamente.

Prof. Dr. PAULO SÉRGIO CERRI
Coordenador da CEUA

À
Profa. Dra. ANA CLÁUDIA PAVARINA
DD. Pesquisadora Responsável
Departamento de Materiais Odontológicos e Prótese

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8.3 – Página inicial do artigo publicado referente ao Capítulo 3.

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Research Article

RESEARCH ARTICLE

Genotoxic effect of photodynamic therapy mediated by curcumin on *Candida albicans*

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One sentence summary: With the widespread use of blue LED for the treatment of human infections such as oropharyngeal candidiasis, concerns about the safety of this technology in photodynamic therapy have been raised.

Editor: Cristina maccioni

ABSTRACT

Photodynamic therapy (PDT) is a promising method for localized and specific inactivation of fungi and bacteria. A nontoxic light-sensitive compound is taken up by cells, which are then exposed selectively to light, which activates toxicity of the compound. We investigated the potential of sublethal PDT using light-sensitive curcumin (CUR) in combination with blue (455 nm) light to promote reactive oxygen species (ROS) formation in the form of singlet oxygen and DNA damage of *Candida albicans*. Surprisingly, CUR-mediated PDT but also light alone caused significantly longer comet tails, an indication of DNA damage of *C. albicans* when compared with the negative control. The intracellular ROS production was also significantly higher for the group treated only with light. However, PDT compared to blue light alone significantly slowed DNA repair. Comet tails decreased during 30 min visualized as a 90% reduction in length in the absence of light for cells treated with light alone, while comet tails of cells treated with PDT only diminished in size about 45%. These results indicate that complex mechanisms may result in PDT in a way that should be considered when choosing the photosensitive compound and other aspects of the treatment design.

Keywords: photoinactivation; fungi; photosensitizer agent; genotoxicity

INTRODUCTION

The species of *Candida* genus are considered as commensal microorganisms that are part of the oral microflora of healthy individuals. Under certain circumstances, these microorganisms can act as opportunistic pathogens, being responsible for the development of infections. Oropharyngeal candidiasis (OPC) has been reported as the most common opportunistic infection caused by an accentuated growth and penetration of fungal species in the oral tissues (Cannon et al. 1995; Pfaller and Diekema 2007). Some predisposing factors such as poorly fit dentures, AIDS and broad-spectrum antibiotic use over extended periods of time can enhance host susceptibility to develop this infection (Samaranayake 1990; Scully, El-Kabir and Samaranayake 1994; Shapiro, Robbins and Cowen 2011). Disseminated candidiasis in immunocompromised patients can cause high mortality rates (Kromery and Barnes 2002; Pfaller and Diekema 2007). Treatments used against infections caused

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Araraquara, 29 de julho de 2015.

JULIANA CABRINI CARMELLO