UNIVERSIDADE FEDERAL DO CEARÁ
FACULDADE DE FARMÁCIA, ODONTOLOGIA E ENFERMAGEM
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

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TERAPIA FOTODINÂMICA ANTIMICROBIANA PARA *CANDIDA ALBICANS*: ESTUDO MICROBIOLÓGICO, BIOMOLECULAR E DE SEUS EFEITOS NA RESINA ACRÍLICA.

FORTALEZA
2016

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Tese apresentada à comissão examinadora aprovada pela coordenação do Programa de Pós-graduação em Odontologia, como pré-requisito para obtenção do título de Doutor em Odontologia.

Área de concentração: Clínica Odontológica.

Orientadora: Prof(a). Dr(a). Karina Matthes de Freitas Pontes.

FORTALEZA

2016

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Biblioteca de Ciências da Saúde

F961t Frota, Bruna Marjorie Dias de Carvalho.

Terapia fotodinâmica antimicrobiana para *Candida albicans*: estudo microbiológico, biomolecular e de seus efeitos na resina acrílica./ Bruna Marjorie Dias Frota de Carvalho. – 2016.

98 f. : 2 il. color., enc. ; 30 cm.

Tese (doutorado) – Universidade Federal do Ceará; Centro de Ciências da Saúde; Faculdade de Farmácia, Odontologia e Enfermagem; Programa de Pós-Graduação em Tecnologia; Fortaleza, 2016.
Área de Concentração: Clínica Odontológica.
Orientação: Profa. Dra. Karina Matthes de Freitas Pontes.

1. Fotoquimioterapia. 2. Desinfecção. 3. Genotoxicidade. 4. *Candida albicans* I. Título.

CDD

617.695

DEDICATÓRIA

À **Deus**, pelas imensas bênçãos derramadas sobre a minha vida. Por estar sempre comigo, principalmente nos momentos mais difíceis. Por sempre me dar força e fazer-me perceber que nenhum obstáculo é grande demais quando confiamos Nele.

Aos meus pais, **Jaime e Valderez**, por todo carinho, amor, companheirismo, apoio, amizade e principalmente por serem exemplos de caráter, de humildade, força e determinação. Quero sempre ser motivo de alegrias e de muito orgulho para vocês.

Aos meus irmãos, **Erica e Filipe,** que são bênçãos na minha vida. Por me mostrarem a função de uma família.

Ao meu esposo, **Fernando**, que sempre me incentivou a lutar pelos meus objetivos. Com todo meu amor, eu agradeço pela sua companhia, paciência e carinho nos momentos mais difíceis.

A **Bel**, por toda companhia e amor incondicional. Saudades!

AGRADECIMENTOS ESPECIAIS

À minha orientadora, **Prof(a). Dr(a). Karina Matthes de Freitas Pontes**, por ter contribuído para minha formação intelectual e científica desde a graduação. Agradeço pelos ensinamentos, conselhos, inteligência, segurança e honestidade em todo esse período. Obrigada pela confiança e apoio dedicados a mim.

A ex-coordenadora **Prof(a). Dr(a). Lidiany Karla Azevedo Rodrigues** e ao atual coordenador do Programa de Pós-graduação em Odontologia da UFC, **Prof. Dr. Vicente de Paulo A. Saboia**, pela atenção dada a todos os alunos e ao programa.

Ao professor **Marcus Aurélio Lima Verde**, por todo o exemplo de paciência, bondade e profissionalismo.

Aos amigos do nosso grupo de pesquisa, **Bruna Albuquerque, Hallison, Nicole, Kadidja, Ivo, Adamastor, Janaína** por toda colaboração e companheirismo.

AGRADECIMENTOS

Aos **Professores do Programa** de Pós-graduação em Odontologia da UFC, que muito me ensinaram. A garra e a capacidade demonstrada por todos foi um grande incentivo em minha carreira, servindo de exemplo em minha vida.

Aos Funcionários do Programa de Pós-graduação em Odontologia, **Lúcia Ribeiro Marques Lustosa** e **Janaíne Marques Leal**, pela constante disponibilidade.

Aos **alunos do Programa**, os quais tive o privilégio de dividir e adquirir conhecimento. Todos são muito especiais para mim.

Aos **Titulares e Suplentes** da banca de defesa, por suas disponibilidade e apoio.

À equipe do Laboratório de Engenharia de Sistema de Computação da UFC, especialmente ao **Prof. Jarbas Aryel** e ao aluno **Mário Wilson**, pela ajuda na confecção do dispositivo do estudo.

Ao professor **Dr. Kyle Tanner**, pela valiosa contribuição à minha formação e orientação durante estágio realizado no Institut de Biologie Phisico-chimique, Paris, França, sobretudo pelas discussões e aprendizado decorrentes do planejamento e execução de um capítulo desta tese.

Aos professores **Rui Oliveira** e **Bjorn Johnasson**, pela ajuda e atenção no desenvolvimento de um capítulo desta tese, realizado na Universidade de Minho, Portugal. Pelas discussões e aprendizado adquiridos ao longo deste trabalho.

A **Central Analítica** da Universidade Federal do Ceará, por fornecer material e profissionais para a análise de imagens fornecidas em um capítulo desta tese.

Às instituições de fomento **CAPES** (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) e **FUNCAP** (Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico), pelos recursos financeiros que propiciaram o nosso estudo.

Aos **meus familiares e amigos** pela compreensão às muitas horas em que estive ausente para à dedicação ao estudo.

O desenvolvimento desta tese foi também uma grande caminhada de crescimento pessoal, possibilitado pelo carinho, apoio e compreensão de muitas pessoas, nem todos puderam ser citados. A todos que, de alguma forma, colaboraram para a concretização deste trabalho.

MEUS SINCEROS AGRADECIMENTOS!

“A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê.”

(Arthur Schopenhauer)

RESUMO

O amplo espectro das candidíases orais e a respectiva importância clínica da infecção impulsionam pesquisas que pretendem identificar tratamentos eficazes seletivos contra *Candida albicans*. Este estudo foi dividido em quatro capítulos, cujos objetivos foram: Capítulo 1) Comparar diferentes tipos de associações de Terapia Fotodinâmica antimicrobiana (TFDa), por meio da determinação da Concentração Inibitória Mínima (CIM), Concentração Fungicida Mínima (CFM), ação em cultura planctônica e biofilme de *Candida albicans*, sobre as seguintes formas de associação: LED 630 nm/ 1000 mW/ 152 J cm^{-2}, associado às soluções de azul de metileno (AM) e azul de orto toluidina (AOT) e LED 520 nm/ 400 mW/cm2/ 152 J cm^{-2} junto à solução de rosa bengala (RB); Capítulo 2) Avaliar possíveis danos à estrutura de superfície da resina acrílica, como alteração de cor, rugosidade e perda de massa, com o uso de fotossensibilizadores (FSs) aplicados nas terapias propostas no capítulo 1, e compará-los com desinfetantes de uso comum na prática clínica; Capítulo 3) Verificar o potencial genotóxico da TFDa e a produção de Espécies Reativas de Oxigênio (EROs), com teste citométrico associado ao composto fluorescente H2DCF-DA, e testes de extensão dos danos no DNA em células de *C. albicans*; Capítulo 4) Desenvolvimento de um projeto de patente de dispositivo confeccionado para TFDa em materiais protéticos. Para os trabalhos realizados com uso de TFDa, os grupos experimentais foram divididos da seguinte maneira: não tratado (FS-L-); somente irradiado (FS-L+); somente corado (FS+L-); corado e irradiado (FS+L+); e controles positivos (Nistatina 100.000U/ml ou Hipoclorito de sódio 1%). Os resultados determinaram que a melhor associação de TFDa foi AOT ou AM com LED 630nm/1W, obtendo redução da viabilidade fúngica em biofilme de 2 a 4 Log. Os FS não apresentaram alterações de rugosidade de superfície, mudança de cor, quando comparados aos desinfetantes químicos nos especimes de resina acrilica. TFDa causou danos ao DNA em células de *C. albicans* (113,7±4,818) quando comparada ao controle negativo (17,03±1,138) ($p<0,0001$), através de análise de ensaios de cometa (μm), houve também aumento da produção de EROS quando o FS era submetido à irradiação (FS+L+), em comparação aos outros grupos. Conclui-se que TFDa utilizada apresenta-se como uma promissora abordagem terapêutica contra *C. albicans*.

Palavras-chaves: fotoquimioterapia, desinfecção, genotoxicidade, *Candida albicans*.

ABSTRACT

The broad spectrum of oral candidiasis and their importance in clinical infection boosted researches intended to clarify the pathogenic mechanisms and identify selective treatments against *Candida albicans*. This study was divided into four chapters, with the following objectives: Chapter 1) Compare different types of antimicrobial photodynamic therapy (PACT) associations, through determination of Minimum Inhibitory Concentration (MIC), Minimum Fungicide Concentration (MFC), action front planktonic culture and biofilm of *Candida albicans*, on the associations: LED 630 nm/1 W/152 J cm-2, associated with methylene blue (MB) and toluidine blue ortho (TBO) and LED 520 nm/400 mW/152 J cm-2 with Bengal Rose (BR); Chapter 2) To evaluate possible damage to the surface structure of acrylic resins, such as color change, surface roughness and mass loss, through the use of photosensitizers (Ps) applyed in therapies proposed in Chapter 1, and compare them with commonly used disinfectants in clinical practice; Chapter 3) Check the genotoxic potential of PACT and the production of Reactive Oxygen Species (ROS), with cytometric test associated with the fluorescent compound H2DCF-DA, and testing of DNA damage on *C. albicans* cells; Chapter 4) Development of a device patent design made for antimicrobial phototherapy (PACT) for disinfection of prosthetic materials. For the studies carried out with use of PACT, the experimental groups were divided as follows: not treated (P-L-); irradiated only (P-L+); only stained (P+L-); stained and irradiated (P+L+); and positive controls. The results determined that the best PACT association was TBO or MB with LED 630 nm/1 W, obtaining reduction of fungal viability of the biofilm from 2 to 4 log. The Ps did not cause surface roughness change, color change, or mass loss, when compared to chemical disinfectants. PACT caused DNA damage on *C. albicans* cells (113.7 ± 4.818), when compared to the negative controls (17.03 ± 1.138) ($p < 0.0001$), by analyzing comet assays (μm). There was an increasing of ROS production when the P was subjected to irradiation (P+L+), compared to other groups. Therefore, PACT showed to be a promising therapeutic approach against *C. albicans*.

Key Words: photochemotherapy, disinfection, genotoxicity, *Candida albicans*.

SUMÁRIO

1 Introdução Geral

1 INTRODUÇÃO

A colonização microbiana dos tecidos dos seres humanos tem início ao nascimento pela exposição de uma variedade de microrganismos presentes no meio ambiente e em outros seres vivos. No entanto, cada superfície possui propriedades físicas e biológicas próprias, levando a aquisição e desenvolvimento natural de uma microbiota diversificada e ao mesmo tempo característica de cada sítio anatômico, que normalmente vive em harmonia com o sítio hospedeiro (OLIVEIRA *et al*., 2010). Entre os sítios anatômicos, a cavidade bucal é um dos mais populosos, e com o uso de métodos moleculares, mais de 700 espécies de microrganismos já foram identificadas (AAS *et al*., 2005). Os fungos constituem uma pequena parte dessa microbiota, da qual a maior proporção é formada por leveduras do gênero *Candida* (MARSH & MARTIN, 1992; SIQUEIRA & BILGE, 2004). Dentre as aproximadamente 200 espécies do gênero *Candida,* oito são reconhecidas como agentes de processos patológicos orais: *C. albicans*, *C. tropicalis*, *C. parapsilosis, C. krusei*, *C. dublinienses*, *C. kefyr*, *C. glabrata*, *C. guilliermondii* (SEGAL, 2005).

As leveduras do gênero *Candida* são patógenos oportunistas, dependentes da habilidade de modular a expressão dos fatores de virulência em resposta a alterações locais associadas à competência do sistema imune do hospedeiro (SOLL, 2002). É importante lembrar que deficiências do sistema imune podem ser observadas em pessoas idosas com maior frequência (SILVA *et al.,* 2008). Muitas vezes também associadas com outro fator importante que é o uso de próteses dentárias. A reabilitação oral através da utilização de prótese dentária visa restabelecer a funcionalidade e resgatar a auto-estima do paciente. Porém, a utilização de aparelhos constituídos de resina acrílica é um sítio favorável à colonização por microrganismos, devido a sua porosidade, esses somados, proporcionam um ambiente favorável para que a *Candida spp* possa assumir a forma parasitária, produzindo doenças bucais conhecidas como estomatite protética (SILVA & SEIXAS, 2008; FALCÃO *et al.*, 2004).

Candida albicans é a espécie mais patogênica do gênero *Candida* e mais frequentemente isolada da cavidade oral, tanto como microrganismo comensal, como patógeno oportunista (NAGLIK *et al*., 2004; SULLIVAN *et al*., 2004). O tratamento da

estomatite protética em pacientes imunocomprometidos é complicada por sua natureza recorrente e sua exposição anterior reduz sua suscetibilidade a antifúngicos convencionais. *C. albicans* e outras espécies de *Candida* podem desenvolver resistência aos antifúngicos utilizados para tratar a candidíase oral (SANCHEZ-VARGAS *et al*., 2005; DELGADO *et al*., 2009). A terapia seletiva tem a vantagem de ser menos danosa à microbiota indígena, que está em risco durante o tratamento antifúngico sistêmico (WAINWRIGHT, 2004), além de ter baixo risco de interações medicamentosas (TEICHERT *et al*., 2002).

TERAPIAS PADRÃO – DESVANTAGEM E VANTAGEM

Atualmente, existem terapias alternativas contra microrganismos patogênicos baseados na fotossensibilização desses, conhecida como terapia fotodinâmica antimicrobiana (TFDa). Essa terapia consiste na associação de um agente corante fotossensibilizador a uma fonte de luz, no intuito de provocar necrose celular e morte microbiana. A ação se dá quando a substância fotossensibilizadora absorve os fótons da fonte de luz e seus elétrons passam a um estado excitado, ocorrendo uma primeira reação com produção de superperóxido, radicais hidroxila e radicais livres. Na presença de oxigênio molecular, ocorre uma segunda reação, em que o fotossensibilizador transfere energia ao mesmo quando retorna ao seu estado natural, formando moléculas de vida curta e altamente reativas, como o oxigênio singlete ($^1O_2$). Todos estes produtos oxidam moléculas biológicas, como proteínas mitocondriais, alterando sua estrutura e atividade, desnaturam proteínas e lipídios da membrana e modificam a estrutura do DNA celular (LYON *et al*., 2011; YAMADA, 2004).

