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PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA
DOUTORADO EM ODONTOLOGIA
ÁREA DE CONCENTRAÇÃO EM CLÍNICA INTEGRADA

Thiago Maciel Cavalcanti

TERAPIA FOTODINÂMICA ANTIMICROBIANA UTILIZANDO FTALOCIANINA DE CLOROALUMÍNIO EM NANOEMULSÃO EM ESPÉCIE DE *Candida albicans*

RECIFE,
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Tese apresentada ao Programa de Pós-Graduação em odontologia, do Ciências da Saúde do Centro de Ciências da Saúde da Universidade Federal de Pernambuco, para obtenção do título de doutor em odontologia. Orientadora: Profª. Dr. Jair Carneiro Leão.

RECIFE,
2016.

Catalogação na fonte:
bibliotecário: Aécio Oberdam, CRB4:1895

C376t Cavalcanti, Thiago Maciel.
Terapia fotodinâmica antimicrobiana utilizando ftalocianina de cloroalumínio em nanoemusão em espécie de *cândida albicans* / Thiago Maciel Cavalcanti. – Recife: o autor, 2017.
95 f.; il.; 30 cm.

Orientador: Jair Carneiro Leão.
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Ciências da Saúde. Programa de pós-graduação em odontologia.
Inclui referências, apêndices e anexos.

1. Lasers. 2. Antifúngico. 3. Terapia fotodinâmica. 4. *Cândida albicans*. 5. Fotossensibilizantes. I. Leão, Jair Carneiro (orientador). II. Título.

617.6 CDD (23.ed.) UFPE (CCS 2018 - 053)

A Deus, que me ilumina e me guia em todos os momentos. A minha esposa, Ana Patrícia, pelos cuidados, apoio, dedicação e compreensão fomentados pelo amor diário.

Aos meus pais, Adailton e Neves, exemplos de vida, não mediram esforços para proporcionar a melhor educação desde meus primeiros anos de vida, sempre com incentivos, palavras, atos e gestos fortalecedores.

Aos meus irmãos, Diego e Rafaella, que cumprem o verdadeiro sentido da família unida, nos fazendo sempre três espelhos com reflexo de vitória, felicidade e amor, um para o outro À minha querida avó Hilda, pelos conselhos, ensinamentos, partilha e orações.

AGRADECIMENTOS

Agradeço à Universidade Federal de Pernambuco, na pessoa do Magnífico Reitor, Prof. Dr. Anísio Brasileiro de Freitas Dourado, por garantir toda estrutura necessária à conclusão deste trabalho. À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) do Ministério da Educação. À Pró-Reitoria para Assuntos de Pesquisa e Pós-Graduação (PROPESQ) da Universidade Federal de Pernambuco.

Aos que fazem o Programa de Pós-Graduação em Odontologia, professores e funcionários, na pessoa da coordenadora Prof. Dra. Alessandra de A. T. Carvalho, pela dedicação e esforço em melhorar sempre a qualidade do Programa.

Ao meu orientador, professor Dr. Jair Carneiro Leão, referência internacional na Odontologia, pelos valiosos conhecimentos transmitidos, confiança e apoio depositados.

A minha co-orientadora, Profa. Dra. Ruthinéia, fundamental para a realização da pesquisa, pela inteligência inspiradora que me impulsiona desde a graduação.

À professora Maria Regina, que tão bem conduziu as etapas laboratorias, pela solidariedade contínua e pela contribuição científica.

Ao colega de profissão Rivonaldo Adriano, por toda contribuição técnicolaboratorial.

À professora Maiara Moraes pela presteza, atenção e colaboração com a pesquisa desde os primeiros momentos

Ao professor Joao Paulo Longo, pela humildade e confiança demonstradas.

Aos familiares das famílias Maciel, Cavalcanti, Aguiar, Feitosa e Toscano pela torcida e orações.

A Jalber Almeida dos Santos pela amizade construída e edificada durante anos

Aos amigos de toda vida por participarem de tantos momentos importantes na minha trajetória.

Às companheiras de doutorado, Marcela e Michelle pelos conhecimentos compartilhados.

Meus irmãos, considerem motivo de grande alegria o fato de passarem por diversas provações, pois vocês sabem que a prova da sua fé produz perseverança. E a perseverança deve ter ação completa, a fim de que vocês sejam maduros e íntegros, sem lhes faltar coisa alguma.

Tiago 1:2-4

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AM (Azul de metileno)

AsGaAl (Arseneto de gálio e alumínio

AGNB (Bacilos Aeróbios Gram-negativos)

ATCC (*American Type Culture Collection)*

ATP (Trifosfato de Adenosina)

BHI (Brain heart infusion)

$Ca(OH)_2$ (Hidróxido de cálcio)

ClAlPc/NE (cloroalumínio ftalocianina em nanoemulsão)

ClAlPc (cloroalumínio ftalocianina)

CFU/mL(Colony Forming Units per mililitre)

DNA (Ácido desoxirribonucléio)

FS (Fotossensibilizador)

FSs (Fotossensibilizadores)

LBP (Laser de baixa potência)

LLL (Low Level Laser)

μg/ml (Micrograma por mililitro)

GePc (Ftalocianina de Germânio)

He-Ne (Hélio-Neônio)

Hz (Hertz)

InGaAlP (Índio Gálio Alumínio e Fósforo)

J (Joules)

J/cm^2 (Joules por centímetro quadrado)

LED (light emitting diode)

mW (MiliWatt)

mM (Milimolar)

µM (Micromolar)

µU (Micromolar)

ml (mililitro)

N/cm (Newton por centímetro)

NDDS (Sistema de entrega de droga nanoparticulado)

Nd:YAG (Neodímio dopado com ítrio granada de alumínio) .

NaOCl (Hipoclorito de sódio)

NiTi (Níquel-Titânio)

nm (Nanômetro)

PC (Ftalocianina)

PDT (Photodynamic Therapy)

Pg (*Porphyromonas gingivalis)*

PS (photosensitizer)

ROS (Especie de oxigênio Reativo)

SDA (Agar Sabouraud Dextrose)

SiPc (Ftalocianina de Silicio)

TBO (Azul de toluidina)

TFD (Terapia fotodinâmica)

UFC (Unidades formadoras de colônias)

UNB (Universidade de Brasília)

UFPE (Universidade Federal de Pernambuco)

UFRN (Universidade Federal do Rio Grande do Norte)

W (Watt)

Resumo

Avaliar *in vitro* a ação antimicrobiana da terapia fotodinâmica (TFD) utilizando ClAlPc/NE, como agente fotossensibilizador (FS), em Candida *albicans.* A atividade antimicrobiana foi verificada pelo método de contagem de UFC/mL *in vitro*. 100µl (10^6 UFC/ml) de *C. albicans* foram depositados em adição ao mesmo volume de ClAlPc/NE em uma placa de microdiluição nas quatro concentrações testadas (40, 20, 10, 5µU). Posteriormente, as placas foram deixadas no escuro em um dos tempos de incubação (1,5,10 e 15min.). Após cada tempo de incubação, as placas foram continuamente irradiadas com Laser de Baixa Potência (LBP) (Grupo A) ou não eram irradiadas (Grupo B). O experimento foi em duplicata. As placas foram incubadas a 37ºC, no escuro, e a contagem das UFC/mL foi iniciada após 48h. O efeito fotodinâmico foi mensurado pela fração de sobrevivência dos microrganismos, entre os números de UFC/mL do grupo teste e o controle negativo.O grupo A da TFD apresentou média menor de UFC/ml em relação ao grupo B, substância sem laser**.** As médias de UFC/ml em $\log_{10}$ dos grupos A e B foram diferentes (valor-*p* = 0,00048). O maior efeito fotodinâmico com comprovação estatística foi apresentado na concentração 20 µU, do grupo de TFD, para um tempo de incubação de 10 minutos**.** A quantidade de UFC/ml, após TFD, diminuiu em relação ao controle negativo em todos os tempos de incubação e concentrações do FS. Desta forma, a TFD antimicrobiana com ClAlPc/NE foi efetiva.

Palavras-chave: Lasers. Antifúngico. Terapia fotodinâmica. Candida *albicans*. Fotossensibilizantes.

Abstract

To evaluate the *in vitro* antimicrobial effect of photodynamic therapy (PDT) using ClAlPc / NE as a photosensitizer agent (FS) in Candida albicans. The antimicrobial activity was assessed by CFU/mL counting method *in vitro*. . 100µl (10^6CFU/ ml) C. albicans were deposited in addition to the same volume of ClAlPc/NE in a microdilution plate in the four tested concentrations (40, 20, 10 and 5µM). Subsequently, the plates were left in a dark for especifc incubation periods (1,5,10, and 15 min.). After each incubation period, the plates Group A were continuously irradiated with LLL, unlike plates group B which were not irradiated. The experiment was duplicated. The plates were incubated at 37 °C in the dark, and the count of CFU/ml was initiated after 48 hours. The photodynamic effect was measured by the microorganisms survival fraction between the number of CFU/ml of the test group and the negative control. Group A with PDT had lower average CFU/ml compared to group B without laser iiradiation. The average CFU /ml in log10 of groups A and B were different (p-value = 0.00048). The highest photodynamic effect with statistical evidence was presented at a concentration of 20 µM, for 10 minutes incubation period in the PDT group. The number of CFU/ml after PDT, decreased compared to the negative control at all incubation periods and all concentrations of FS. Thus, the antimicrobial PDT ClAlPc/NE was effective.