Vantagens que podem ser observadas com o uso da inativação fotodinâmica são: seletividade do fotossensibilizador, focalização da luz apenas na região de interesse, possibilidade de repetição da terapia sem efeitos tóxicos cumulativos, não invasivos e de baixo risco (TEICHERT *et al*., 2002; DOVIGO, 2007; PERUSSI, 2007).

Há vários fatores que influenciam o dano frente essa terapia, incluindo o tipo, a dose, o tempo de incubação e localização do fotossensibilizador, a disponibilidade de oxigênio, o comprimento de onda da luz (nm), a densidade de potência de luz (mW cm^{-2}) e a densidade de energia da luz (J cm^{-2}) (ROLIM *et al*., 2012; SOUKOS & GOODSON, 2011; AZIZI *et al*., 2016). O oxigênio singlete ainda pode ter efeito direto nas moléculas extracelulares devido a sua alta reatividade química de modo que polissacarídeos presentes na matriz extracelular de biofilmes orais também sejam susceptíveis ao fotodano (KONOPKA & GOLINSKI, 2007).

Resultados de pesquisas experimentais demonstraram que leveduras podem ser mortas pela ação fotodinâmica empregando fotossensibilizadores fenotiazínicos, porfirinas e

fitalocianinas (COSTA *et al*., 2012; RODRIGUES *et al*., 2013; DONNELLY *et al.,* 2007). Corantes têm a habilidade de absorver luz visível com eficiência e são capazes de induzir ou participar de reações fotoquímicas. O fotossensibilizador ideal caracteriza-se por baixa toxicidade após a administração, não induzindo reação alérgica e deve absorver a luz no espectro determinado. Deve também ser facilmente sintetizado, ser um composto puro, hidrossolúvel e ser eliminado facilmente pelo paciente (CASTANO, DEMIDOVA & HAMBLIN, 2004). Ainda, deve ser biologicamente estável, fotoquimicamente eficaz, seletivo e minimamente tóxico aos tecidos normais ALTERAR (GARCEZ *NUNEZ E,,,,,*., 2013).

A literatura também apresenta três principais tipos de fontes luminosas para a realização da terapia fotodinâmica antimicrobiana: os lasers, os diodos emissores de luz (LEDs) e as lâmpadas de luz halógena. Os LEDs passaram a ser empregados na TFDa nos últimos anos (COSTA *et al*., 2012; MELO *et al*., 2010). Entre as principais vantagens da utilização da luz LED em relação às fontes laser são seu baixo custo, pequeno porte e facilidade de configuração para irradiações diferentes (NAGATA *et al*., 2012). Além disso, por não apresentarem tanta colimação e coerência, resultam em bandas de emissão de luz mais largas, favorecendo, assim, a complementaridade com o fotossensibilizador utilizado (GIUSTI *et al*., 2008).

Agentes desinfetantes são importantes na higiene, controle da adesão da placa bacteriana e remoção de biofilme em superfícies da resina acrílica. Entre os métodos mais difundidos de desinfecção para próteses acrílicas estão a imersão em hipoclorito de sódio, a imersão em clorexidina e o uso de tabletes de perborato de sódio (KUHAR & FUNDUK, 2005). Existem diversos trabalhos acerca de seus efeitos nas propriedades físico-químicas da resina acrílica (DA SILVA *et al*., 2008; PAVARINA *et al*., 2003), no entanto os fotossensibilizadores utilizados na TFD ainda não foram avaliados quanto a esses possíveis efeitos.

Dentro desta abordagem podemos destacar que o estudo de novas terapias alternativas aos antifúngicos e desinfetantes tradicionais, com maior e mais seletiva ação farmacológica, menor poder toxicológico e maior rapidez de ação, induzindo menor possibilidade de resistência celular, apresenta grande interesse clínico e científico na área da odontologia.

2 Proposição

2 PROPOSIÇÃO

Essa tese de doutorado será apresentada em capítulos, tendo como objetivos:

CAPÍTULO 1: Avaliar *in vitro* a atividade antimicrobiana de fontes de luz de 520nm e 630nm associadas com diferentes fotossensibilizadores em cultura planctônica e biofilme de *C. albicans*;

CAPÍTULO 2: Identificar possíveis alterações na resina acrílica submetida a aplicação de fotossensibilizadores comparados a desinfetantes químicos, quanto a rugosidade, perda de massa e alteração de cor;

CAPÍTULO 3: Avaliar o potencial genotóxico da terapia fotodinâmica antimicrobiana, utilizando a associação do azul de ortotoluidina e luz LED (630nm/1W) em suspensão de *C. albicans* e à produção de Espécies Reativas de Oxigênio (EROs) intracelular;

CAPÍTULO 4: Desenvolver através dos resultados obtidos nos trabalhos prévios, projeto de patente de novo dispositivo, portátil, de irradiação de luz vermelha ou azul (1W), para terapia fotodinâmica antimicrobiana em espécimes de resina acrílica de uso protético;

3 Capítulos

3 CAPÍTULOS

Esta tese está baseada no artigo 46 do regimento Interno do Programa de Pós-graduação em Odontologia da Universidade Federal do Ceará, que regulamenta o formato alternativo para dissertações de Mestrado e teses de Doutorado, e permite a inserção de artigos científicos de autoria ou co-autoria do candidato. Dessa forma, esta tese composta por quatro capítulos, contendo artigos a serem submetidos para publicação em revistas científicas, conforme descrito abaixo:

Capítulo 1

"*In Vitro* Comparative Evaluation of Photodynamic Antimicrobial Chemotherapy protocols against *Candida albicans*". Bruna Marjorie Dias Frota de Carvalho, Bruna Albuquerque Garcia, Ramille Araújo Lima, Karina Matthes de Freitas Pontes. Este artigo será submetido para publicação no periódico "*Photochemistry and Photobiology*".

Capítulo 2

"Effects of photosensitizers and disinfectant solutions on color stability, mass loss and surface roughness of denture base acrylic resins". Bruna Marjorie Dias Frota de Carvalho, Hallisson Wander de Mâcedo, Bruna Albuquerque Garcia, Karina Matthes de Freitas Pontes. Este artigo será submetido para publicação no periódico "*International Dental Journal*".

Capítulo 3

"Effect of antimicrobial photodynamic therapy mediated by ortho-toluidine blue on *Candida albicans* DNA damage". Bruna Marjorie Dias Frota de Carvalho, Karina Matthes de Freitas Pontes, Rui Oliveira, Bjorn Johansson. Este artigo será submetido para publicação no periódico "*Photodiagnosis and Photodynamic Therapy*".

Capítulo 4

"Dispositivo fototerápico com LED de alta potência associado a fotossensibilizadores específicos para desinfecção de materiais protéticos." Bruna Marjorie Dias Frota de Carvalho,

Karina Matthes de Freitas Pontes, Jarbas Aryel Nunes da Silveira, Mário Wilson Paiva Pereira.

Capítulo 1

In Vitro Comparative Evaluation of Photodynamic Antimicrobial Chemotherapy protocols against *Candida albicans suspesions and biofilm*.

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Este artigo está escrito segundo às normas de publicação de artigos da revista Photochemistry and Photobiology

http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)17511097/homepage/ForAuthors.html

ABSTRACT

Photodynamic antimicrobial chemotherapy (PACT) is an alternative treatment to infections based on the use of photosensitisers (Ps) and visible light. Several PACT protocols have been used for *Candida albicans* without standardization. In the present study, blue (400mW;152J/cm^2; 5min) and red (1W;152J/cm^2; 120sec) light emission diodes (LEDs) probes were used to activate different concentrations of ortho toluidine blue (TBO), methylene blue (MB) and bengal rose (BR) against *Candida albicans*, searching to identify the best protocol. Initially, the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) were determined, with values between 88 – 0.687 μM, followed by the analysis in planktonic culture and formed biofilm, with 135 disk-shaped acrylic resin specimens. For these tests the following groups were designed: no photosensitizer or light (P-L-); photosensitizer and light (P+L+); just photosensitizer (P+L-); just light (P-L+); nistatin 100,000U/ml (NT); sodium hypochlorite 1% (CH). After the treatments, the 1/100 and 1/1000 dilutions were plated and the number of colony-forming units (CFU) was calculated after 48 h. Data were analyzed by Kruskal-Wallis and Dunn's tests for those which didn't follow the normality curve (nao paramétricos), and ANOVA and Tukey's test for the others (α=0.05). The MIC/MFC results for TBO, MB and BR were, respectively, 5.5/11μM, 22/44μM, 22/22μM. For the planktonic culture, statistical difference was observed between the groups P+L+ and P-L- ($p<0.0001$) and no difference between NT and P+L+ ($p>0.05$). For the biofilm, all associations showed difference between P+L+ and P-L- ($p<0.0001$). Reduction values for TBO, MB, BR were respectively: 4.22, 2.28, 1.48 Logs. CFU reduction was also observed when the red LED 1W was used alone ($p<0.0001$). This study demonstrated that all associations of PACT exhibited antifungal effects, but TBO and MB with red LED 1W proved to be the best association.

Key-words: Photochemotherapy, *Candida albicans*, biofilms.

INTRODUCTION

Denture stomatitis (DS) is a chronic inflammatory response of the mucosa to a harmful stimuli, is widespread in edentate individuals and is considered to be the determinant of oral health in this population.[1] It is also the most common mucosal lesion associated with removable prostheses,[2,3] affecting one in every three complete denture wearers.[4] Several risk factors have been reported to be associated with denture stomatitis, including trauma,[5] poor hygienic habits, continuous and nocturnal denture wear [6] and fungal infections, particularly *Candida albicans*. [7]

Antifungal agents are commonly used to treat DS. Despite their effectiveness, the recurrence of infection after treatment is very common[8] and the major problem associated with the use of antifungal agents is the development of resistant species.[9] Because these agents do not eradicate microorganisms that colonize the denture,[10] it is also necessary to improve denture hygiene, discontinue nocturnal denture wearing, and eventually re-line or replace the dentures. Nonetheless, the effective removal of denture plaque by brushing requires a certain degree of manual dexterity which is commonly compromised in the elderly. In addition, the irregularities and porosities present on the acrylic resin surface may also contribute to penetration of micro-organisms into the dentures, making it difficult to be cleaned by brushing.[11,12]

Photodynamic antimicrobial chemotherapy (PACT) is a promising alternative to conventional antifungal treatment. For such therapy, three components are essential: the light source, the photosensitizer and oxygen. There are different types of associations in literature with different photosensitizers and light sources. Although its efficiency in the planktonic

form of *Candida* is well studied,[13-15] the protocols for decontamination in the more resistant biofilm form still need to be established.[16,17]

The sensitization depends on the parameters related to the light, such as wavelength, power density or light intensity, the energy density and also on the type of photosensitizers. Experimental research showed that yeast may be killed by photodynamic action employing phenothiazines, porphyrins and phthalocyanines.[18]

In the present study, *Candida albicans in vitro* susceptibility to different photosensitizers and light sources on planktonic culture and biofilm was investigated.

MATERIAL AND METHODS

Experimental design

This study consists in experimental analysis of different combinations of photosensitizers (P) and light sources for the inactivation of *C. albicans ATCC*. For this, combinations of Ps and light were used as follow: ortho toluidine blue (TBO) and methylene blue (MB) photoactivated by LED 1W - 152J/cm^2 - 120seg and bengal rose (BR) associated with LED 400mW - 152J/cm^2 - 5min, correspondent to the P+L+ group, in the experiments described below:

- Evaluation of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of these Ps;
- Analysis of antimicrobial effect of PACT against planktonic culture (n=5);
- Analysis of antimicrobial effect of PACT against biofilm formed on acrylic resin specimens (n= 9).

Microorganism and culture condition

Candida albicans ATCC 10231 was reactivated in sabouraud dextrose agar plates (Difco, Detroit, USA) at ± 35 °C for 24 hours. Five colonies of the product from the reactivation were collected with a sterile loop in Falcon tube containing 10mL of YNB broth culture medium (Yeast Nitrogen Base, Difco, USA) supplemented with 50mM glucose and incubated under stirring at ± 35 °C, for 18-20 h, to reach the exponential growth phase of the microorganism.[19]

After that, the suspentions were centrifuged for 5 minutes at 5000 rpm at room temperature and cells were washed twice with 10mL sterile PBS (137mM NaCl, KCl 2.7mM, 4.3mM Na2HPO4, 1.47mM KH2PO4, pH 7.4). Then an aliquot of centrifuged cells was added to tubes containing YNB supplemented with 100mM glucose. The cell suspension was adjusted using a spectrophotometer in a corresponding density of 10^7 cells/mL (Optical Density, OD = 0.38). The resulting suspensions were used for all the further procedures.[19]

Investigation of photosensitizers concentration

In this initial phase, the minimum inhibitory concentrations (MIC) and minimum fungicidal concentration (MFC) were analyzed by broth microdilution technique, recommended by Clinical and Laboratory Standards Institute (CLSI) document M27-A3.[20]

The *Candida albicans* susceptibility to photosensitizers was determined using 96-well microtiter plates, and each well received the inoculum, the medium culture and solutions of TBO, MB or BR (Sigma-Aldrich, St. Louis, Missouri, EUA) for a final volume of 210µL, activated by light irradiation 630nm/1W or 520nm/400mW. The range of concentrations tested was 88µM to 0.687µM. Five types of control conditions were used: without photosensitizer and irradiation (P-L-), only with P in the dark (P+L-), only with irradiation (P-L+), with nystatin solution (100,000 IU/mL) and control of sterility for the broth.

The initial OD (0.38) established containing equivalent to 10^7 cells/mL, was diluted to 1/10 and 1/100 in PBS solution, then to 1/20 in Sabouraud broth, according to the CLSI standard, yielding a final concentration of inoculum of $5.0x10^3$ at $2.5x10^3$ cells/mL. After the different treatments, the microplates were incubated at 35°C for 24h in the dark. Then, MIC was determined to all associations, by visual identification and ELISA test confirmation.

To determine the MFC, 25μL of dilution were removed, representing the MIC, and streaked onto Sabouraud agar plates, in triplicate, and incubated at 35°C for 24h. Two dilutions above (more concentrated) and two dilution below (less concentrated) were also streaked.

Planktonic cultures and PACT

Aliquots of 100μL of *Candida albicans s*tandardised suspension (exponential growth phase) were individually transferred to separate wells of a 96-well microtitre plate. After inoculation, an equal volume of diluted TBO, MB and BR solutions (100μL) was added to the wells to give final concentrations of 11, 22 and 44μM, RESPECTIVELY. For each photosensitizer, two concentration were chosen based on the MIC/MFC tests. After dark incubation of 5min, the samples were irradiated with the red LED ($152J/cm^2$ – 1W) for 2 min or with the green LED ($152J/cm^2$ – 400mW) for 5 min, which corresponded to (P+L+). To determine whether the tow LED's alone had any effect on cell viability, additional samples were made with no P (P−L+). The effect of Ps alone was also determined by exposing the yeast suspensions to the P with no light exposure (P+L−). The cultures which were not exposed to LED light or P acted as overall control (P−L−). All experiments were performed three times on three independent occasions. The nystatin solution (100,000 IU/mL) acted as a therapy control.

Ten-fold serial dilutions (1/10, 1/100 and 1/1000) were generated from the fungal suspensions and plated on Sabouraud Dextrose Agar (SDA), in triplicate. The plates were incubated at 35 °C for 48 h. After incubation, CFU mL^{-1} were determined.

Biofilm and PACT

Preparation of acrylic resin specimens

One hundred and thirty five disc specimens were made from thermo-polymerized acrylic resin (Classico Dental products Classic Ltda. São Paulo, SP, Brazil), measuring 10 mm in diameter and 2 mm in thickness. The resin was manipulated, packed, pressed into the mold and polymerized according to the manufacturer's instructions. After polymerization, the specimens were removed from the molds and immersed in distilled water at 37 ± 1°C for two days for residual monomer release. Then the surfaces were finished using 220, 400 and 600-grit sandpapers (Norton; Saint-Gobain Abrasivos Ltda., Guarulhos, SP, Brazil), and polished with a felt cone and pumice with white spain powder.

The polished specimens were marked with identifying codes and their dimensions were confirmed with a digital caliper (CD-6 - CSX-B; Mitutoyo Sul América Ltda, Suzano, SP, Brazil). Maximum tolerated differences between the dimensions of matrices and specimens were ± 0.2 mm. Afterwards, the specimens were packaged and sterilized in hydrogen peroxide plasma (Embraester; Fortaleza, Brazil).

Temperature evaluation of irradiated specimens

All measurements were performed at room temperature (23°C±1°C). The temperature was analyzed by a thermopar attached to a digital thermometer (SmartMether, Novus, Porto Alegre, RS, Brazil) fixed to the opposite irradiated surface of the acrylic resin specimens,

during the period of irradiation. The temperature gradients (ΔT) were calculated (ΔT = Final Temperature – Initial Temperature) for each group.

Biofilm formation

Under aseptic conditions, the specimens were horizontally placed into a 24-well culture plate containing *Candida albicans* cell suspension (10^7 cells/mL) prepared in YNB supplemented with 100mM glucose, and incubated aerobically under agitation at ± 35ºC for 1.5h (adhesion phase). The specimens were washed twice with PBS and individually transferred to plates containing fresh YNB medium.

These sets were incubated under agitation for 48 h at 35ºC. At the end of each 24h period, the specimens were washed with PBS, and fresh medium was added. After 48h the biofilm was formed, then the specimens were removed and treated in accordance with the experimental group.[19]

Treatments

Aliquots of 1 mL of each P, at best concentration obtained by MIC, MFC and planktonic culture, TBO (22μM), MB (44μM) and BR (44μM), were added to each appropriate well directly onto the specimens with formed biofilm. The experimental conditions were identical to those of the planktonic cultures: P+L+, P-L+, P+L- and P-L-. For the positive control was used the 1% sodium hypochlorite (Biorad; Hercules, Califórnia, EUA). After the treatments the discs were transferred to a new sterile tube with 500uL PBS and then subjected to sonication at a frequency of 40 kHz (Branson Ultrasonic Cleaner; Branson Ultrasonics, Danbury, CT) for 20 sec/ 3 times, on ice. Serial dilutions of suspension were made (1/10, 1/100 and 1/1000), which were spread in SDA plates and incubated at 35°C for 48h. All experiments were performed three times, on three independent occasions. In order to estimate the viability of the yeasts and the effects of PACT, CFU/mL were determined and log-transformed ($\log_{10}$).

Data were analyzed by Kruskal-Wallis and Dunn's tests for those which did not follow the Shapiro-Wilk normality curve, and ANOVA and Tukey's test for the others (α=0.05).

RESULTS

Concentration of photosensitizers

The MIC/MFC results for TBO, MB and BR were, respectively, 5.5/11 μM, 22/44 μM, 22/22 μM, also presented at Table 01.

PACT for planktonic culture

For the culture suspension, statistical difference was observed between the groups (P+L+) and P-L- ($p<0.0001$). There was no difference between Nistatin and P+L+ groups ($p>0.05$) (Figure 1).

For the TBO association, when only the photosensitizer at 22μM was used (P+L-), significant difference was observed, in comparison to P-L- ($p= 0.0031$). The same results could be observed for MB therapy, with the higher concentration of 44μM ($p=0.0074$). Therapies using BR, had the same results, when P+L+ was compared with P-L- group ($p<0.001$) and Nistatin ($p>0.05$). There was no difference between the groups of treatment P+L+, when different concentrations of BR were used (22 and 44μM) ($p=0.4$) (Figure 1).

PACT for biofilm formed on acrylic resin specimens

The temperature gradients (ΔT) did not show any significant difference of variation, while the LEDs 1W and 400mW were in use ($p>0.05$). The use of this two equipments, at the times calculated in a fluence of 152J/cm^2, is safe, and did not cause visual damages at the acrylic specimens.

As regard the biofilm (Figure 2), all associations showed difference between P+L+ and P-L- ($p<0.0001$). Reduction of Log values for TBO, MB, BR experiments were

respectively: 4.22, 2.28 and 1.48. CFU reduction was also observed when the red LED 1W (630nm) was used alone (P-L+) ($p<0.0001$).

The TBO and MB groups showed a significant reduction in CFU/mL following irradiation (P+L+ groups). When the BR was used as photosensitizer, less reduction was found in comparison with the blue dyes associated with red LED ($p < 0.05$).

DISCUSSION

PACT efficacy depends on the microorganism, the Ps and the light used. According to our results, TBO and MB combined with LED 1W/630nm showed higher antimicrobial photodynamic effect for *Candida albicans* than BR photosensitizer, whereas, this could be due to the fact that the higher potency of the spectrum emission of the lamp is associated with the blue dye groups.

In general, *Candida* biofilms are more resistant to oxidative stress than planktonic fungal cells due to limited photosensitizer diffusion and further cellular resistance mechanisms.[21,22]

Different photosensitizers combined with red light had been indicated as a promising and safe alternative for decontamination in several clinical dental procedures, against *Candida albicans,*[23-27] but the choice of the P and the radiant energy of the light source is of crucial importance on the outcome of the treatment.

The results suggested that antimicrobial photodynamic therapy using bengal rose (44 μM) with a pre-irradiation period of 5 min and LED for 5 min was enough to cause a significant reduction in planktonic culture and biofilm of *C. albicans*. But when compared with the TBO and MB therapies, it presented lower antimicrobial activity, as in the work of Silva *et al.* (2016).[28]

In Mima *et al*. (2011) study, the PACT mediated by erythrosine and LED of 520nm significantly reduced planktonic cultures and biofilms of yeasts, such as *C. albicans*.[23]

Although our study used different photosensitizers was also obtained decrease in growth of this specie in planktonic culture and biofilm. Demonstrating that there is a series of different protocols with good results on inactivation of this microorganism, but does not exist yet a standard form of treatment.

Among photosensitisers (Ps) examined for antifungal use, the phenothiazinium derivatives methylene blue and toluidine blue are the most commonly examined, mainly due to their low toxicities and other clinical uses.[28,29] Both Ps have been shown, previously, to be effective in the inactivation of *C. albicans*.[30-35]

The phenothiazine derivatives, methylene blue (MB) and toluidine blue O (TBO) presented photodynamic inactivation of planktonic cultures and biofilm formation on acrylic resin substrates. Rodrigues *et al*. (2013)[36], investigated the photodynamic inactivation on *Candida* species with phenothiazine photosensitizers and red light, and they obtained the same result as ours, but about the cytotoxicity, the new methylene blue N presented the high values and alteration of the yeasts cells.

In this study significant differences between the two light emission devices were observed, which correspond to the wavelength of absorption of each photosensitizer. The LED 1W/630nm used for 2 min at 152J/cm^2 alone (P-L+) presented potential antimicrobial activity against *Candida albicans* biofilm formed on acrylic specimens. This higher potency LED provides reduction on short irradiation time, improving the future treatment of the patients with this therapy.

The concentration of TBO (22μM) was smaller that MB (44μM), for the planktonic culture and biofilm inactivation, proving been more effective and having more advantage in clinical use because the decrease of possibles changes in staining of acrylic resins devices.

Photodynamic antimicrobial therapy is an important alternative approach to treat microbial diseases.[36] The absence of reported microbial resistance makes PACT an important tool to be used in medicine and dentistry.[37,38]

Unfortunately the consensus about the best protocol for photoinactivation of *Candida* species was not yet reported in the literature, due to the great variation of techniques and products used. Therefore the clinical use of this treatment still does not have definition inside the dentistry area.

CONCLUSION

This study demonstrated the effectiveness of *in vitro* PACT mediated by bengal rose (44μM) associated to LED 520nm/400mW and methylene blue (44μM)/ toluidine blue O (22μM) associated to LED 630nm/1W, against *Candida albicans* contamination. The association of blue dyes and red light showed better results. TBO was the most effective photosensitizer.

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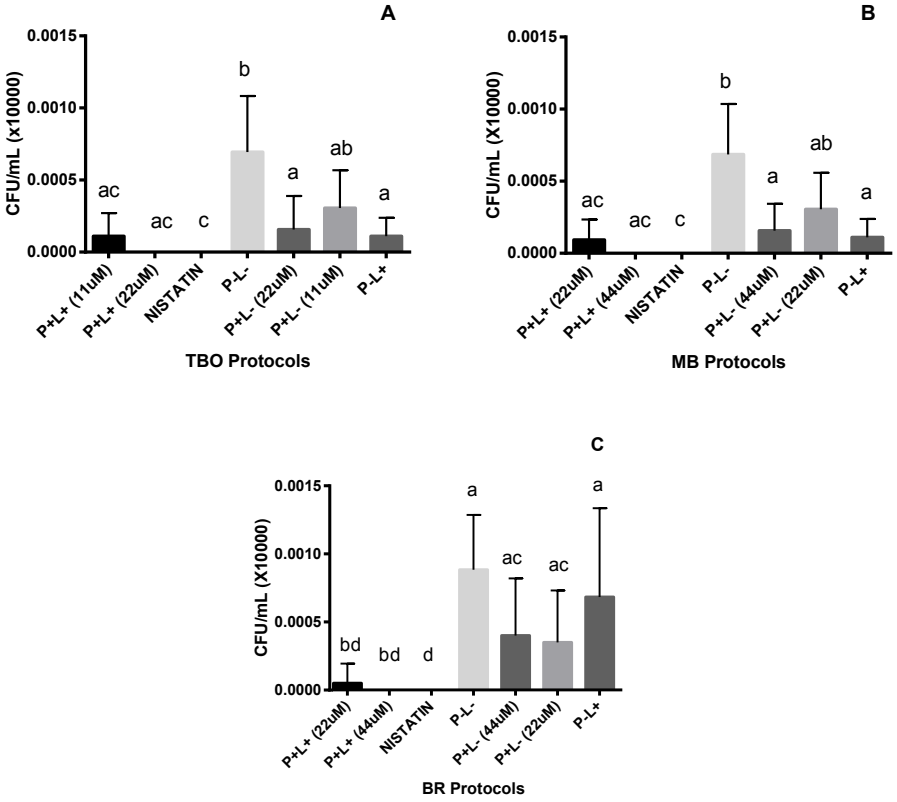
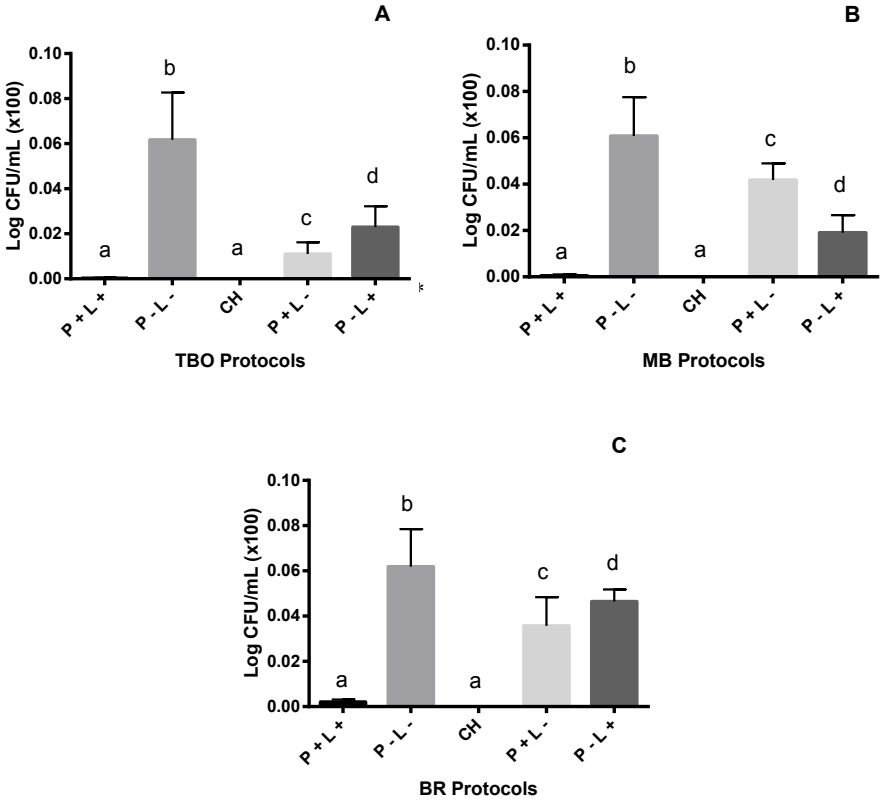
Table 1. Results for the MIC and the MFC for the three different photosensitizers in the photodynamic associations (TBO/MB + LED 1W/630nm/2min; BR + LED 400mW/520nm/5min).