Keywords: Lasers. Antifungal. Photodynamic therapy. Candida albicans. Photosensitizers.

Sumário

1. INTRODUÇÃO

O aparecimento de doenças bucais depende essencialmente da presença dos microrganismos, que durante o metabolismo dos carboidratos formam ácidos e produtos metabólicos [1]. Esse processo só ocorre quando há um desequilíbrio no ecossistema bucal. Atualmente os estudos na área Odontológica têm sido norteados pela valorização do aspecto preventivo e desse modo uma manutenção da saúde bucal [2].

Fungos oportunistas podem provocar infecções superficiais ou invasivas graves, especialmente em pacientes debilitados ou imunocomprometidos. Devido a dificuldades no diagnóstico e a disponibilidade limitada de drogas antifúngicas eficazes, representam uma ameaça crescente para a saúde humana, podendo resultar em alta mortalidade [3].

A candidíase é uma infecção comum em pacientes que usam próteses. Além disso, fatores dietéticos, higiene oral inadequada, uso de drogas por via oral, radioterapia, xerostomia, tabagismo e doenças sistêmicas podem aumentar a probabilidade de desenvolvimento desta doença [4].

Várias espécies de Candida podem causar candidíase. A *C. albicans* continua sendo a mais comum espécie responsável pela doença. Por esta razão os crescentes casos de resistência à terapêutica antifúngica atual é um motivo de preocupação, pois ocorre a diminuição da eficiência das terapias convencionais, que na maioria das vezes são tratamentos demorados, os quais exigem maiores orçamentos e cuidados com a saúde. Além disso, estas drogas atuais apresentam toxicidade, espectro de ação limitado e as interações medicamentosas são comuns [5].

Os esforços para controlar as infecções microbianas foram prejudicados pelo surgimento e proliferação de patógenos resistentes às drogas, sendo necessária a busca de abordagens complementares à base de fármacos atuais. Além disso, a escassez de terapias eficazes para muitas infecções importantes a nível mundial realça a necessidade do desenvolvimento de novas abordagens para o tratamento ou prevenção da infecção [6].

Infecções causadas por Candida, como por outros fungos, continuam a representar um problema significativo de saúde pública, principalmente as espécies resistentes aos antifúngicos tradicionais, azóis e polienos, como fluconazol e nistatina, respectivamente. Por isso, é necessário o desenvolvimento de pesquisas científicas que busquem ampliar as formas de tratamento das lesões de Candida, possibilitando um tratamento efetivo dos casos de insucesso e melhorando o bem estar dos pacientes acometidos por esta doença [4].

Diante das limitações das terapias convencionais para o tratamento de infecções cutâneas superficiais causadas por *C. albicans*, tratamentos alternativos devem ser estimulados. A terapia fotodinâmica antimicrobiana pode ser uma alternativa agente eficaz para eliminar estes microrganismos [5].

A reação fotodinâmica (TFD) envolve a administração de um agente fotossensibilizador (corante) que é ativado por luz de um comprimento de onda específico, resultando na geração de radicais, altamente reativos. Estas espécies ativas podem causar destruição das células [7].

O termo “terapia fotodinâmica” é relativamente novo, mas a modalidade de tratamento que combina a administração da droga e subsequente exposição à luz solar é antiga. Existem relatos do uso de combinação de plantas ou extratos ingeridos oralmente e a luz do sol para

tratar doenças de pele como vitiligo e psoríase com sucesso na Índia, Egito e China há 4000 anos [8,9].

A primeira demonstração de fotossensibilização letal de células data de 1900, quando Raab verificou que baixas concentrações de acridina que, no escuro, não possuíam efeito sobre o Paramecium, podiam sob exposição à luz normal do dia, determinar a inativação do microrganismo[10].

Von Tappeiner e Albert Jesoniek, em 1903, realizaram o tratamento de carcinomas com aplicação de solução de eosina a 5% por via tópica e posterior exposição a lâmpadas ou a luz do sol [11]. E, no mesmo ano, publicaram um curto, mas notável artigo sobre o tratamento de herpes, psoríase e câncer de pele por aplicação tópica de eosina e subseqüente exposição à luz do Sol. Von Tappeiner e seus colegas biomédicos afirmaram que essas reações eram altamente dinâmicas e diferiam da fotossensibilização de chapas fotográficas por certos corantes. Então o termo *Photodynamische Wirkung* (efeito fotodinâmico) foi cunhado, por Von Tappeiner e Jodlbauer, para todas as reações fotobiológicas envolvendo um fotossensibilizador que ocorrem na presença de oxigênio molecular e levam à destruição de tecidos [12,13].

A reação fotodinâmica envolve três componentes: a luz, um fotossensibilizador e o oxigênio. Após a irradiação com luz de um comprimento de onda específico, o fotossensibilizador passa por uma transição de uma energia de baixo nível, de estado fundamental para um estado singleto excitado. Posteriormente, o fotossensibilizador pode voltar para seu estado fundamental, com emissão de fluorescência, ou pode sofrer uma transição para o estado de nível de energia mais alto, o estado triplete. Neste estado, pode

ocorrer uma reação do oxigênio endógeno produzindo o oxigênio singleto e outras espécies de radicais livres, causando uma rápida destruição seletiva do tecido alvo [14].

Existem dois mecanismos pelos quais o fotossensibilizador no estado triplete pode reagir com biomoléculas (figura 1) [5].

O Tipo I envolve transferência de elétrons/hidrogênio diretamente do fotossensibilizador, produzindo íons ou elétrons/hidrogénio removidos de uma molécula de substrato para formar os radicais livres. Estes radicais reagem rapidamente com o oxigênio, resultando na produção de espécies de oxigênio altamente reativas (superóxido, hidroxila radicais, peróxido de hidrogênio).

As reações do tipo II produzem o estado eletronicamente excitado e altamente reativas de oxigênio conhecido como o oxigênio singlete. Na TFD é difícil distinguir os dois mecanismos de reação. A contribuição de ambos os tipos indica que o mecanismo de dano é dependente tanto da tensão de oxigênio quanto da concentração do fotossensibilizador [14].

Embora os fungos sejam alvos mais complexos do que as bactérias, evidências indicam que o comportamento de tais células frente a processos fotodinâmicos é mais simples em comparação às bactérias [15]. Por este motivo, as células Candida podem ser destruídas por FSs aniônicos como Photofrin. Além disso, as células fúngicas podem ser mortas por taxas fotodinâmica mais baixas, o que significa que uma "janela terapêutica" está disponível [16].

O mecanismo fotodinâmico destrói as células fúngicas através das perfurações provocadas pelas espécies reativas de oxigênio (ROS) nas paredes e membranas celulares, permitindo assim que o FS passe para o interior celular. Uma vez dentro da célula, espécies

oxidantes geradas pela excitação da luz induzem destruição de organelas celulares internas e finalmente ocorre a morte celular [5]. Múltiplos alvos celulares estão disponíveis para o efeito foto-oxidativo causando inativação de enzimas e proteínas e peroxidação dos lipídeos, levando à lise das membranas celulares, lisossomos e mitocôndrias. Assim, o oxigênio singleto gerado pela excitação do FS é um agente de oxidação não específica, e não há defesa celular contra ele [15].

O uso da terapia fotodinâmica envolvendo o laser de baixa potência (LBP) tem crescido de forma constante nas últimas duas décadas. Várias aplicações têm se desenvolvido e alcançado êxito, dentre elas, a terapia fotodinâmica (TFD), que se revelou ser um poderoso método terapêutico para o tratamento seletivo de tumores malignos e muitas outras doenças [17].

No ato da irradiação, o corante, substância fotossensibilizadora, tem suas moléculas convertidas em estado de excitação e nesse estado, a energia transferida da fonte para moléculas do corante resulta na formação de moléculas reativas como o oxigênio singleto que podem danificar e ainda matar células bacterianas [18].

O uso da TFD se concentra em compostos ou substâncias que apresentam dentre suas características a baixa toxicidade (no escuro) causando danos às células apenas sob irradiação; absorção na faixa de comprimento de onda entre 630 e 680 nm, propriedade requerida para a penetração da luz em células e o número de fótons absorvidos, uma vez que o fluxo de luz absorvido diminui exponencialmente com a distância; estabilidade in vivo limitada para rápida eliminação dos tecidos tumorais; permitir derivações para otimização das propriedades; fluorescência característica que permite a localização; composição química bem definida [19].

Fazendo parte da primeira geração de fotossensibilizadores tem-se o photofrin (utilizado na prática clínica), constituído de derivados oligoméricos de hamatoporfirinas, uma composição complexa que dificultou a reprodução da sua síntese.

Na segunda geração foram desenvolvidas substâncias simples como as principais famílias de corantes mais utilizadas na TFD, **as ftalocianinas**, cianinas e as fenotiazinas.

Lisossomos, mitocôndrias e membranas plasmáticas, em células de mamíferos, são os principais alvos para TFD, enquanto em células microbianas, o dano à membrana externa desempenha um papel importante, pois pode impedir o dano ao DNA [20].

Formalmente, as ftalocianinas são porfirinas tetrabenzil (Figura 2). O aumento do caráter aromático explica o motivo da absorção de luz ser mais intensa do que no núcleo central da porfirina. Em termos de potencial fotossensibilizante, as ftalocianinas dão maior rendimento oxigênio singleto, superando fotossensibilizadores como o azul de metileno e azul de toluidina [21, 22].

A Ftalocianina (PC) é um dos mais recentes fotossensibilizadores de segunda geração. São compostos quimicamente puros que possuem alto poder de absorção de luz com comprimentos de onda na faixa de 650-680 nm, penetrando mais nos tecidos, em comparação com os derivados de hematoporfirina (1ªgeração). As ftalocianinas apresentam uma variedade de íons de metal na sua estrutura química, que é responsável pela propriedade particular de cada molécula. Alumínio e zinco são dois íons metálicos usados para melhorar a fototoxicidade da ftalocianina desenvolvendo um bom rendimento quântico de produção de estados tripleto [21,23].

As propriedades fotofísicas de ftalocianinas são fortemente dependentes deste íon metálico central. Dentre as metaloftalocinanina, a ftalocianina de zinco (ZnPC) e Cloroaluminio Ftalocianina (ClAlPc) (figura 2) são as que apresentam as propriedades fotofísicas mais favoráveis para aplicação em TFD, estados singletos com longa duração (3-8 ns) e estados tripletos com alto rendimentos quânticos [17].

Além disso, os corantes à base de ftalocianina podem se acumular e se eliminar seletivamente muito mais eficientemente a partir do tecido alvo, com quase nenhuma fluorescência observada nas células 24 h após a administração da droga [21].

A solubilidade em água da ClAlPc é baixa, sendo desta forma, o maior obstáculo para a avaliação das propriedades farmacológicas e para a administração tópica ou parenteral desta PC. Sistemas de administração de fármacos nanoestruturados (NDDS) como nanoemulsões, lipossomas e nanocápsulas estão sendo utilizados como veículos para o FS hidrofóbico, como a PC [24].

O sistema de entrega de droga nanoparticulado (NDDS) constitui um dispositivo farmacêutico versátil, pois pode facilmente incorporar manipulação externa com várias vantagens em relação às terapias convencionais. A Preparação é principalmente com base em métodos de nanoencapsulação, que inclui o modelo de hospedagem, a fase polimérica nanométricos, estruturas biologicamente ativas (medicamentos e vacinas), e agentes vetoriais [26].

As nanoemulsões são dispersões óleo-em-água (o/w), possuindo gotículas com tamanho de 10-600 nm, projetado para transportar materiais solúveis em óleo que podem ser dissolvidos na fase de óleo de uma emulsão ou em fármacos anfifílicos, que são aderidos na interface óleo-água [25].

As nanopartículas, têm sido o foco de intensa investigação na última década, revelando a sua capacidade de transporte da droga mantendo os níveis terapêuticos necessários ao alvo. Além da capacidade de melhorar o manejo de medicamentos insolúveis em água, podem controlar a liberação do fármaco, reduzindo efeitos secundários, protegendo moléculas bioativas antes de atingirem o seu local de ação aumentando a penetração intracelular e melhorando a acumulação-alvo da droga [24].

Este estudo teve como principal objetivo avaliar *in vitro* a ação antimicrobiana da terapia fotodinâmica (TFD) frente à espécie de fungo Cândida *Albicans* utilizando cloroalumínio ftalocianina em nanoemulsão (ClAlPc/NE) como agente fotossensibilizador.

2. MATERIAIS E MÉTODOS

2.1 Caracterização do Estudo

Foi realizado um estudo experimental, analítico e quantitativo. Para tanto, procedeu-se um estudo *in vitro* da atividade antimicrobiana da terapia fotodinâmica (TFD) utilizando ClAlPc como fotossensibilizador em espécie de cepas isoladas de *C. albicans*, obtidas do banco de amostra do laboratório de Microbiologia do curso de Odontologia da UFRN. A Candida *albicans (*AB/Ambiente bucal 92) foi obtida da coleção de cultura do Laboratório de Microbiologia do Departamento de Análises Clínicas e Toxicológicas da UFRN (Comitê de Ética em Pesquisa do Hospital Universitário Onofre Lopes - CEP-HUOL protocolo nº. 152/07 (ANEXO A)

O estudo *in vitro* foi realizado no Laboratório de Microbiologia do departamento de Odontologia da Universidade Federal do Rio Grande do Norte

2.2 Reativação e inóculo das Cepas

As cepas de *C. albicans* isoladas foram inoculadas com o auxílio de uma alça de platina, estéril, em um tubo de ensaio contendo 5 mL de caldo BHI. Os tubos foram incubados a 37ºC por 24 horas e após este período observou-se a turvação nos meios de cultura, indicando viabilidade microbiana. Para condução do estudo, suspensões fúngicas, sob a concentração 10^6 microrganismos/mL, foram preparadas em solução salina, comparável ao tubo 0,5 da Escala de MacFarland.

2.3 Meios de cultura

Para a realização dos experimentos foi utilizado o meio de cultura Agar Mueller Hinton - AMH (fabricante DIFCO) a 37ºC por 48h. O meio de cultura foi preparado e utilizado segundo as recomendações do fabricante. Foram utilizados 65g de pó para 1L de água destilada. O recipiente contendo esta solução foi levado ao autoclave, com 121ºC por 15 minutos. Após esta esterilização, cerca de 20ml foram colocados nas placas de petri estéreis, com proximidade da chama do bico de Bunsen. Após solidificação do meio, todas as placas de Petri foram identificadas e guardadas sob refrigeração.

3. TERAPIA FOTODINÂMICA

3.1 Divisão e descrição dos grupos

As placas de petri foram divididas em grupos experimentais de acordo com o procedimento a ser executado:

Grupo A _ TFD (**ClAlPc/NE)**

Grupo B _ *uso isolado do* **ClAlPc/NE**;

Grupo C _ controle negativo: Solução Salina (0,9%)

Grupo D _ controle positivo: Nistatina (100.000 U.I/mL)

Segue a descrição dos grupos:

3.2 Grupo A

A atividade antimicrobiana foi verificada pelo método de contagem de unidades formadoras de colônias. Com agitação constante, alíquotas de 100µl da suspensão do fungo (10^6 UFC/ml) foram transferidas individualmente para um poço de uma placa de microdiluição com 96 poços. Em seguida, foi acrescentado o mesmo volume de fotossensibilizador Cloroalumínio ftalocianina em nanoemulsão (ClAlPc/NE) em uma das quatro concentrações testadas (40, 20, 10, 5µM).

Posteriormente, as placas contendo as suspensões resultantes foram deixadas em repouso, no escuro, durante um dos tempos de incubação (1,5,10 e 15min.). Após cada tempo de pré-irradiação(incubação), as placas de 96 poços foram abertas e as células da levedura foram continuamente irradiadas, a partir do topo da placa de microtitulação, com um diodo laser (comprimento de onda de 660nm; potência de 100mW e dose de 320 J/cm^2). A ponteira ótica do transdutor ficou a uma distância de 1cm da amostra, o conteúdo dos poços foi constantemente agitado durante a iluminação para assegurar que as células da levedura não se depositam no fundo dos poços

Após exposições, as suspensões foram agitadas e diluídas (a 10^{-3}), e 100µl de cada diluição foram espalhados na superfície das placas de petri (90x15mm) contendo o meio de cultura AMH. O experimento foi em duplicata. As placas foram incubadas a 37ºC, no escuro, e a contagem das Unidades Formadoras de Colônia (UFC) foi iniciada após 48h.

O efeito fotodinâmico foi mensurado pela fração de sobrevivência dos microrganismos. S/S0, sendo o S o número de colônias registrado e S0 o número de colônias do grupo controle, não submetido à terapia. Quanto menor a fração de sobrevivência, maior a eficácia do tratamento.