	MIC	**MFC**
TBO	5.5μM	11μM
MB	22μM	44μM
BR	22μM	22μM



* Different letter means statistically different values ($p < 0.001$).

Figure 01. Effect of photodynamic inactivation in reduction of number of viable cells of *Candida albicans* for each treatment group in planktonic culture. (P-L-) control without any treatment; (P-L+) Light Emitting Diode (LED) irradiation; (P+L-) only photosensitizers; (P+L+) LED with the photosensitizers. (**A**) Therapy with TBO (11 and 22μM) + LED 1W. (**B**) MB (22 and 44μM) + LED 1W. (**C**) BR (22 and 44μM) + LED 400mW.



Different letter means statistically different values ($p < 0.001$).

Figure 02. CFU/mL values of each treatment group for biofilm formed on acrylic resin specimens. (P-L-) control without any treatment; (P-L+) Light Emitting Diode (LED) irradiation; (P+L-) only photosensitizers; (P+L+) LED with the photosensitizers. (**A**) TBO at 22µM with LED 1W/630nm. (**B**) MB, 44µM and LED 1W/630nm. (**C**) BR, 44µM and LED 400mW/520nm. All associations showed difference between P+L+ and P-L- ($p<0.0001$). CFU reduction was also observed when the red LED 1W was used alone ($p<0.0001$).

Capítulo 2

Effects of photosensitizers and disinfectant solutions on the color stability, mass loss and surface roughness of denture base acrylic resins.

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Running Tittle: Photochemotherapy and surface properties.

Key Words: disinfection, acrylic resin, photochemotherapy, surface properties.

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Word count: 3.273 (including all tables, legends and references)

Este artigo está escrito segundo às normas de publicação de artigos da revista International Dental Journal.
http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1875595X/homepage/ForAuthors.html

ABSTRACT

Photodynamic antimicrobial chemotherapy (PACT) has been used against denture stomatitis, but the effects of the photosensitizer on the acrylic resin in comparison to commonly disinfectants are not yet known. This study evaluated the color stability, mass loss and surface roughness of a heat polymerized acrylic resin after immersion in orto toluidine blue (TBO), methylene blue (MB) and bengal rose (BR) - used in PACT, compared to immersion in 1% sodium hypochlorite (NaOCl), 2% chlorexidine digluconate (CHX), sodium perborate (SP), 4% acetic acid (AA), 2% peracetic acid (PA), distilled water (control). Ninety shaped disks (10 x 2mm) specimens were prepared and randomly distributed (n=5) into groups of different products. Three situations were tested, simulating disinfection protocols during treatment for denture stomatitis: 1) immersion for 20 min daily, during 2 weeks (5 h), for the group of chemical disinfectants; 2) immersion for 5 min, twice a week, for one month (40 min), for the group of photosensitizers; 3) immersion for 20 min daily, during 180 days, for all groups (60 h). Color was measured by a portable colorimeter. All the samples were weighed before and after exposure to treatments, in order to analyze mass loss. Five parallel lines (1 mm) were registered on each specimen to measure the surface roughness. Data were analyzed by ANOVA and T-test (α=0.05). There was significant difference in color change (ΔE) for the AA group 60 h ($p=0.0025$). None of the groups presented mass loss ($p>0.05$) under any situation. About surface roughness (Ra), there was difference for 1% NaOCl, before (0.072±0.012) and after (0.157±0.021) the immersion ($p<0.0001$), as well as for 2% CD group, before (0.095±0.046) and after (0.11±0.041) treatment ($p=0.0045$), for 5h of treatment. Analysis of the treated surfaces was also conducted through Scanning Eletronic Microscopy (SEM) and more irregular surfaces were observed on 1% NaOCl and 2% CD groups. It may be concluded that immersion in solution of 4% acetic acid disinfectant had influence in the color stability, while the specimens treated with 1% NaOCl and 2% CD showed alterations in surface roughness, in relation to photochemical therapy.

Key words: Disinfection, Photochemotherapy, Acrylic Resins, Surface Properties.

INTRODUCTION

Heat cured polymethyl methacrylate (PMMA) polymers is a conventional denture base materials and considered the most popular material for oral rehabilitation. The presence of bacterial and fungal biofilms is the main etiological factor of denture stomatitis.[1] According to Ramage *et al*. (2004)[2] 11% to 67% of complete denture users present candidiasis. The surface topography of the denture has been shown to greatly influence adhesion and subsequent retention. The adherence of *C. albicans* to inert surfaces would be a pioneering step for successful colonization and development of the disease.[3]

The treatment of lesions associated with denture stomatitis encompasses denture and oral cavity hygiene instructions, removal of the irritant factor (prosthetic device), cleansing, use of antifungal agents and, if necessary, acquisition of a new denture.[4] Therefore, indication of denture cleansing is of paramount importance. Denture cleaning may be achieved mechanically by brushing, chemically by use of chemical agents or by the association of both methods.[5] Numerous disinfectants have been suggested for the disinfection of prostheses, however there is no ideal one. Several studies report their toxicity or ability to modify structure of the prosthesis.[6-10]

Photodynamic antimicrobial chemotherapy (PACT) involves the association of a photosensitizing agent to a light source in order to cause microbial cell damage or death. It has been used in various studies *in vitro* and *in vivo* as disinfection method of acrylic device and as an alternative treatment for denture stomatitis.[11-17] However their effects on these devices have not been investigated yet.

Since the chemical method for disinfection of dentures is widely used by patients and it is one of the processes for prevention or even treatment of denture stomatitis, this study investigated the effects of chemical and PACT disinfection methods on the surface roughness, mass loss and color stability of denture base resin.

MATERIAL AND METHODS

Specimen fabrication

Ninety specimens, disk-shaped, were prepared (10 x 2 mm thickness) of heat polymerized acrylic resin (Artigos Odontológicos Clássico Ltda., São Paulo, SP, Brazil). The resin was manipulated, packed, pressed into the mold and polymerized according to the

manufacturer's instructions. After polymerization, the specimens were removed from the molds and immersed in distilled water at 37 ± 1°C for 50 h ± 2 h for residual monomer release. The excess resin was trimmed and one of the surfaces was finished using 220, 400 and 600-grit sandpapers (Norton; Saint-Gobain Abrasivos Ltda., SP, Brazil), and polished with slurry of pumice followed by white spain pouder. The polished specimens were marked with identifying codes and their dimensions were confirmed with a digital caliper (CD-6 - CSX-B; Mitutoyo Sul América Ltda, SP, Brazil). Maximum tolerated differences between the dimensions of matrices and specimens were ± 0.2 mm.

Experimental design

The specimens were randomly distributed into 9 groups (n=10), for one of the following solutions: 1) 1% sodium hypochlorite (ASFER; Ind. Química Ltda); 2) 2% chlorexidine digluconate (RIOHEX; Rioquímica); 3) Sodium Perborate (Corega Tab), 4) 4% Acetic Acid (Jade Ponte, São Paulo, Brazil); 5) 2% peracetic acid (Sekusept®aktiv; Henkel Ecolab GmbH, Germany); 6) 22 μM orto toluidine blue; 7) 44 μM methylene blue; 8) 44 μM bengal rose (Sigma Aldritch); 9) Distilled water (control group). Later, each group was divided into two subgroups (n=5), with reference to immersion time of treatment (Table 1).

The experiments simulated treatment protocols for denture stomatitis, with immersion of prosthetic devices: 1) 20 min daily, during 2 weeks (5h) for chemical disinfectant solutions; 2) 5 min,, twice a week, for one month (40 min) for photosensitizers (Nunez, Ribeiro and Garcez, 2013)[19]; 3) 20 min daily, during 180 days (60 h) for all groups.

Specimens were submitted to immersion cycles at room temperature (23 ± 2 ºC). After each cycle, the soaking solution was discarded, and specimens were thoroughly washed in running water and dried with absorbent paper. For the simulation test of 180 days, a total of 45 cycles were performed daily, immersion for 10 min and 10 min in distilled water, with renovation of the solution in each time. Specimens were immersed in distilled water at room temperature when the cycles were not carried out, regardless the group.

Color Analysis

The 90 shaped-disk specimens were submitted to color analysis. Color measurements were determined by a portable colorimeter (Color-guide 45/0; BYKGardner, Columbia,

USA), at the same side of the identifying codes on each specimen, in a white background. Color changes (ΔE) were analyzed between the measurements (before and after immersion in disinfectant solutions) and were calculated with the use of CIELab color space.

To relate the color differences (ΔE) to a clinical environment, the color data were quantified by the National Bureau of Standards (NBS) units through the formula NBS units = ΔE x 0.92.[20]

Mass loss

For this experiment the specimens were previously saturated in its liquid absorption capacity. They were immersed for 7 days in distilled water at room temperature (23 ± 2 ºC) and weighted daily, until the dough was constant. Then the treatments were initiated.

To analyze mass loss (ML), all the samples were weighed before and after exposure to a disinfectant solution on a digital weighing scale to the nearest 0.1 mg (Bioprecisa, Eletronic Balance, FA2104N, São Paulo, Brazil), recording W1: weight before exposure to each solution and W2: weight after the procedure. The percentage of mass loss (ML) for each sample was calculated with the following equation: [W1- W2 /W1] X 100.[1, 18]

Surface Roughness Test

A stylus profilometer (Hommel Tester T1000, Hommelwerke GmbH, Schwenninger, Germany) was used to measure the surface roughness of each specimen before and after treatments (n=90). The resolution of the record data was 0.01 μm. The stylus moved across the specimen surface and 5 lines were recorded with a distance of 1 mm between each scanning line. The mean Ra was calculated from 5 lines as the mean roughness of the specimen.

Scanning Electronic Microscopy (SEM)

Scanning Electronic Microscopy (SEM, INSPECT 50 - FEI, Hillsboro, USA) was employed to analyze the topography of the 60 h samples, at 200x, 1000x and 2000x. The assessed surfaces were cleaned in distilled water in an ultrasonic device during 10 minutes.

After this procedure, the samples were placed on the aluminum cylinders ("stubs") and gold coated for observation by SEM.

Statistical analysis

Statistical analysis of the numerical data was performed by Analysis of Variance (ANOVA) and paired T test (95% reliability level) through the Graphpad Prism 6 software (Graphpad Software Inc., La Jolla, EUA).

RESULTS

About the color stability (mean ΔE) with the treatments at different protocols of time, no significant difference was found among almost all the tested treatments. The only solution that presented significant alteration of color before (17.75±0.2485) and after (15.90±0.1377) the treatment was the 4% acetic acid ($p=0.0025$), simulating 20 min of disinfection daily, during 180 days (60h).

For mass loss (ML), there were no significant differences among all the groups of treatment ($p>0.05$) (Table 2).

Table 3 presents the results for surface roughness (ΔRa), as mean of five measures. Significant differences were found only in the groups of chemical disinfectants, as 1% sodium hypochlorite and 2% chlorhexidine digluconate, before and after each treatment for the two different periods (5 h and 60 h), $p<0.05$.

Surface topography after immersion in the disinfection solutions showed different patterns of changes (Fig.1). The irregular surface produced by 2% chlorhexidine digluconate and 1% sodium hypochlorite is characterized by the presence of more defects.

DISCUSSION

The occurrence of oral infections caused by fungal species, particularly *Candida* can be associated with the use of dental prosthesis and it is mostly reported in individuals with poor general health and immunosuppression. These infections are called denture stomatitis.[1] Many *Candida spp.* are able to penetrate the acrylic resin used in the prosthetic devices at depths ranging from 1 to 2 μm, thus highlighting the need for a product that allows removing the biofilm without harming the mechanical properties of the resin.[5]

Brushing associated with toothpastes is the main method for cleaning dentures, but it can cause harmful alterations on materials, as mass loss, which can cause structural damage and aesthetic changes. The toothbrushing abrasion can also result in increased surface roughness, promoting bacterial adhesion and biofilm retention.[21]

The use of chemical solutions are also recommended for denture cleansing as an adjuvant method. Nishi *et al.* (2012)[22] reported that daily soaking of dentures in a denture cleanser was effective method for reducing the quantities of microorganisms adhering to dentures. The guidelines outlined by the American College of Prosthodontics recommend that dentures should be cleaned daily by soaking and brushing with an effective, nonabrasive denture cleanser.[23]

This study evaluated the disinfectant capacity of three photodynamic associations, with photosensitizer derivatives of phenothiazine and porphyrin families and compares these with the common chemical disinfectant solutions. Similarly to this study, others evaluated the effects of disinfectant solutions on physical and mechanical properties of acrylic denture base resins, i.e. roughness, hardness and surface morphology. [1,24-27]

Roughness affects the patient's comfort and prosthesis longevity. A smoother surface leads to better esthetic results and less biofilm retention. Several authors emphasized that irregular surfaces increase retention of the microorganisms and may affect oral health.[25,28,29] According to Bollen *et al.* (1997),[30] higher ridges to 0.2 μm may favor the adherence of microorganisms. In this study, before the treatments all the specimens presented surface roughness close to the value of 0.2 μm. The roughness values increased in the samples that were disinfected with 1% sodium hypochlorite and with 2% chlorexidine digluconate, but did not differ from the other groups. The results are also in accordance with those of Lima *et al.* (2006),[31] with alterations in surface roughness of acrylic resin after immersion in NaOCl among other cleansers. NaOCl is able to cause structural changes in the polymeric matrix of acrylic resins.[32]

Mass loss evaluation on the acrylic resins devices into the immersion groups for 5 h/ 40 min or 60 h in all the disinfectant solutions revealed no statistically significant difference after treatment. Carvalho et al. (2012) also identified no mass loss in their study with similar disinfectant solutions in acrylic resin devices.

Although the acetic acid is not frequently used in dentistry as a disinfectant,[10] it is preferred as a promising alternative disinfectant in several areas because of its low toxicity and low cost .[33] Acetic acid of white vinegar was frequently used in 50% and 100% concentrations to disinfect toothbrushes and acrylic resins. [10] Da Silva et al. (2008)[10] and

Yildirim-Bicer *et al.* (2014)[34] reported that the white vinegar showed effective antimicrobial activity against *Candida albicans* in 100% concentration for acrylic resins. But in our study this solution showed color alteration for the treatment after 60 h of immersion. Therefore, this solution can change the color properties of acrylic resin devices in prosthetic rehabilitation.

Surface morphology analysis showed changes in the groups that were immersed in disinfectant solutions for 60 h, mainly in samples immersed in sodium hypochlorite. Pore formation was observed in the 1% NaOCl and 2% DC, as in the study of Carvalho *et al.* (2012).[26]

Pereira *et al.* (2011)[13] and Ribeiro *et al.* (2012)[35] reported the efficacy of PACT against *C. albicans* biofilms by using higher and lower concentrations of dye and different levels of light energy. This study sought to use a low dye concentration in order to avoid discoloration of the acrylic resin.

The photosensitizer used in this study (TBO, MB and BR) did not present surface roughness alterations, nor mass loss of the acrylic samples, nor difference of color before and after the treatments, simulating the protocol for candidiasis and also as a disinfectant of daily use during 180 days ($p>0.05$). This promising outcome suggests that PACT may be used for reducing the microbial load in dentures as an adjuvant treatment for denture stomatitis and maybe as a future daily cleaning procedure for the patient.

These findings suggest that components of some disinfectant solution can penetrate the resin base material and cause partial dissolution and color change of the surface. Further studies will be necessary to investigate the benefit involved in their use during a longer immersion period. Also there are the necessities of clinical works with this same purpose, since the prosthesis use can cause changes of texture and the biofilm will be present in association with this devices.

CONCLUSION

From the results obtained, and within the limitations of this *in vitro* study, it was concluded that: there were no significant mass loss for all groups; significant differences of surface roughness for 1% sodium hypochlorite (NaOCl) and 2% chlorexidine digluconate (CD) were observed; and color alteration in 4% acetic acid group, simulating 180 days (60h) was verified. The photosensitizers toluidine blue o, methylene blue and bengal rose resulted in no changes of color or surface properties of heat-polymerized acrylic resins.

This study suggests that photodynamic therapy, immersion in 2% peracetic acid and immersion in sodium perborate for 6 months treatment, are secure methods for dentures.

DISCLOSURE

There was not any source of funding in this study. There is no potential conflict of interests between the authors either.

ACKNOWLEDGEMENTS

To the Analytic Center of the Federal University of Ceará, Brazil, that provided the analysis in Scanning Electron Microscopy (SEM).

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Table 1. Description of the different treatments in the study (n=5).

TREATMENTS	PRODUCTS		TIME
Simulating denture stomatitis treatment.	Disinfectant solutions	1% sodium hypochlorite; 2% chlorexidine digluconate; Sodium perborate (Corega Tab); 2% Paracetic acid; 4% acetic acid; distilled water.	20min/daily, during 2 weeks (5h)
	Photodynamic therapy	Ortho toluidine blue (TBO-22 μM); Methylene blue (MB-44 μM); Bengal rose (BR-44 μM).	5 min before irradiation (LED) twice a week for one month (40min)
Simulating treatment of denture stomatitis during six months.	Disinfectant solutions	1% sodium hypochlorite; 2% chlorexidine digluconate; Sodium perborate (Corega Tab); 2% Paracetic acid; 4% acetic acid; distilled water.	Immersion for 10 min, 45 times by day, simulating 180 days of treatment (60h)
	Photodynamic therapy	Ortho toluidine blue (TBO-22 μM); Methylene blue (MB-44 μM); Bengal rose (BR-44 μM).	Immersion for 10 min, 45 times by day, simulating 180 days of treatment (60h)

Table 2. Mass Loss (ML) of acrylic denture base resin specimens after the immersions tests expressed in mean with standard deviation of the difference, in percentage, (n=5). Data were analyzed by ANOVA and T-test (α=0.05).

	Immersion of 5h or 40min		**Immersion of (60h)**	
	Mean and SD of differences(%)	***P* value**	Mean and SD of differences(%)	***P* value**
BR + LED	0.0844 ± 0.01284	= 0.199	0.09211 ± 0.006213	>0.05
TBO + LED	0.09279 ± 0.008324	= 0.3874	0.0894 ± 0.01260	>0.05
MB + LED	0.08964 ± 0.0141	= 0.4544	0.07933 ± 0.01027	>0.05
1% NaOCl	0.08698 ± 0.009263	= 0.6112	0.08868 ± 0.00645	>0.05
2% CD	0.08898 ± 0.00762	= 0.5221	0.09036 ± 0.01390	>0.05
2% PA	0.08994 ± 0.01344	= 0.4324	0.09344 ± 0.01211	>0.05
4% AA	0.0931 ± 0.008255	= 0.4583	0.08934 ± 0.01544	>0.05
Corega Tab	0.08954 ± 0.01252	= 0.3211	0.09981 ± 0.00821	>0.05
Distilled Water	0.0871 ± 0.009488	= 0.4232	0.089912 ± 0.00916	>0.05

Table 3. Surface roughness (ΔRa) of acrylic denture base resin specimens before and after the immersions tests expressed in mean value with standard deviation (n=5). Data were analyzed by ANOVA and T-test (α=0.05).

	Immersion of 5h or 40min		**Immersion of 60h**	
	ΔRa	***P* value**	ΔRa	***P* value**
BR + LED	0.0064 ± 0.0453	= 0.0575	0.00204 ± 0.0359	= 0.0687
TBO + LED	0.0056 ± 0.0127	= 0.3896	0.002 ± 0.01215	= 0.1631
MB + LED	0.0014 ± 0.0098	= 0.4468	0.0072 ± 0.03285	= 0.0871
1% NaOCl	0.0792 ± 0.00748	< 0.0001 *	0.0688 ± 0.0067	= 0.0019 *
2% CD	0.0252 ± 0.0197	= 0.0045 *	0.0664 ± 0.01125	= 0.0021 *
2% PA	0.0077 ± 0.00589	=0.0631	0.006 ± 0.0001	>0.05
4% AA	0.0044 ± 0.00025	=0.0625	0.0072 ± 0.00045	>0.05
Corega Tab (SP)	0.0072 ± 0.001	>0.05	0.0094 ± 0.001335	>0.05
Distilled Water	0.0028 ± 0.01028	= 0.1422	0.0021 ± 0.00115	=0.134

* showed statistical difference with the treatment.

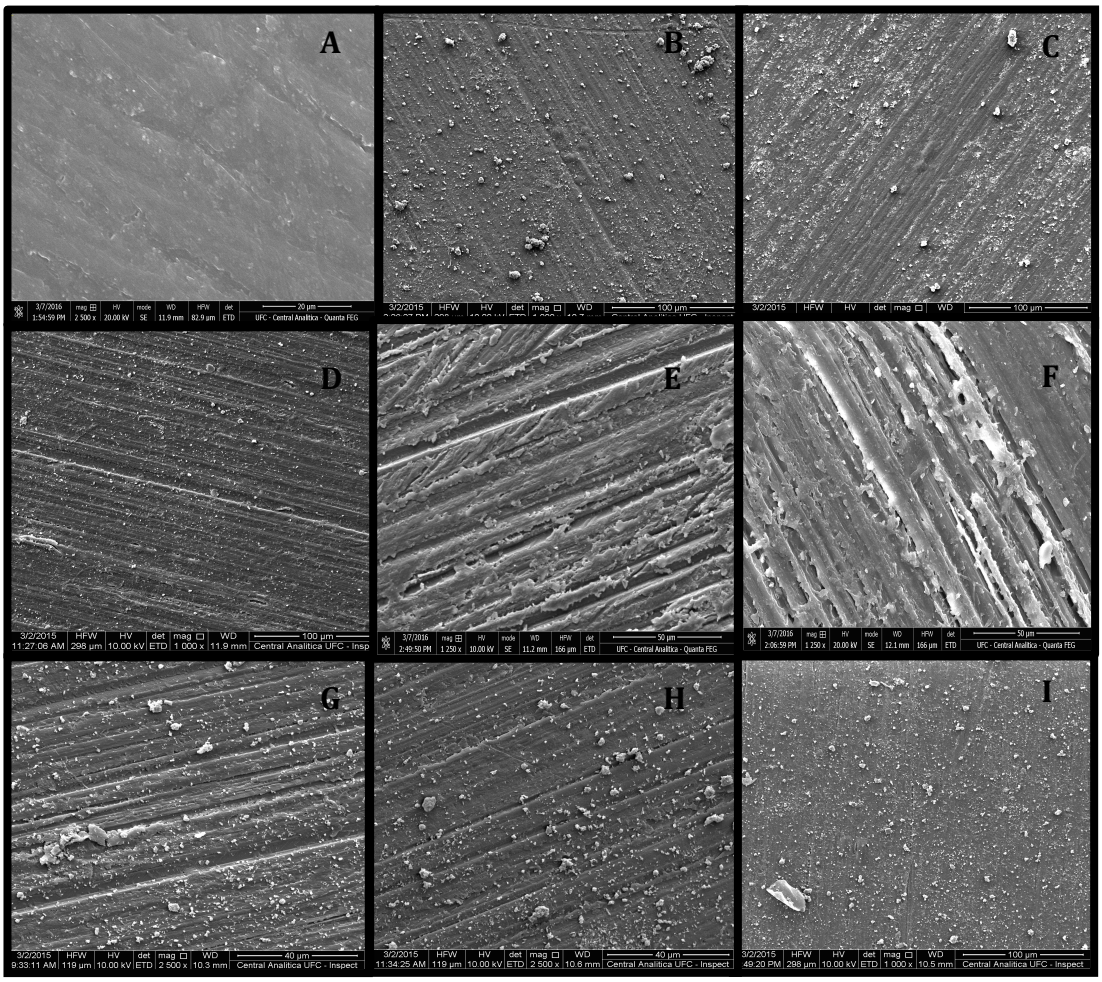

Figure 1. Images of the groups after the treatments in Scanning Electronic Microscopy (SEM) 1000X, after the immersion for 60h. (**A**) distilled water (control group); (**B**) 22 μM orto toluidine blue (TBO); (**C**) 44 μM methylene blue (MB); (**D**) 44 μM bengal rose (BR); (**E**) 1% sodium hypochlorite (NaClO); (**F**) 2% chlorexidine digluconate (CD); (**G**) 2% peracetic acid (PA); (**H**) 4% acetic acid (AA); (**I**) sodium perborate (SP).

Capítulo 3

Effect of antimicrobial photodynamic therapy mediated by ortho-toluidine blue on *Candida albicans* DNA damage.

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Este artigo está escrito segundo às normas de publicação de artigos da revista Photodiagnosis and Photodynamic Therapy. https://www.elsevier.com/journals/photodiagnosis-and-photodynamic-therapy/1572-1000/guide-for-authors

ABSTRACT

Background: Photodynamic antimicrobial chemotherapy (PACT) is a promising method in the inactivation of pathogenic microorganisms. Their genotoxic properties are poorly elucidated, probably, because of various types of associations. Methods: In this study it was investigated genotoxic effect, associating a Light Emitting Diode (LED) 630 nm/1 W and a photosensitizer (P) ortho-toluidine blue (TBO), to promote the formation of ROS (reactive oxygen species) under as singlet oxygen and to cause possible damage to the DNA of *Candida albicans*. For this analysis it was performed cytometric test, ROS production associated with the fluorescent compound H2DCF-DA, and DNA damage tests by Comet assay, in which the chromosomal DNA migration distance correlates with the extent of damage of the DNA in low-melting agarose (LMA), during electrophoresis. 100 µL pre-culture of *Candida* (10^6 cells mL^{-1}) was inserted in random wells culture plate treated according to the different groups: 0.6 µM TBO (5 min of pre-irradiation) + LED 630 nm/1W, for 2 minutes (P+L+); only irradiated (P-L+); stained (P+L-); distilled water (negative control); group 10 mM H_2O_2 (positive control). Data were analyzed by ANOVA and Tukey-test ($\alpha = 0.05$). Results: The production of intracellular ROS was higher for the P+L+ group. PACT caused significant increase in comet tails length (113.7 ± 4.818 µm) when compared to the negative control, 17.03 ± 1.138 µm, ($p < 0.0001$). Conclusions: These results indicate that the mechanisms of PACT cause genotoxicity to the microbial cells of *Candida albicans*.

Key words: genotoxicity, *Candida albicans*, photochemotherapy.

INTRODUCTION

In comparison to antibacterial therapy, antifungal treatment is limited to a very small number of drug substances. Treatment is prolonged, serious side effects and drug–drug interactions are often common [1]. In many cases, treatment is also ineffective [2]. In addition, the incidence of growth of several multidrug-resistant yeast strains has led to researches to find more effective alternative therapies to control these infections [3].

Photodynamic antimicrobial chemotherapy appears as a promising therapeutic system for this purpose. This technique involves the administration of a photoactive substance (photosensitizer, PS), activated in the presence of visible light, at a specific wavelength, for a certain period of time, and in the presence of oxygen. The reaction between the activated PS

(triplet state) and oxygen results in reactive oxygen species (ROS) formation, such as superoxide and hydroxyl radicals, and singlet oxygen. The photo reactions promote the destruction of the target organism by oxidation mechanisms that lead to microbial membrane lysis and protein degradation [4]. In this way, topical PACT can be employed for treating infections, providing a less toxic alternative when compared with other topical antimicrobial treatments, besides being a non-invasive treatment [5].

Among photosensitisers (PS) examined for antifungal use, the phenothiazinium derivatives methylene blue (MB) and ortho toluidine blue (TBO) are the most commonly examined, mainly due to their low toxicities and other clinical uses [6-8]. Both PS have been shown previously to be effective in the inactivation of biofilms and cell suspension culture of *C. albicans* [9-14]. Soares *et al*. (2009) assessed the effects of TBO and LED (630nm) on *C. albicans* strains that were both sensitive and resistant to fluconazole and they observed an average reduction of 3.41 log10 and a 55% reduction in adhesion to buccal epithelial cells [15].

There are few information about the genotoxicity on fungal cells of PACT, PS or just radiated [16]. Various processes and agents can result in DNA damage, they constitute the most efficient way to kill the cells. The extent of DNA damage can be assessed by single cell gel electrophoresis, in which the chromosomal DNA migration distance correlates with the extent of DNA damage [17]. This technique has been used for a variety of applications with several organisms, such as testing for genotoxicity, ecological monitoring and human biomonitoring [18].

Reactive oxygen species (ROS) generated by a photodynamic reaction induce damage to multiple cellular structures including the cell membrane, cell wall, and nucleic acids, which contributes to genetic instability and possible mutations [19]. More information about the genotoxic potential of PACT and/or of its compounds (PS and light) on the cells is needed. The study was performed to assess the potential of PACT mediated by TBO in causing DNA damage on *Candida albicans* cells.