3.3 Grupo B

Seguindo a mesma metodologia do grupo A, após cada tempo de pré-irradiação, não se aplicou o laser de baixa potência, permanecendo as amostras, no escuro. As suspensões foram agitadas e diluídas (a 10^{-3}), e 100µl de cada diluição foram espalhados na superfície das placas de petri (90x15mm) contendo o meio de cultura AMH. O experimento foi em duplicata. As placas foram incubadas a 37ºC, no escuro, e a contagem das Unidades Formadoras de Colônia (UFC) foi iniciada após 48h.

3.4 Grupos controle negativo e positivo

No grupo controle negativo, 100 µL da suspensão (10^6 UFC/mL) de células de *C. albicans* receberam o mesmo volume de solução salina à 0,9% em cada poço de microplaca. As placas ficaram em repouso no escuro pelos mesmos tempos de incubação dos grupos anteriores (1, 5, 10 e 15 min.). As suspensões foram agitadas e diluídas (a 10^{-3}), e 100µl de

cada diluição foram espalhados na superfície das placas de petri (90x15mm) contendo o meio de cultura AMH, em duplicata.

Enquanto no grupo controle positivo, as células de *C. albicans* receberam tratamento, ou seja, com a suspensão de Nistatina na concentração de 100.000 U.I/mL, já utilizada comercialmente. 100 µL da suspensão (10^6 UFC/mL) foram transferidos para os poços da placa de microtitulação e, em seguida, o mesmo volume da Nistatina foi depositado em cada poço onde ficou em repouso no escuro pelos mesmos tempos de incubação dos grupos anteriores (1, 5, 10 e 15 min.). Em seguida as placas foram levadas em duplicata à estufa.

4. EQUIPAMENTO LASER

Nos grupos correspondentes à terapia fotodinâmica foi utilizado o equipamento *Laser*, da marca DMC®, com os seguintes parâmetros:

- Meio ativo InGaAlP, saída de 100 mW, comprimento de onda 660 nm, laser vermelho e emissão contínua.
- Dosimetria: 320J/cm^2 para cálculo em uma área de incidência de 1cm^2.
- Tempo: A incidência da luz laser foi perpendicular ao fundo do poço e teve duração de 90 segundos

O aparelho foi aferido e calibrado previamente ao início dos trabalhos.

Fotossensibilizador Cloroalumínio Ftalocianina em Nanoemulsão(ClAlPc/NE)

Foi sintetizado pelo laboratório de Química e bioquímica da UNB, gentilmente cedida pelo professor João Paulo Longo.

5. ANÁLISE ESTATÍSTICA

Os resultados desta pesquisa foram obtidos através dos testes estatísticos: teste Shapiro-Wilk, teste F e teste T de Student. Na análise feita com o teste Shapiro-Wilk os p-valores foram considerados significativos, inferiores a 0,05. Posteriormente, encontrada uma diferença significativa entre os grupos como um todo, foram aplicados os demais testes (teste F e teste t de Student), para verificar onde ocorreram estas diferenças. Os dados foram digitados no programa Excel versão 2013 e exportados para o programa R(versão 3.3.1), onde foram realizados as análises e testes estatísticos.

6. RESULTADOS

Os resultados para cada grupo, de acordo com a metodologia, foram obtidos, e os números de UFC/mL de espécie de Candida *albicans* foram transformados em logaritmo decimal (log_{10}). A tabela 1 representa os resultados dos grupos A e B, o grupo C (controle negativo) e o grupo D (controle positivo).

O teste de normalidade Shapiro-Wilk verificou que as observações provêm de uma população normal, permitindo assim, a aplicação dos demais testes, comparação de variáveis (Teste F) e comparação de médias (teste T).

No efeito fotodinâmico mensurado pela fração de sobrevivência dos microrganismos utilizando o teste T, as médias da quantidade de UFC/ml em logaritmo decimal (log_{10}) dos grupos A e B em relação à concentração e ao tempo de incubação da Cloroalumínio Ftalocianina em Nanoemulsão(ClAlPc/NE) são diferentes estatisticamente (valor-*p* =

0,00048). O grupo A da TFD apresentou média de fração de sobrevivência de UFC/ml menor em relação ao grupo B, substância sem laser.

Especificamente, verifica-se pela tabela 2 que as médias no tempo de 1 min e 10 min apresentam diferenças estatísticas entre as médias da quantidade de UFC/ml nos grupos A e B com um nível de significância de 5%. Já em relação às concentrações, houve diferença estatística entre as médias da quantidade de UFC/ml dos grupos A e B para as concentrações de 10 e 20 µU com um nível de significância de 5%.

No grupo A em relação ao tempo, é perceptível que no período de 1 minuto os dados estão bem concentrados próximo da média (0,8567) e sua variância corresponde à cerca de 0,0001, já no tempo de 10 minutos, os dados apresentam ter uma variância igual 0,0004 com média correspondente a 0,8291 (gráfico 1). A fração de sobrevivência em relação à concentração do grupo A (gráfico 2), percebe-se concentrações de 10 e 20 µU, variâncias de 0,0012 e 0,0007 respectivamente. Já em relação às médias, na concentração de 10 µU foi de 0,8592 e na de 20 µU 0,8193. (gráficos 1 e 2).

No grupo B em relação ao tempo, é perceptível que no período de 1 minuto os dados estão bem concentrados próximo da média (0,8878) e sua variância corresponde à cerca de 0,0001, já no tempo de 10 minutos, os dados apresentam ter uma variância igual 0,0005 com média correspondente a 0,8837, (gráfico 3 e 4).

Pelo controle positivo (antifúngico Nistatina), apenas a média do número de UFC/ml do Grupo A foi estatisticamente significativo (p= 0,0001243), com a melhor e menor média de UFC/ml após a TFD com ClAlPc/NE. A fração de sobrevivência para o grupo B e controle positivo não foi significativo (p = 0,563) (tabela 3, Gráficos 5 e 6).

7. DISCUSSÃO

Numerosos medicamentos antifúngicos tópicos e sistêmicos são administrados para tratar infecções. O surgimento de formas resistentes do fungo Candida é uma preocupação crescente, podendo aumentar a prevalência da Candidíase. Além disso, os efeitos colaterais dessas drogas, como hepatotoxicidade, limitam longos períodos da terapia em alguns casos. Assim, novas abordagens terapêuticas, como a TFD antimicrobiana, estão sendo estudadas para reduzir as complicações e aumentar a eficácia do tratamento [27].

Neste grande complexo microbiano, optou-se por utilizar uma espécie de Candida *albicans* por ser o agente etiológico mais predominante, representando 70-80% de todos os microrganismos isolados a partir de lesões orais. A candidíase oral é uma manifestação comum de infecções fúngicas, especialmente em imunocomprometidos, como AIDS e pacientes que receberam radioterapia [28,29].

Em terapia fotodinâmica antimicrobiana, inúmeras pesquisas foram realizadas *in vitro* e *in vivo*, em espécies de Candida [28,30–32]. Entre os fotossensibilizadores, os derivados de fenotiazínicos, como azul de metileno e azul de toluidina, são os mais comumentes usados na inativação de *C. albicans* em diversos estudos [3,22,33–39].

Apesar da eficácia clínica deste grupo de FS, a baixa capacidade de geração de ROS em comparação com outros é relatada como contraponto. Para resolver este problema, FS de 2ª geração, tais como derivados de ftalocianinas, caso deste estudo, têm sido propostos como uma alternativa para a aplicação em odontologia. A Pc já apresenta excelentes resultados na diminuição da toxicidade de células tumorais, TFD antitumoral [23,40–42].

Este PS tem alta atividade fotodinâmica, é o mais eficaz na geração de ROS após a ativação da luz e tem menos capacidade para corar estruturas dentais, em comparação aos fenotiazinicos. Apesar destas propriedades positivas, a maioria destas ftalocianinas é formada por compostos hidrofóbicos prejudicando sua aplicação em ambientes hidrofílicos, como ambiente bucal.

Para superar esses obstáculos, moléculas de ftalocianina podem ter associações específicas com o NDDS, como as nanoemulsões que contêm compartimentos hidrofóbicos úteis para solubilizar a ftalocianina nas soluções hidrofílicas, melhorando assim, a cinética de inativação microbiana. Desta forma, nanotecnologia tem sido agregada também à TFD, nos últimos anos [3,21,40,41].

Poucos estudos utilizaram a ftalocianina como FS para TFD antimicrobiana em espécie de Candida [23,42–45]. Nestes trabalhos, variações do anel central da molécula foram encontradas nas ftalocininas: SiPc1, GePc1 ClAlPc, ZnPc, Pc-4, BAMSiPc. As nanoemulsões foram utilizadas como sistemas de entrega de drogas nanoparticulados, assim como incorporações de tensoativos ou surfactantes, permitindo solubilidade em meio aquoso e viabilizando a interação com os microrganismos.

Este trabalho avaliou *in vitro,* a terapia fotodinâmica antimicrobiana utilizando ClAlPc/NE em espécie de Candida *albicans*. A TFD antimicrobiana foi avaliada através de média de contagem de UFC/ml.

Dentro deste contexto, neste trabalho, optou-se pelo LBP com 100mW de potência total, com comprimento de onda de 660nm, dentro do espectro de absorção do FS utilizado, o ClAlPc/NE. Vários estudos utilizaram o comprimento de onda de 660nm ou dentro da margem deste espectro visível para TFD [28,31,38–40,46].

Objetivando potencializar o efeito da TFD em um menor tempo de trabalho sem prejuízo na qualidade do tratamento, quatro diferentes tempos de incubação e quatro concentrações de ClAlPc/NE foram testadas e comparadas.

A hipótese era descobrir qual o melhor custo-benefício para TFD antimicrobiana em relação à concentração de ClAlPc/NE e o tempo em que esta substância necessitaria ficar em contato com as células de Candida *albicans* para produzir um efeito antimicrobiano satisfatório.

Várias pesquisas realizaram tal metodologia de testes de tempos e concentrações diferentes para outros fotossensibilizadores [39,47–51].

Neste trabalho, constatou-se que a TFD antimicrobiana utilizando ftalocianina foi eficiente em espécie de Candida, corroborando com outros trabalhos [47–52].

A quantidade de UFC/ml, após TFD, diminuiu em relação ao controle negativo em todos os tempos de incubação e concentrações do FS. A média de UFC/ml para todos as concentrações e tempo de incubação do grupo A (TFD) foi estatisticamente menor em relação ao grupo B que utilizou a ClAlPc/NE isolado. Desta forma, a TFD antimicrobiana com ClAlPc/NE foi efetiva.

O maior efeito fotodinâmico com comprovação estatística foi apresentado na concentração 20 µU, do grupo de TFD, para um tempo de incubação de 10 minutos, sendo assim, o melhor custo-benefício para terapia.

Quando se comparou a TFD antimicrobiana (ClAlPc/NE) com o controle positivo representado pelo fármaco Nistatina (suspensão), já utilizado na comunidade científica, verificou-se que a média do primeiro foi estatisticamente menor em relação ao segundo.

Alguns estudos verificaram a eficiência da TDF antimicrobiana em Candida *albicans* utilizando ftalocianinas com metais, porém não houve efetividade quando apresentada sem NDDS[23,42].

A susceptibilidade de microrganismos fúngicos para a terapia fotodinâmica tem sido demonstrada in vitro e in vivo. No entanto, não há evidências conclusivas para confirmar um protocolo claro e preciso para a destruição fotodinâmica de fungos patogênicos. É necessário mais estudos [28,53–58].

Pode-se dizer que a TFD antimicrobiana foi eficiente na redução de UFC/ml de espécie de Candida *albicans* e o melhor custo-benefício para a terapia foi usando o tempo de 10 minutos e concentração de 20μU do ClAlPc/NE.

Figuras

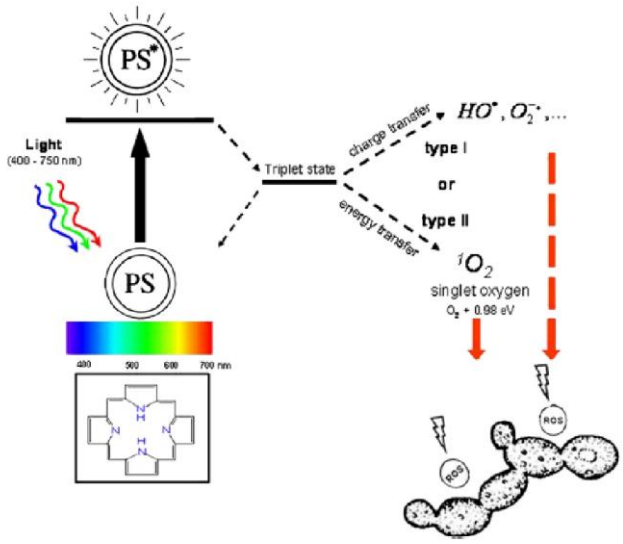

Figura 1 - Representação esquemática da reação fotodinâmica e terapia fotodinâmica.

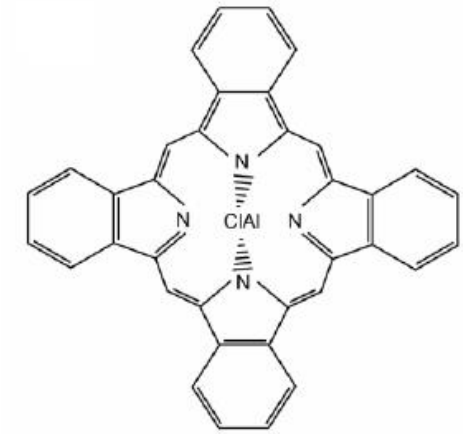

Figura 2 - Estrutura química da ftalocianina de cloroalumínio.

Quadros

Quadro 1: Representação das concentrações e tempos de incubação para os grupos grupos A e B

TERAPIA FOTODINÂMICA LASER InGaAl (660 nm; 100 mW; 320 J/cm^2; 70s)			
Grupo A	Fotossensibilizador		Incubação com ClAlPc/NE
	Concentração ClAlPc/NE	Subgrupos	Tempo
	5 µU	TFD – A	1 min.
			5 min.
			10 min.
			15 min.
	10 µU	TFD – B	1 min.
			5 min.
			10 min.
			15 min.
	20 µU	TFD – C	1 min.
			5 min.
			10 min.
			15 min.
	40µU	TFD - D	1 min.
			5 min.
			10 min.
			15 min.
LASER DE BAIXA POTÊNCIA NÃO SERÁ APLICADO			
Grupo B	Fotossensibilizador		Incubação com CLALPC/NE
	Concentração CLALPC/NE	Subgrupos	Tempo
	5 µU	ClAlPc/NE– A	1 min.
			5 min.
			10 min.
			15 min.
	10 µU	ClAlPc/NE – B	1 min.
			5 min.
			10 min.
			15 min.
	20 µU	ClAlPc/NE – C	1 min.
			5 min.
			10 min.
			15 min.
	40µU	ClAlPc/NE – D	1 min.
			5 min.
			10 min.
			15 min.

Quadro 2: Representação das concentrações e tempos de incubação das substâncias do eontrole negativo e do positivo

PROTOCOLO DO LASER: Não será aplicado		
Grupo C	Solução salina	Incubação com solução salina
	Concentração	Tempo
	0,90%	1 min.
		5 min.
		10 min.
		15 min.
PROTOCOLO DO LASER: Não será aplicado		
Grupo D	Nistatina	Incubação Nistatina
	Concentração	Tempo
	100.000 U.I/ml	1
	100.000 U.I/ml	5
	100.000 U.I/ml	10
	100.000 U.I/ml	15

Tabelas

Tabela 1: Valores de UFC/ml de espécie de Candida *albicans* para os grupos A, B e C,D.

Concentração da ClAlPc/NE (µU)	**Tempo de incubação (minutos)**	**Grupo A (UFC/mL em log10)**	**Grupo B UFC/mL em log10)**
5	1	4,7782	5,0792
	5	5,0000	5,3802
	10	4,8451	5,0000
	15	5,0792	5,2304
10	1	4,8451	5,0414
	5	5,0792	5,2304
	10	4,6021	5,1761
	15	4,9031	5,0792
20	1	4,8451	4,9542
	5	4,4771	5,0000
	10	4,6021	4,9031
	15	4,6021	4,8451
40	1	4,9031	5,0000
	5	5,0000	5,0000
	10	4,6990	4,9031
	15	4,6990	4,8451
Grupo C - Concentração da Solução Salina	**Tempo de incubação (minutos)**	**(UFC/mL em log_{10})**	
0,90%	1	5,6532	
	5	5,6628	
	10	5,6721	
	15	5,6435	
Grupo D - Concentração da Nistatina	**Tempo de incubação (minutos)**	**(UFC/mL em log_{10})**	
100000 U.I/mL	1	5,0000	
	5	5,0414	
	10	4,9542	
	15	5,0792	

Tabela 2: Médias da fração de sobrevivência dos grupos A e B e comparações em relação ao tempo e concentração.