MATERIAL AND METHOD

Experimental design

For the experiments, we used ortho-toluidine blue, TBO (Sigma-Aldrich, St. Louis, MO). A stock TBO solution (44 μM) was prepared in 200mL PBS (137mM NaCl, 2.7mM

KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4, pH 7.4). The red LED device was developed by Cromatek (SP; Brazil) and made by the Computer System Engineering Laboratory (LESC, Federal University of Ceará, CE; Brazil). It consists of a piece that provided uniform emission of 630 nm with light energy equivalent to 1W cm^{-2}.

A pilot study was made previously to determine the best concentration of photosensitizer for the comet assay test (0.6 μM) and for ROS production (2.75 μM).

Planktonic cultures of *C. albicans* and PACT

Candida albicans strain ATCC 10231 (American Type Culture Collection) was used in the study. It was cultured in YPD medium (1% w/v yeast extract, 2% w/v peptone and 2% w/v dextrose) at 37°C, 200 rpm and frozen at -80°C. For the experiments, cultures of yeast were grown aerobically at 37 °C for 48 h on solid YPDA medium (YPD with 2% w/v agar).

A pre-culture of *C. albicans* was prepared in a tube containing 5 mL YNB medium (Yeast Nitrogen Base; Difco) was supplemented with 2% (w/v) glucose and incubated in an orbital shaker at 30 °C at 200 rpm for 18 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 (0.38 optical density at 600 nm, which corresponds to 10^6 cells mL^{-1}). The cells were then centrifuged at 18000×g, washed twice for 2 min and suspended in zymoliase (20,000 U/g; ImmunO™) buffer (1.0 M sorbitol, 25 mM KH_2PO_4, 50 mM β mercaptoethanol and 500 U mL^{-1} zymoliase). The suspension was incubated at 30 °C for 30 min. After this incubation, the cells were centrifuged (18 000×g) and washed twice with S buffer (1.0 M sorbitol and 25 mM KH_2PO_4, pH 6.5).

To conduct the PACT, 100 μL aliquots of the suspension were placed in 96-well culture plate and the experimental groups were determined. The samples of the group treated with PACT were exposed to 0,6 μM TBO for 5 min in the dark (pre-irradiation time) followed by light emission for 2 min, giving a total light irradiance of 152 J cm^{-2} (group P+L+); the others groups were divided in: no light or photosensitizer (P-L-); stained and not irradiated (P+L-); just irradiated (P-L+). The H2O2 group consisted of samples treated with 10 mM H_2O_2 (8.8 M; 30%), 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 performing the different types of treatments, each sample was removed from the

wells and placed in microtubes, which were centrifuged at 18 000×g for 2 min. Then, the supernatants were discarded and the pellets were resuspended in 100 µL of S buffer centrifuged again (18 000×g/ 2 min), then resuspended 1.5% (w/v) low melting agarose (LMA) at 30°C, placed on glass slides, covered with coverslips and incubated at 4°C for 20 min for gelation of agarose. After this period, the coverslips were removed and the slides were first immersed in lysis buffer (30 mM NaOH, 1.0 M NaCl, 50 mM ethylenediaminetetraacetic 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^{-1} for 10 min, and then 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 finally air-dried. For visualization in a fluorescence microscope (Leica DM5000 Microsystems DM fluorescence) slides were stained with GelRed (1g mL^{-1}; Biotium Uniscience) and representative images were acquired at magnification of 400x in a fluorescence microscope equipped with excitation filter of 475–490 nm. At least 50 representative images of each of the glass slides were analyzed with the help of the free edition of the CometScore software version 1.5. 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 [17].

Analysis of ROS production

To assess the amount of ROS produced by the groups tested, it was used flow cytometry with the fluorescent probe 2.7-dichlorofluorescein diacetate (H2DCF-DA; Life Technologies, San Francisco, CA, USA). Five mL of *Candida* cells from exponentially growing cultures were harvested as above and washed twice with the same volume of ice-cold PBS. Then, 500µL of untreated cells were removed for evaluation of autofluorescence and kept in the dark at 4°C (AF group). H2DCF-DA (50 µM) was added to the remaining cell suspension and incubated at 37°C for1h in the dark. The cells were washed twice with the same amount of PBS and 500 µL aliquots were distributed to different experimental conditions treatment. It was used 2.75 µM TBO associated with LED 1W/630nm, for 2 min, in the PACT (P+L+), only light (P-L+), only TBO (P+L-) or 10mM H2O2. The control consisted of H2DCF-DA-loaded cells without any treatment (P-L-). After treatments, the cells

were washed twice with PBS and quantification of ROS was analyzed using an Epics®XLTM cytometer (Beckman Coulter) equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. The green fluorescence was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 225 nm band-pass filter (used for eukaryotes). The data were analyzed and histograms were made with the Flowing Software (Turku, Finland).

Statistical analysis

Each experiment was done at least in three independent experiments and the results are presented as the mean value and standard deviation. In each replica of the comet assay, a corresponding mean value of at least 50 comets was used and then analyzed using GraphPad prism version 6. Statistical analysis was performed using analysis of variance (ANOVA) and Tukey's multiple comparison test. P-Values less than 0.05 were considered to be significant.

RESULTS

Cells were treated with 0.6 μM TBO and red LED (152 J cm^{-2}, for 2 min; group P+L+), and the DNA damage was analyzed by the comet assay. Controls were included in the analysis: no treatment (P- L-), TBO only (P+ L-), LED only (P-L+) and 10mM H_2O_2 (group H_2O_2).

Microscopic inspection of the comets after the treatments did not present, as expected, considerable comet tails for P-L- (Fig. 1A) unlike P+ L+ group, which displayed longer comet tails (Fig. 1B), indicating that this PACT association 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 Fig. 2, a statistically significant difference was observed between the group that received PACT (113.7±4.818 μm) and the group without treatment (P-L-), 17.03±1.138 μm, ($p < 0.0001$). It also presented the same DNA damage potential that the positive control (H_2O_2), $p=0.576$.

Interestingly, red LED only was able to promote DNA damage (see P-L+ and P-L- in Fig. 2) and also was the TBO group (P+L-), suggesting that this compound may be genotoxic; however, when these groups are compared with P+L+ and H_2O_2, they also presented statistical difference, ($p<0.0001$), indicating that their properties are increased when they act together.

Photochemistry sensibilization is usually mediated by the production of ROS when the photosensitizer is excited by light. To investigate if the genotoxicity of PACT is caused by ROS after irradiation, cells were loaded with the redox sensitive fluorochrome H2DCF-DA and then treated as shown in the experiment method. The apolar nature of the fluorochrome allows free permeation through biological membranes; however, once inside the cells, esterases deacetylate H2DCF-DA, yielding the polar compound dichlorofluorescein (H2DCF), which becomes trapped.

Upon oxidation H2DCF is converted into the fluorescence-emitting oxidized form. Therefore, fluorescence of H2DCF-DA loaded cells is proportional to the amount of ROS produced during the treatments. Fig. 3 shows that the fluorescence emitted by cells treated with PACT (P+L+) was significantly higher than that of the groups P- L+, P+L-, P-L-, which were similar among them, it means that both the photosensitizer and irradiation alone do not cause intracellular oxidation, i.e its effect is equal to cells without any treatment.

Assay (P+L+) cause an increase in fluorescence of the cell population (deviation to the right) which means that the combination of L and P causes intracellular oxidation. As the scale of the x-axis is logarithmic, those deviations correspond fluorescence huge increases (intracellular oxidation). Figure 3 also shows the difference in the fluorescence cytometry of cells that had undergone oxidative stress of the P+L+ and P-L- groups, which corroborated the intracellular origin of the fluorescence.

DISCUSSION

This *in vitro* experimental study evaluated the susceptibility of *C. albicans* to PACT using TBO 0.6 μM photosensitizer along with LED 1W/630 nm. The results of these treatment modalities were analyzed, to the possible, DNA damage through comet assay and ROS production. The PACT applied in this study proved to cause genotoxicity in cell treated with this therapy, the same damage found in the control group treated with H_2O_2. Similar result was observed in Carmello *et al*. (2015) study, that found DNA damage in *C. albicans* cells submitted to PACT with curcumin and LED (455 nm). In their work the application of the therapy needed a long time exposition of light (20 min), and in our work we determined a better clinical application with the same results, because the irradiation time was reduced, due to higher power rating of the LED.

Previous studies have recommended PACT as an alternative to antifungal medications with positive results, using the association of phenothiazines, as ortho-toluidine blue (TBO) and methylene blue (MB), with red LED (630 nm) [20-22].

Several parameters in the protocol of PACT may influence the results such as the light parameters, photosensitizers and the method of light irradiation [20]. The peak absorbance of the photosensitizer must match the radiated wavelength to ensure generation of singlet oxygen and ROS, which are responsible for elimination of pathogenic strains [21,23]. TBO is a water soluble cationic photosensitizing agent from phenothiazine chemical group, with an absorption peak between 632 ± 8 nm [24]. It was chosen as a photosensitizing agent due to its intrinsic molecular cationic charge, its good photodynamic properties for killing diverse microorganisms *in vitro*, and its lower cytotoxicity for human cells, as keratinocytes and fibroblasts, when compared to microbial cells. Also, the antimicrobial efficacy of PACT may depend on the concentration of photosensitizer as well [25].

The P+L+ association in this study had the same result that H_2O_2 group, causing significative DNA damage. These results are probably 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 [26]. ROS are dispersed from the mitochondrial electron transport chain and peroxisomes, diffusing freely through the cell membranes, attacking other components inside [27]. Endogenous ROS production added to those produced by light associated with TBO may have been responsible for the genetic damage of the *C. albicans* cells found in this work.

Fluorescence assessment with H2DCF-DA revealed that PACT and H_2O_2 provoked oxidative stress. Studies confirmed that TBO generate reactive oxygen species, indicating type I phototoxicity metabolism of microorganisms [28]. A low concentration of TBO was used in the present study; we suggest that light might have enhanced the effect of TBO in the cell, for getting this result. Thus, it is possible to mention that low concentration of TBO associated with red LED light with higher potency altered the cellular metabolism, which can result in high quantity of ROS produced in the mitochondria during the cell respiration.

The study proved a considerable production of ROS, involved in the genotoxicity in the intracellular metabolism, with an association of low concentration of the photosensitizer and higher LED energy, with reduced time of exposition, what contributes with future works in clinical application of this therapy.

It is possible that virulence factors of this microorganism might also be affected upon

PACT, which would exacerbate the therapeutic effect [16]. Therefore, it is important to investigate the effects of PACT also in *in vivo* studies by monitoring DNA damage and virulence factors. It will help to understand the mechanism of action of this therapy on *C. albicans* cells that will allow implementing efficiently this therapeutic approach.

CONCLUSION

This study suggest that PACT used (TBO + red LED1W) caused extensive DNA damage with great production of intracellular ROS on planktonic cells of *Candida albicans*. Nonetheless, more research in this filed are necessary and important for understand the mechanisms and future clinical application of this technique.

ACKNOWLEDGEMENTS

The authors are thankful to Research Centre of Molecular and Environmental Biology (CBMA), University of Minho, in Portugal.

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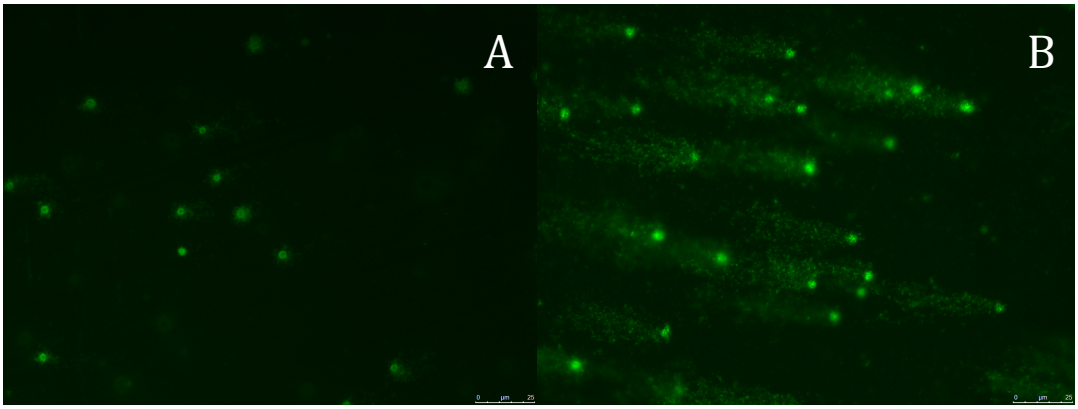

Figure 1. Image by fluorescence microscopy of yeast comets, upon DNA staining with GelRed, representative of the group P– L– (**A**), showing the absence of comets; (**B**) group P+ L+ showing the presence of comets in the *C. albicans* cells; Magnification: 400%.

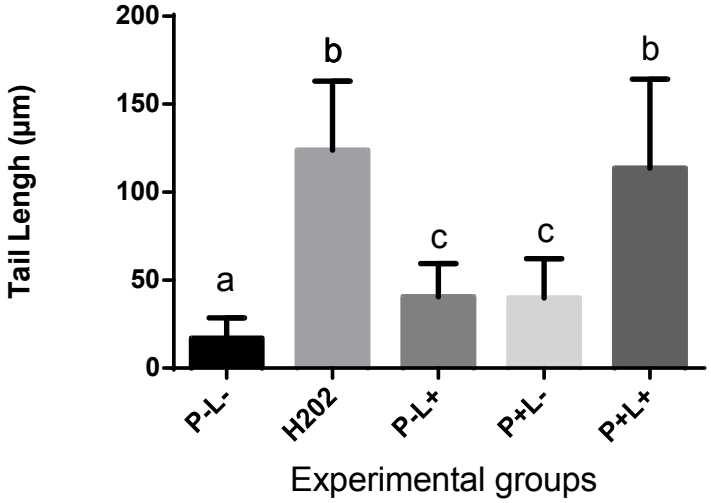

Figure 2. Mean and standard deviation of the length of the tails formed by the exposure of the *C. albicans* cells to PACT (group P+L+), (P+ L-), (P-L+), or 10 mM hydrogen peroxide (H_2O_2). Negative control (P-L-) refers to untreated cells. Different superscript letters on the bars denote statistical difference among groups (Tukey test; $p< 0.05$).