Tempo	**Médias**		**Valor-p**
	Grupo A	**Grupo B**	
1	0,8567	0,8878	0,00319
5	0,8648	0,9115	0,16570
10	0,8291	0,8837	0,01169
15	0,8528	0,8844	0,25540
Concentração	-	-	-
5 µU	0,8713	0,9150	0,06385
10 µU	0,8592	0,9078	0,04364
20 µU	0,8193	0,8713	0,01278
40 µU	0,8535	0,8733	0,23490
Média Geral	0,8501	0,8911	0,00048

Tabela 3: Médias de UFC/ml dos grupos A e B em comparação ao controle positivo

Controle positivo	**Médias de UFC/ml**	**Valor-p**
Grupo A	0,9584	0,000124
Grupo B	1,0046	0,563

Gráficos:

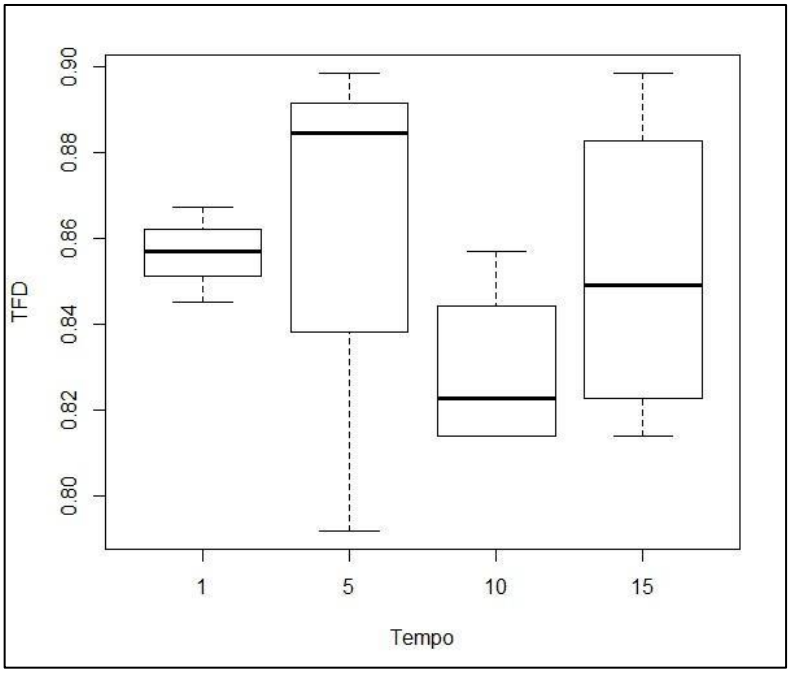

Gráfico 1: Resultados da fração de sobrevivência do grupo A relacionados ao tempo.

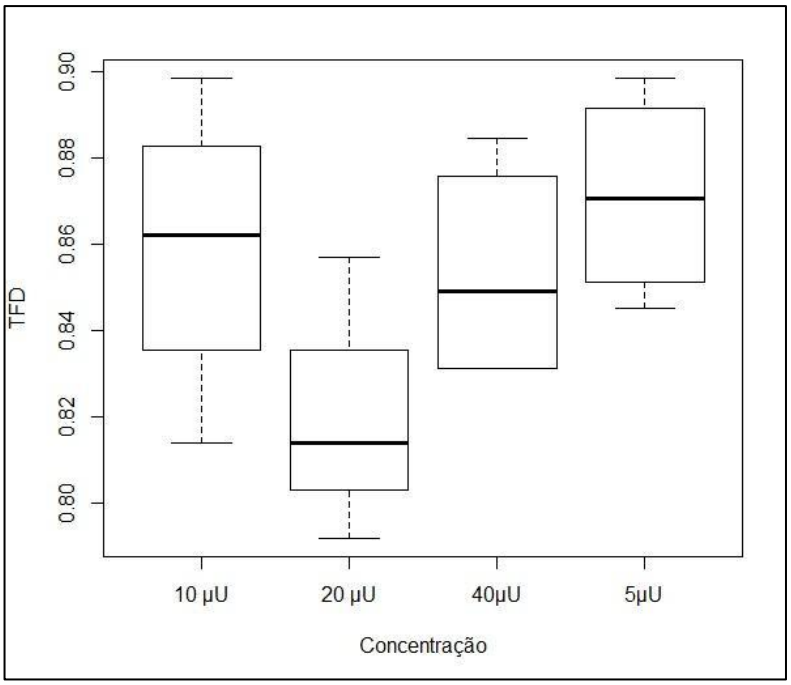

Gráfico 2: Resultados da fração de sobrevivência do grupo A relacionados à concentração.

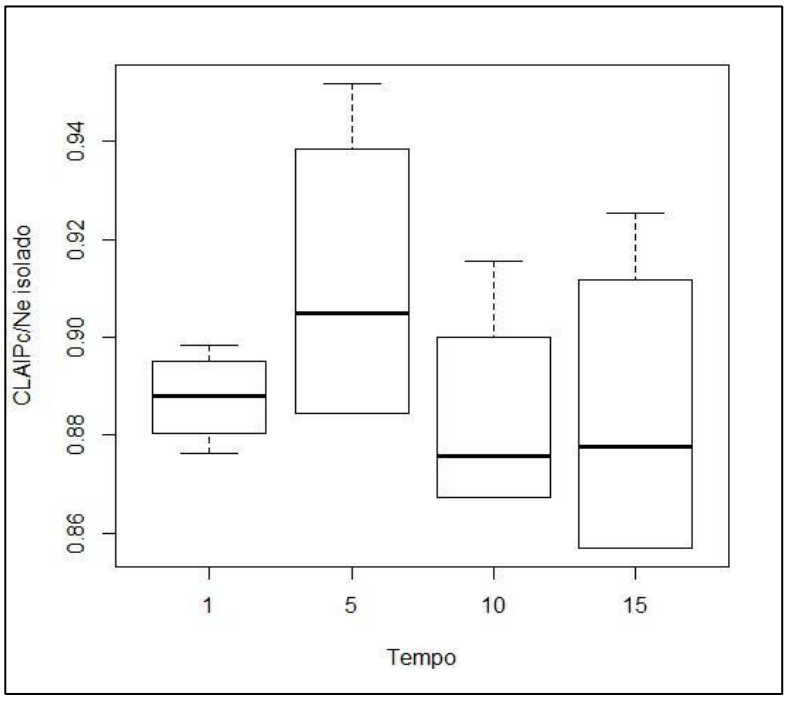

Gráfico 3: Resultados da fração de sobrevivência do grupo B relacionados à variável tempo.

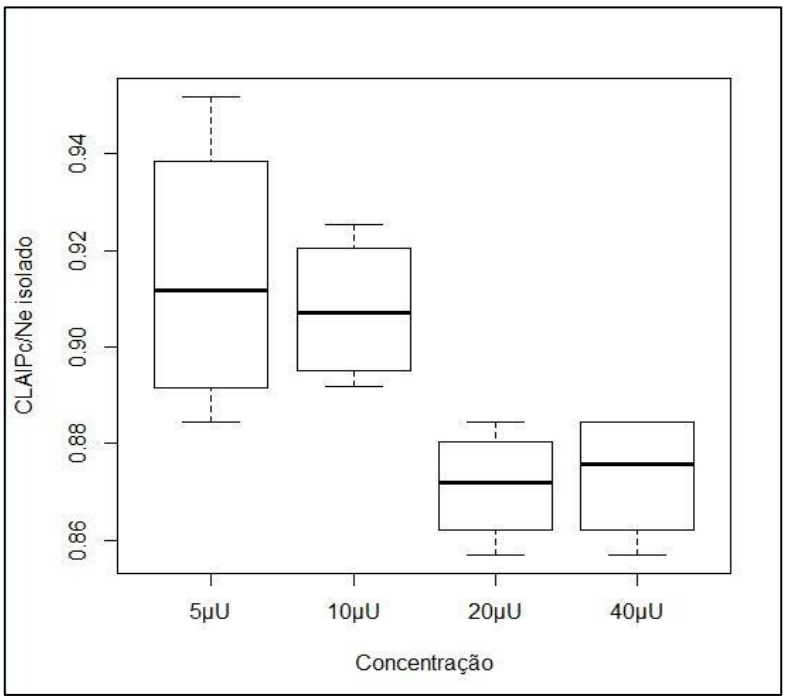

Gráfico 4: Resultados da fração de sobrevivência do grupo B relacionados à variável concentração.