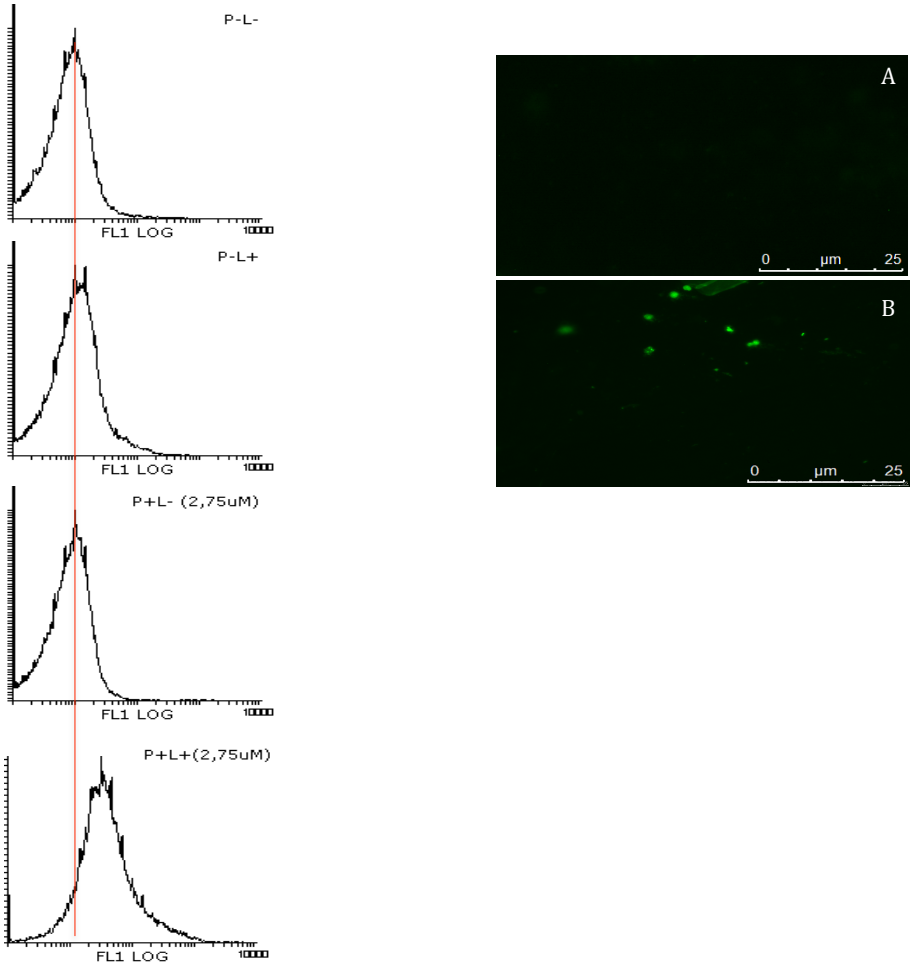

Figure 3. Histogram of intracellular oxidation of *C. albicans* cells exposed to H2DCF-DA for 1h, submitted to PACT (P+ L+), TBO, 2.75µM (P+ L-), or LED 1W (P- L+), in comparison with the control group (P-L-) and AF Group with untreated cells and without fluorochrome. Result analyzed by flow cytometry. Data are from a representative experiment from three independent replicas. (**A**) represent the cell whitout the fluorescence compound and (**B**) loaded with H2DCF-DA, photographed by fluorescence microscopy (Leica DM5000). Zoom 400x.

Capítulo 4

Relatório Descritivo da Patente de Invenção para **"DISPOSITIVO FOTOTERÁPICO COM LED DE ALTA POTÊNCIA ASSOCIADO A FOTOSSENSIBILIZADORES ESPECÍFICOS PARA DESINFECÇÃO DE MATERIAIS PROTÉTICOS".**

[001] Refere-se o presente pedido de patente de modelo de utilidade a um dispositivo adaptador constituído de LED (Light Emitting Diode) de diâmetros muito pequenos dispostos em uma caixa, de forma a se organizarem em formato de um cubo. O LED é um componente eletrônico semicondutor, ou seja, um diodo emissor de luz, mesma tecnologia utilizada nos chips dos computadores, que tem a propriedade de transformar energia elétrica em luz. Tal transformação é diferente da encontrada nas lâmpadas convensionais que utilizam filamentos metálicos, radiação ultravioleta e descarga de gases, dentre outras. Nos LEDs, a transformação de energia elétrica em luz é feita na matéria, sendo, por isso, chamada de Estado sólido.

[002] O dispositivo será composto de 8 LEDs de 1W, na cor vermelha (625-740 nm) ou azul (440-485 nm) dispostos 1 em cada face lateral (posterior, anterior, esquerda e direita) e 2 na face superior e os outros 2 na inferior. Os LEDs são fixados em uma caixa transparente, em que a peça protética é posicionada. Uma outra caixa opaca e escura envolve o restante do circuito, evitando assim que a luz saia. Os LEDs são alimentados por uma fonte de 5V/ 2A e acionados através de resistores limitadores de corrente.

[003] Na área de reabilitação oral, a redução do número de microrganismos favorece o controle da infecção cruzada entre consultório e laboratório de prótese, na necessidade de esterilização de próteses imediatas e guias cirúrgicos para implantodontia, que devem ser utilizados em ambiente cirúrgico asséptico. Há um interesse clínico na procura de um método alternativo que seja simples e eficaz na desinfecção ou, até mesmo, esterilização de dispositivos confeccionados em resina acrílica, uma vez que problemas como infecções cruzadas entre laboratório e consultório e o uso de próteses imediatas e guias cirúrgicos não estéreis em cirurgias orais, têm sido

recorrentes na odontologia. O presente aparelho apresenta-se como solução para esse problema e torna fácil e eficiente a técnica de desinfecção baseada na terapia antimicrobiana fotodinâmica.

[004] Alguns agentes químicos são utilizados para desinfetar dispositivos protéticos acrílicos, como o hipoclorito de sódio, o glutaraldeído, a clorexidina e o álcool 70%. Apesar da sua eficiência como um desinfetante, o hipoclorito de sódio 1% tem algumas desvantagens, incluindo a sua atividade corrosiva nas superfícies metálicas, o efeito irritante sobre a pele e outras células, destruição de tecidos, incluindo *cotton*, e alteração de cor de resinas (BELL *et al.,* 1989). O glutaraldeído 2% é frequentemente utilizado em odontologia (CARDOSO *et al*., 2000). A principal vantagem desse produto é que ele não é inativado quando em contato com materiais orgânicos, não é corrosivo e não degrada plásticos ou borracha (SILVA *et al*., 2004), no entanto, devido à sua potencial toxicidade, eles devem ser manipulados com cuidado e, de acordo com a Resolução RDC 15 de 03/2012 ficou proibida a utilização do glutaraldeído na esterilização em consultório odontológico e a desinfecção em alto nível com esta substância segue normas específicas regulamentadas por esta Resolução (ANVISA, 2012).

[005] A clorexidina tem sido uma das mais estudadas substâncias antimicrobianas. Ela é considerada a melhor escolha entre os antissépticos para o controle de biofilme dental, eficaz para a prevenção da cárie dentária, gengivite e estomatite. Sua atividade antimicrobiana tem sido descrita, principalmente, em bactérias Gram-positivas (GUIMARÃES, 2001). No entanto, Pavarina *et al.,* (2003) observaram alteração na rugosidade superficial de resina acrílica após imersão em digluconato de clorexidina e em hipoclorito de sódio.

[006] A TFD (Terapia Fotodinâmica) consiste na associação da ação de um agente corante fotossensibilizador a uma fonte de luz, no intuito de provocar necrose celular e morte microbiana. A ação se dá quando a substância fotossensibilizadora absorve os fótons da fonte de luz e seus elétrons passam a um estado excitado, ocorrendo uma primeira reação com produção de superperóxido, radicais hidroxila e radicais livres. Na presença de oxigênio molecular, ocorre uma segunda reação, em que o fotossensibilizador transfere energia ao mesmo quando retorna ao seu estado natural, formando moléculas de vida curta e altamente reativas, como o oxigênio

singleto ($^1O_2$). Todos estes produtos oxidam moléculas biológicas, como proteínas mitocondriais, alterando sua estrutura e atividade, desnaturam proteínas e lipídios da membrana e modificam a estrutura do DNA celular. Devido a este mecanismo de ação, é muito improvável que um microrganismo desenvolva resistência a este tipo de terapia (MACHADO, 2000; WOOD *et al.,* 2006; PERUSSI, 2007).

[007] Outras vantagens também podem ser observadas com o uso da inativação fotodinâmica: seletividade do fotossensibilizador, focalização da luz apenas na região de interesse, possibilidade de repetição da terapia sem efeitos tóxicos cumulativos, não invasivos e de baixo risco (TEICHERT *et al*., 2002; DOVIGO, 2007; PERUSSI, 2007).

[008] Não há, atualmente, nenhuma tecnologia, seja em artigos ou em bases de patentes, cuja aplicação seja a mesma do dispositivo, objeto do presente pedido de patente.

[009] Há, entretanto, a patente CN203247266U que se refere a um aparelho que utiliza também LEDs de 1W, dispostos em uma caixa. No entanto, apresentam-se em dois comprimentos de onda (azul ou vermelho), dispostos em maior quantidade que a preconizada no trabalho em questão e somente na porção inferior da caixa, com a finalidade de facilitar a irradiação de células em laboratório, já o nosso dispositivo apresenta irradiação em 360 graus. Outro trabalho de patente que utiliza idéia semelhante seria o US20050112021, neste os autores propõem a confecção de um também dispositivo de LED para auxílio na terapia fotodinâmica antimicrobiana em fluídos sanguíneos.

[010] Com a utilização do presente dispositivo submetido à patente estes problemas encontrados nas técnicas tradicionais de desinfecção, como toxicidade, corrosão e alteração de superfície dos materiais, seriam resolvidos e os dentistas poderiam utilizar o dispositivo associado com fotossensibilizador específico (de acordo com o comprimento de onda do LED) o que possibilitaria uma eficiente inativação dos microrganismos patogênicos, sem causar danos à estrutura dos materiais.

[011] Como já mencionado a utilização de fotossensibilizadores, em presença de oxigênio junto aos LEDs de 1W possui ação antimicrobiana em espécies bacterianas e fúngicas, sem causar danos aos materiais protéticos. Dessa forma, a presente invenção traz para a Odontologia moderna uma alternativa inovadora para

um procedimento corriqueiro realizado em consultórios odontológicos com materiais conhecidamente danosos.

[012] Além de possuir uma aplicação diferenciada, o presente aparelho distingue-se tecnicamente do estado da arte levantado. No dispositivo, objeto desse pedido de patente, os LEDs são dispostos em mesmo comprimento de onda e potência, de modo que a luz seja transmitida por toda sua extensão em 360 graus. Outras diferenças técnico-construtivas são as quantidades dos LEDs, disposição desses e quantidade de luz no interior das camadas do dispositivo, cobrindo completamente, materiais como próteses totais.

[013] Devido a estas características inovadoras, além da aplicação também inovadora que possibilita uma nova técnica para o tratamento de superfície de dispositivos acrílicos protéticos, caracteriza-se o presente pedido possuidor de seus requisitos de patenteabilidade, quais sejam, novidade, ato inventivo e aplicação industrial, conforme determinações da lei de propriedade industrial, em seu artigo 9º.

[013] Refere-se o presente pedido de patente de modelo de utilidade a um dispositivo para fotoinativação de microrganismos em materiais protéticos resinosos dentro de uma caixa, constituído basicamente de:

- Um sistema de LEDs dispostos em um cubo;
- Uma porção receptora;
- Uma porção emissora.

[014] A área iluminada é um cubo de 10 cm de lado da caixa interna, 13 cm de lado na caixa externa, cada qual com espessura de 0,5 cm. A caixa externa constituirá de um material opaco escuro, para impedir a passagem ou saída de luz, com tampa superior móvel de encaixe, o restante fixado permanentemente entre si. Conterá em sua caixa interna uma quantidade de LEDs, um de cada lado do cubo e dois na parte superior e inferior, mantendo uma distância de 1.5 cm entre si, através de um modelo de potência luminosa e dissipação de calor, também com tampa superior móvel de encaixe. Um dos fatores limitantes do projeto é a temperatura do objeto iluminado no centro do cubo, que não poderá ultrapassar os 60ºC. Os dispositivos utilizados no projeto são LEDs da série S com 1W de potência e sem dissipador próprio.

[015] Externo ao cubo há uma placa de controle alimentada por uma fonte chaveada, devido à necessidade de uma maior eficiência na potência dissipada. Essa placa de controle será composta de um microcontrolador ARM (Advanced RISC Machine), que comandará o drive de potência para acionamento dos LEDs através de um PWM (Pulse-Width Modulation- modulação por largura de pulso) regulado pelo usuário. Os valores de potência fornecida aos LEDs bem como o tempo de exposição deverão ser exibidos ao usuário através de um display (Visor).

[016] Será confeccionada uma placa de circuito para controle do equipamento, contendo uma fonte, microcontrolador, drive de potência, display e potenciômetro para regulação do PWM. A placa de controle será conectada a outras 6 placas menores que conterão apenas os LEDs de potência, posicionados sobre um substrato térmico de dissipação, que serão fixadas nas faces internas do cubo.

[017] Para fins de validação, o cubo será preenchido internamente por um conjunto de sensores de temperatura em diferentes locais do cubo, possibilitando assim um completo monitoramento da temperatura interna do dispositivo.

[018] Dessa forma, a luz é dissipada em 360° por toda a extensão da porção emissora de luz do dispositivo que pode adaptar o material protético em seu interior e junto ao fotossensibilizador de escolha fornecer a redução ou até eliminação dos microrganismos, evitando possíveis patologias associadas ao uso desses aparelhos protéticos ou até contaminação cruzada entre laboratório, cirurgião-dentista e paciente. A principal vantagem desse dispositivo é permitir a iluminação de toda a área do aparelho protético de forma igual e sem danos a sua estrutura de superfície, mesmo em áreas de reentrâncias, de maneira mais rápida, promovendo eficiente forma de inativação de microrganismos patogênicos.

[019] A descrição que se segue e as figuras associadas, tudo dado a título de exemplo não limitativo, farão compreender melhor a invenção:

[020] A figura 1 compreende os dispositivos de acrílico externo opaco (a) e interno transparente (b) com suas arestas, de 13 e 10 cm, respectivamente.

[021] Na figura 2 é possível observar a disposição dos LEDs de 1W, um em cada lateral (b) das paredes do dispositivo de acrílico (a) e dois na parte superior e inferior (c).

[022] A figura 3 configura um exemplo de como seria uma caixa inserida na outra, a presença de um visor, onde conteriam as informações de potência, tempo e temperatura, um potenciômetro (a) e sensor de temperatura (b). Sendo necessária alimentação por fonte externa de energia.

[023] A figura 4 demonstra a fotoinativação de uma prótese total com emissões luz uniforme e cobertura de 360 graus da peça submetida ao procedimento de desinfecção com o aparato de patente.

[024] O presente pedido de patente de modelo de utilidade é baseado em dois componentes que não podem ser separados. Os componentes são a porção receptora e a porção emissora de luz. O adaptador transfere a energia obtida através de uma fonte 5V/1A para os diodos emissores de luz (vermelha ou azul) que estão distribuídos no interior de um dispositivo acrílico em forma de cubo, com uma porção interna (transparente), a qual permite a passagem da luz irradiada e outra parte externa opaca (preta), esta não permite a entrada ou saída de luz para que não hajam interferências no processo de fotoinativação.

[025] Numerosos estudos *in vitro* demonstraram que a TFD é altamente efetiva na destruição de vírus e protozoários, assim como bactérias Gram-positivas, Gram-negativas e fungos (PERUSSI, 2007; DONELLY *et al.,* 2007; DOVIGO, 2007, DE FREITAS PONTES et al,, 2014).

[026] O Diodo Emissor de Luz (LED) tem sido apontado como fonte de luz promissora em relação à luz *laser*, pois apresenta como vantagens menor custo, maior flexibilidade no tempo de irradiação, facilidade de operação e aparelhos com formatos menores e mais leves. O LED é utilizado para polimerização de materiais restauradores e, comprovadamente, não é nocivo para os tecidos bucais (KONOPKA & GOSLINSKI, 2007).

[027] Corantes têm a habilidade de absorver luz visível com eficiência e são capazes de induzir ou participar de reações fotoquímicas. O fotossensibilizador ideal caracteriza-se por baixa toxicidade após a administração, não induzindo reação alérgica nem hipotensão e deve absorver a luz no espectro determinado. Deve ser

biologicamente estável, fotoquimicamente eficaz, seletivo e minimamente tóxico aos tecidos normais e materiais (GARCEZ *et al*., 2003).

[029] O dispositivo acrílico do aparato submetido à patente consegue arcar com as variações de umidade e temperatura a que estará sujeito, sem apresentar deformações.

[030] As variáveis construtivas do presente modelo de utilidade podem girar em torno das diferentes distâncias da porção emissora em relação aos aparelhos protéticos que exibem diferentes tamanhos. O aumento no número de LEDs promoveria o aumento do diâmetro da caixa e maior controle de temperatura, o que poderia danificar a estrutura de resina caso a temperatura durante o procedimento fosse maior que o limiar de alteração de superfície do acrílico.

REIVINDICAÇÕES

“DISPOSITIVO FOTOTERÁPICO COM LED DE ALTA POTÊNCIA ASSOCIADO A FOTOSSENSIBILIZADORES ESPECÍFICOS PARA DESINFECÇÃO DE MATERIAIS PROTÉTICOS”.

1. Dispositivo em formato de cubo, com duas porções interna e externa, caracterizado pela disposição de 8 diodos emissores de luz fria (LED), com 1W de potência com luz vermelha (625-740 nm) ou azul (440-485 nm).

2. Vantagens da terapia fotodinâmica para controle de contaminação cruzada ou desinfecção/esterilização de aparelhos protéticos, caracterizada por ser rápida e simples, podendo ser realizada tanto pelo profissional cirurgião-dentista, como pelo próprio paciente.

3. A inserção de um método seguro e rápido para o tratamento de superfície desses materiais, através desse dispositivo, caracterizada por não causar danos físicos às estruturas protéticas.

4. Diminuição do tempo clínico durante a fase de desinfecção, caracterizada por diminuir a contaminação e auxiliar no tratamento e prevenção de possíveis patologias.

5. Consiste em um dispositivo portátil, caracterizado pela facilidade de transporte e poder se transformar em um dispositivo de uso pelos próprios pacientes, pois seus componentes não possuem toxicidade

6. Material não tóxico, caracterizado pelo acrílico, fotossensibilizador e LED.

7. Devido a presença de um potenciômetro na parte interna da caixa, garante-se a correta irradiação, caracterizada pela placa de controle de potência e visualização de seu valor, através do Visor (Display), na porção externa da caixa.

8. Presença de sensores de temperatura, caracterizados por indicar possíveis alterações que podem levar a danos na estrutura do dispositivo e na peça a ser tratada.

9. Mais facilidade de limpeza dessa estrutura, caracterizada pelo encaixes entre as peças.

10. O processo de confecção desse dispositivo é caracterizado pela produção de uma caixa em formato de cubo de 13 cm de aresta, opaca e escura, ambas as

partes superiores em forma de encaixe conectadas, promovendo vedamento. E de uma caixa interna de 10 cm também em formato de cubo, onde facilmente caberá os materiais protéticos, que necessitarão de desinfecção em clínica odontológica. Essa caixa interna irá se encaixar na caixa externa e maior, com adaptação junto à estrutura de LEDs distribuídos, um em cada lateral do cubo e dois nas regiões superior e inferior, onde se encontram as maiores superfície das peças protéticas. Os LEDs que irão compor a parte ativa do tratamento nesse dispositivo poderá ser de dois comprimentos de onda, ou toda a caixa interna estará associada a um LED de cerca de 630 nm (vermelho) ou a LEDs azuis (440-485 nm).

11. A utilização desses dispositivos é caracterizado pelas reinvidicações 2, 3, 4, 5, 6, 7, 8 e 9 o que determina maior segurança e proporciona avanços nas técnicas de desinfecção de peças protéticas, tanto pelo professional como pelo paciente.

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FIGURA 1

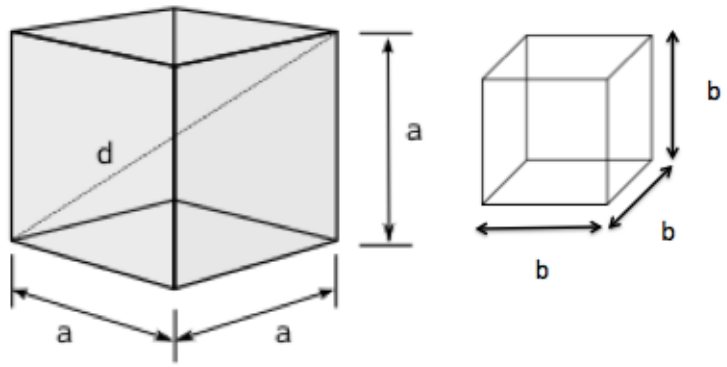


FIGURA 2

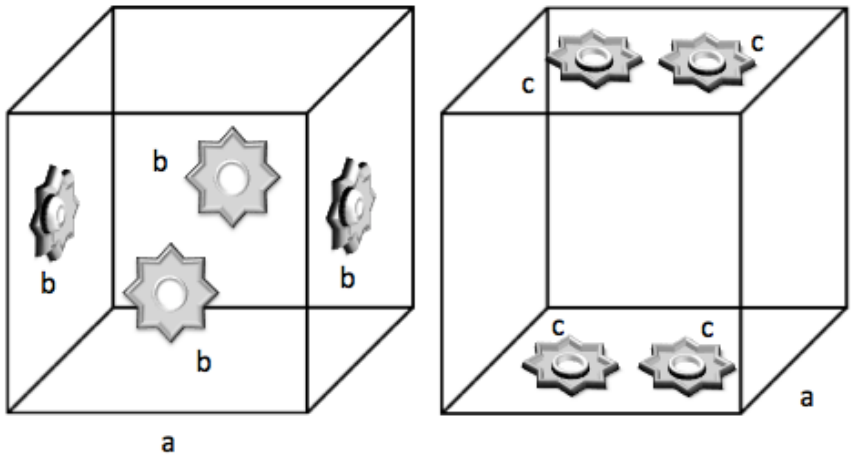


FIGURA 3

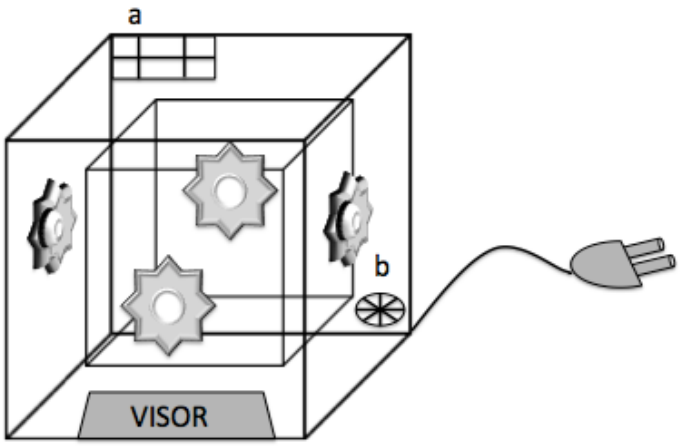
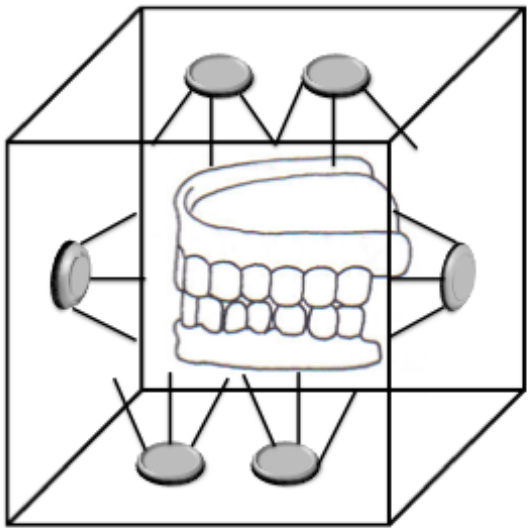


FIGURA 04.



RESUMO

Patente de Invenção**: "DISPOSITIVO FOTOTERÁPICO COM LED DE ALTA POTÊNCIA ASSOCIADO A FOTOSSENSIBILIZADORES ESPECÍFICOS PARA DESINFECÇÃO DE MATERIAIS PROTÉTICOS".**

O modelo de utilidade apresenta um aparelho fotoirradiador para dispositivos protéticos, que inclui dois corpos de caixa associados com uma fonte de luz, em que a fonte de luz são placas equipadas com LEDs (Diodos emissores de luz) posicionado em todas as partes da caixa; uma caixa externa opaca e escura, com região superior de encaixe, serve como aparato para uma caixa interna de menores proporções; um potenciômetro fixamente disposto na parede lateral da caixa, ligado a um ecrã de visualização de potência óptica, tempo e temperatura; as fontes de luz de LED (1W) conectados eletricamente a uma fonte de alimentação externa. A célula de fotoirradiação utilizada para fonte da terapia fotodinâmica, como método de desinfecção de aparelhos protéticos, tem vantagens, como: fontes de luz em vermelho (625-740 nm) ou azul (440-485 nm), presença de sensores que evitam a geração de calor durante o processo de irradiação; apresenta estrutura externa que bloqueia a passagem de luz e uma interna que permite a irradiação em 360 graus; fonte segura e eficaz de tratamento.

4 Conclusão Geral

4 CONCLUSÃO GERAL

Com base nos resultados dos estudos que compõem esta tese, pode-se concluir que:

I. Os fotossensibilizadores azul de metileno (AM) e azul orto de toluidina (AOT) associados à fonte de LED de alta potência (1W/630nm) reduziram significantemente a viabilidade fúngica de culturas planctônicas e de biofilmes de *Candida albicans*. No entanto, o AOT apresentou maior redução da contagem de células, em comparação ao AM. O rosa bengala irradiado com LED (400mW/520nm), apresentou poder de inativação fúngica inferior às anteriores associações. O LED de 1W/630nm compreendeu uma melhor alternativa para a realização da irradiação da terapia fotodinâmica antimicrobiana, devido o seu reduzido tempo de trabalho (120 segundos) e superior efeito contra *C. albicans*, quando comparado ao LED de 400mW/520nm.

II. Os fotossensibilizadores azul orto de toluidina, azul de metileno e rosa bengala não causaram alterações de cor, aumento de rugosidade de superfície, alterações na topografia e perda de massa na resina acrílica termopolimerizável.

III. A terapia fotodinâmica antimicrobiana no parâmetro testado mostrou ser capaz de gerar altos níveis de estresse oxidativo intracelular, e danos ao DNA celular em *C. albicans*.

Os estudos que compreendem esta tese permitiram o desenvolvimento de um aparelho fotoirradiador para dispositivos protéticos, para realização de terapia fotodinâmica antimicrobiana. É um equipamento portátil, de baixo custo de fabricação, permitindo um tratamento barato e atóxico, além de ser um produto inovador, cujo pedido de patente foi encaminhado ao Instituto Nacional da Propriedade Industrial (INPI).

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6 Anexo

< Uso exclusivo do INPI >

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Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas:

1. Depositante (71):

1.1 Nome: UNIVERSIDADE FEDERAL DO CEARA

1.2 Qualificação: INSTITUIÇÃO DE ENSINO SUPERIOR

1.3 CNPJ/CPF: 07272636000131

1.4 Endereço Completo: AV DA UNIVERSIDADE,2853 BENFICA

1.5 CEP: 60020180

1.6 Telefone: 85-33669434 1.7 Fax:

1.8 E-mail: javam@ufc.br

☐ continua em folha anexa

2. Natureza: ☒ Invenção ☐ Modelo de Utilidade ☐ Certificado de Adição

3. Título da Invenção ou Modelo de Utilidade (54):

"DISPOSITIVO FOTOTERÁPICO COM LED DE ALTA POTÊNCIA ASSOCIADO A FOTOSSENSIBILIZADORES ESPECÍFICOS PARA DESINFECÇÃO DE MATERIAIS PROTÉTICOS "

☐ continua em folha anexa

4. Pedido de Divisão: do pedido Nº **Data de Depósito:**

5. Prioridade: ☐ Interna (66) ☐ Unionista (30)

O depositante reivindica a(s) seguinte(s):

País ou Organização do depósito	Número do depósito (se disponível)	Data de depósito

☐ continua em folha anexa