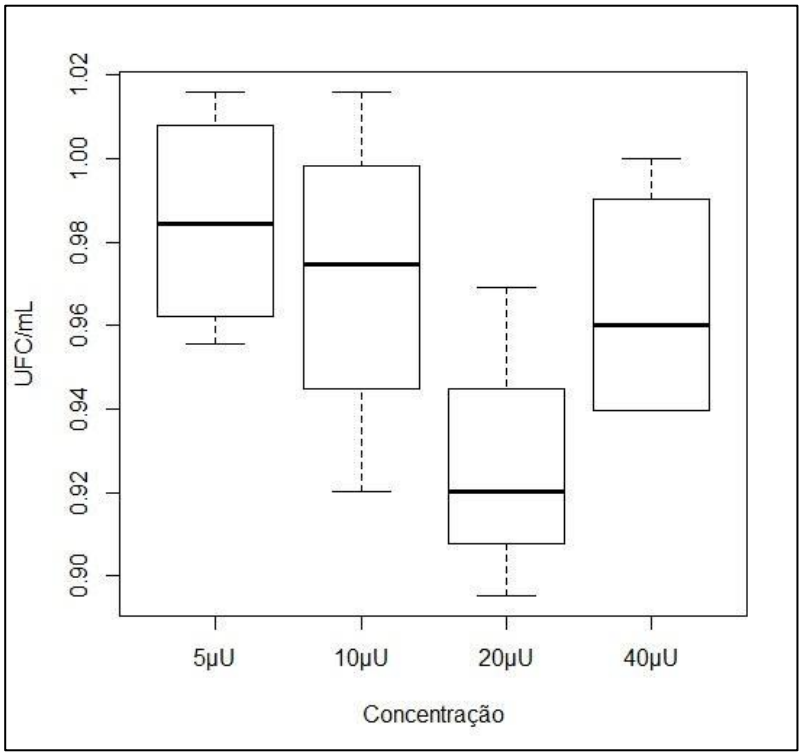

Gráfico 5: Resultados para variável tempo de incubação, segundo o grupo A comparando ao grupo controle positivo

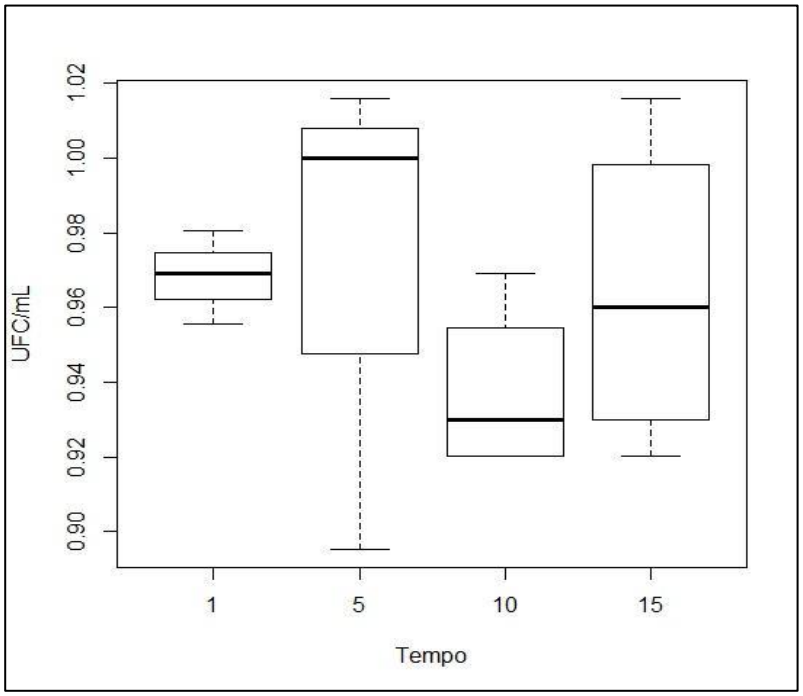

Gráfico 6: Resultados do grupo A comparando do grupo controle positivo pela variável concentração.

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Apêndices

ARTIGO CIENTÍFICO 1

Terapia fotodinâmica antimicrobiana utilizando ftalocianina de cloroalumínio em nanoemulsão em espécie de Candida *albicans*

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1. **APÊNDICE**

PRODUÇÃO DISCENTE

ARTIGO CIENTÍFICO 2

Original Research

Effect of low-level laser therapy on wounds in intra-oral surgery

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Abstract: During the last two decades, much has been written in both the scientific literature and the popular press about lasers and their use in dentistry. Both soft- and hard-tissue applications have been discussed, including frenectomy, gingival contouring, caries removal and bleaching. The aim of this study is evaluate and compare the effects of laser therapy on the surgical wound repair process and painful symptoms in patients undergoing bilateral teeth extractions at the Department of Dentistry, State University of Paraiba. Three sessions of laser therapy (GaAlAs) were performed for each patient. The first session was immediately after the surgical procedure, the second occurred 48-72h after the first session, and the third and final at 7 days after surgery, coinciding with the removal of the sutures. Of the patients examined, 65.5% at the first assessment (after the second laser therapy session) and 75% at the second assessment (after the third session) exhibited a better degree of repair on the side treated with laser (STL) compared to the side not treated with laser (SNTL). There was no difference in edema between the STL and SNTL. Regarding pink tissue colour, there was an increase in its frequency, from 25% to 62.5% of cases, on the STL between the first and second assessments. Bleeding also showed an increased frequency on the STL and decreased on the SNTL in the second assessment. Regarding painful symptoms, the results revealed that, on the STL, they decreased, from 62.5% to 0% between the first and second assessments, and on the SNTL, from 87.5% to 12.5%. Hence, low-power laser use appeared to be effective for the repair of intraoral surgical wounds, accelerating the degree of repair, improving tissue colour and reducing pain symptoms.

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Introduction

Undoubtedly, one of the great advances in medicine in the twentieth century was the development of laser devices [1-4]. Laser radiation has been used in surgical procedures with the objective of increasing surgical benefits and improving clinical outcome [5,6]. It has some advantages such as disinfection of the operative field, the absence of vibration, the vaporisation of lesions, tissue destruction accuracy, minimal damage to adjacent tissues, a haemostatic effect, anti-inflammatory and biostimulation properties, reduced pain and edema, and greater comfort for patients [7,8-10]. Lasers commonly used in dentistry have gallium arsenide and aluminium (GaAlAs) as the active medium, with wavelengths ranging from 790 and 850 nm, outside the visible range of the light spectrum (infrared) and aluminium-gallium-indium phosphate (InGaAIP), with a shorter wavelength, within the visible range (red) [11-14]. The desired therapeutic effects on irradiated tissues are obtained when there is absorption of the laser beam.

Low-power lasers have analgesic effects, stimulating the release of endorphins; inhibiting nociceptor signs; and reducing painful symptoms, antiinflammatory effects, edema and hyperemia [3,5,9,11,13]. They also have biostimulation properties, accelerating wound healing, stimulating bone repair and remodelling, recovering neural function after injury and modulating immune system cells, promoting repair [3,5,10,15,16]. The biological effects (analgesic, anti-inflammatory and biostimulant) that lowpower lasers have on tissues are primarily due to light energy, which is absorbed and turned into primary (direct), secondary (indirect), and general therapeutic effects. These effects control the production of substances released during pain and inflammation, such as prostaglandins, prostacyclins, histamine, serotonin, bradykinin, and leukotrienes [13,17-20]. Furthermore, they modify normal enzymatic reactions, excitation and inhibition, as well as ATP production and the synthesis of prostaglandins. Of the many phenomena that characterise the living organism, wound healing is one of the most interesting. Low-power laser treatment has been effective for wound healing, accelerating the physiological repair process and reducing pain [13,21-23]. This type of treatment is still little known by many professionals, but it is a promising new tool.

Laser therapy has been assessed over the last 10 years, being the focus of numerous studies [1,2,5,9,20,23-25]. The use of laser in the tissue repair process was first studied by Mester et al. (1971) and since then the concern of researchers has been to evaluate the biostimulation effect of the laser beam on the tissue repair process [4,26]. The biological effects of laser radiation on tissues occur in different ways: specifically, by inducing mitotic activity of epithelial and endothelial cells, modifying the capillary density, stimulating the local microcirculation and, especially, increasing collagen synthesis *in vitro* or *in vivo* [3,5,11,17]. Statistical data on the clinical effects of laser radiation on humans are still very

scarce and its cost-benefit ratio requires confirmation before laser therapy use can be extended to the general population [6,12].

Extractions of teeth are invasive surgical procedures that cause postoperative discomfort to patients, often disabling their social life at least temporarily. However, a large number of patients need to undergo extractions[21-13]. Most of these patients are workers and need biopsychosocial balance to continue to accomplish their activities. Thus, minimizing postoperative problems is important for a faster reintegration of the patient into society and a fast return to normal activities. In this context, we evaluated the efficacy of laser therapy in the repair process of intra-oral surgical wounds, including the following variables: degree of repair or closure of wounds, edema, bleeding, erythema, and pain symptoms.

Materials and Methods

Study protocol

According to Resolution 196/96, this experiment was duly registered with the National System of Research Ethics - SISNEP: 0240.0.133.000-07 and submitted to the Ethics Research Committee of the State University of Paraíba (UEPB), Campina Grande, Brazil. This was an experimental study using a laser device and was a blinded *in vivo*, prospective, descriptive, intervention study, with a clinical randomised, controlled, split-mouth type trial. The study universe consisted of all patients between 18 and 64 years old, followed at the clinics of the Department of Dentistry, UEPB, with indications for extractions from July 2008 to May 2009. The sample was composed of eight patients undergoing bilateral extractions, who met the following inclusion criteria: indications for bilateral extractions of teeth from the same group and same surgical etiology, who underwent drug therapy based only on analgesics (dipyrone), and who signed the informed consent form. Patients with systemic diseases and

conditions that could affect the repair process, allergies to the selected medication, and those requiring antibiotic therapy were excluded.

Experimental model

The laser application was performed in a timely manner in three regions of the edges of surgical wounds: central, mesial, and distal. The laser used was an infrared type, at a wavelength of 808 nm, and a dose of 100 J/cm², adopting the area of the laser pointer for fluency calculation, with gallium arsenide and aluminium (GaAlAs) as the active medium. In each session, three applications of 22s and 2.2J of energy were performed (Table 1). Each patient underwent three sessions of laser therapy: the first was performed on the day of surgery, soon after it. The second occurred 48–72 h after the first session and the third and final occurred 7 days after surgery, coinciding with the removal of the sutures. The side of laser application was randomly chosen by the operator and called the STL, while the opposite side, which was not treated with the laser was called the SNTL.

Low-level laser therapy analysis

Pain symptoms were analysed using a questionnaire that asked the patient about the presence or absence of pain in the STL and SNTL after the second and third sessions of laser treatment. Assessments of surgical wounds, including the degree of repair or closure of wounds, edema (absent or present), erythema (reddish/bluish tissue colouration), and bleeding (absent or present in mild, moderate, or severe form) were performed by an examiner (blinded) using digital photographs taken by the laser equipment operator at the end of the last two sessions of laser therapy. Only the degree of repair or closure of wounds and edema, when present, were evaluated comparatively between the test (STL) and control (SNTL), being classified as superior or inferior to the other side.

Statistical analysis

For decision-making, the Mann-Whitney U-test for independent samples and the Wilcoxon test for dependent or paired samples were used, adopting a significance level of 5%. Data were analysed using the SPSS software (ver. 13.0).

Results

In the first evaluation, performed after the second session of laser therapy (48– 72h after surgery), edema was found in 50% of the wounds on the STL, which was lower than that on the SNTL. In addition, of the wounds on the STL (SNTL), 37.5% (12.5%) were not associated with painful symptoms and 37.5% (50%) had bleeding. Moreover, the degree of repair or closure of wounds was higher in the laser-treated group than in the control in 62.5% of cases (Figures 1, 2). Figure 2B showing points at which the laser was applied. Erythema (reddish/bluish colour) was more evident in the untreated group, corresponding to 87.5% of cases. However, when the Mann-Whitney U-test was applied at a significance level of 5%, there was no statistically significant difference in pain ($p = 0.264$), bleeding ($p = 0.626$), or erythema ($p = 0.535$) between the groups 48–72h after the surgical procedure (Table 2, 3).

In the second evaluation, performed after the third session of laser therapy (7 days after surgery), all patients in both groups (100%) showed edema. However, 7 days after surgery, there was less edema on the treated than on the untreated side in 75% of the wounds. None of the wounds of the laser-treated group showed pain symptoms, while 12.5% of those of controls did. Bleeding was more common in the test group, present in the majority (62.5%) of cases, while only 25% of the untreated group showed bleeding. There was greater repair on the treated than on the untreated side in 75% of the wounds (Figures 3, 4). Regarding erythema, the majority (62.5%) of wounds exhibited pink tissue colouration in the test group,

while in the control group, half (50%) of the cases exhibited a pink colour and the other half had a reddish/bluish colour. Applying the Mann-Whitney U-test at a significance level of 5%, there was also no statistically significant difference in pain ($p = 0.317$), bleeding ($p = 0.143$), or tissue colouration ($p = 0.626$) between the groups 7 days after surgery (Table 2, 3). A slight increase in the degree of repair or closure of wounds in the laser-treated group compared to the untreated group was observed, because in the first evaluation, 62.5% of wounds in the test group showed a higher degree of repair than the control group; this percentage increased to 75% in the second evaluation. Between 48 and 72h after surgery, only 12.5% of wounds showed no edema; however, at postoperative day 7, all wounds showed some degree of edema. It was also observed in the first assessment that 25% of the edema cases were in the laser-treated group than in the untreated group, while in the second evaluation, only 12.5% of edema cases were in the test group. Also, in the second evaluation, 75% of edema on the side treated with the laser were inferior to those on the side not treated with the laser and this value was 50% in the first assessment. However, applying the Wilcoxon test at a 5% significance level, there was no statistically significant difference in the degree of surgical wound repair ($p = 0.317$) or edema intensity ($p = 0.334$) between the two evaluation periods.

Discussion

GaAlAs laser therapy showed effectiveness in tissue repair of surgical wounds in the first evaluation (48–72h after bilateral extractions), showing superiority over wounds not subjected to laser therapy in 62.5% of cases. In the second evaluation (at day 7 after surgery), the comparative frequency of the repair of surgical wounds increased to 75% of cases, indicating that low-power laser treatment seemed to improve the healing of surgical wounds

when used immediately after surgery, and 48–72 h and 7 days after surgery. However, we found no statistical evidence that the degree of wound repair differed significantly between the assessment periods. Patients who exhibited edema in the regions of surgical wounds were submitted to a comparative analysis of the intensity of this variable on both sides. However, the degree of edema did not differ between the groups at the first assessment, although it showed improvement in the treated group but not the untreated group at the second evaluation. This suggests that low-power laser treatment improved edema, but this result was not statistically significant. In the first assessment, erythema was rosy (compatible with a normal state) in 25% of wounds in the treated group and in 13.5% of wounds in the untreated group.

In the second evaluation, the values were 62.5% and 50%, respectively, reinforcing the results regarding the efficacy of low-power laser in improving tissue colouration, as reported previously [3-7,11-13,17]. However, as untreated wounds also showed improvement, it would appear that the repair process was unrelated to laser treatment. The presence of bleeding was found in only 37.5% of wounds in the treated group and in 50% of wounds in the untreated group at first assessment. However, at the second assessment, the wounds of the test group showed increased bleeding in 62.5% of cases, while those of the control group showed less bleeding in 25% of cases. These results may be explained by the low-power laser stimulating local microcirculation [5].

Patients with the presence of bleeding on either side were also tested to assess the intensity of this variable, which at first assessment was mild in 100% of cases in the treated group and in 75% of cases in the untreated group; the rest of the sample (25%) showed moderate bleeding. In the second evaluation, the test group showed mild and moderate bleeding intensity in 33.3% and 67.7% of cases, respectively, which may also be explained by

the laser activating local microcirculation, while in the wounds of the control group, bleeding was mild in 50% of cases and intense in the other 50%. Low-power lasers exhibit analgesic effects, because they stimulate the release of endorphins and inhibit nociceptor signs, thus reducing painful symptoms [3,6,7,11-13, 17]. In the present study, 37.5% (12.5%) of patients reported no pain in treated (untreated) wounds at first assessment, whereas 100% (87.5%) of cases reported no pain at second assessment, consistent with other reports [9,10,18]. Based on previous literature and the results of the present study, laser therapy is an important method for the rehabilitation of the maxillofacial complex and healing of wounds in soft tissues [2-6,8-12,17,18,25]. Furthermore, low-power laser seems to help control pain [10]. However, there is as yet no consensus on the ideal laser protocol for use in oral surgery.

Conclusions

Low-power laser irradiation has a positive effect on the repair of intraoral surgical wounds, accelerating the degree of repair, improving erythema, and reducing painful symptoms. However, further studies are needed to confirm its clinical efficacy.

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FIGURE LEGENDS:

Fig 1. Degree of wound repair - SNTL - 48h after surgery
Fig 2. Degree of wound repair - STL - 48h after surgery
Fig2B. Points of application of laser in STL
Fig 3. Degree of wound repair - SNTL - 7 days after surgery
Fig 4. Degree of wound repair - STL - 7 days after surgery

TABLE LEGENDS:

Table 1. Specifications for Laser Parameters
Table 2. Statement of the results in first avaliantion, in %.
Table 3. Statement of the results in second avaliantion, in %.

Device information	
Manufacturer	DMC
Model identifier	Whitening Lase II
Emitter type	GaAlAs
Irradiation parameters	
Center wavelength (nm)	808 nm
Operating mode	Continuous
Pulse on duration (sec)	60 ns
Beam shape	Circular
Treatment parameters	
Beam spot size at target (cm^2)	1 mm^2
Exposure duration (sec)	22 sec

Radiant exposure (J/cm^2)	100 J/cm^2
Radiant energy (J)	2,2 J
Number of points irradiated	3
Area irradiated (cm^2)	1 cm^2
Application technique	Without skin contact
Number and frequency of treatment sessions	3 sessions, performed in 1 week
Total radiant energy (J)	18 J per session, 54 J over all sessions

Table 1. Specifications for Laser Parameters

1st Avaliation	**Edema**	**Absence of pain**	**Positive bleeding**	**Tissue repair**	**Erythema**
Laser	50	37,5	37,5	62,5	13,5
No Laser	50	12,5	50	37,5	87,5

Table 2. Statement of the results in first avaliantion, in %.

2nd Avaliation	**Edema**	**Absence of pain**	**Positive bleeding**	**Tissue repair**	**Erythema**
Laser	25	100	62,5	75	37,5
No Laser	75	12,5	25	25	50

Table 3. Statement of the results in second avaliantion, in %.

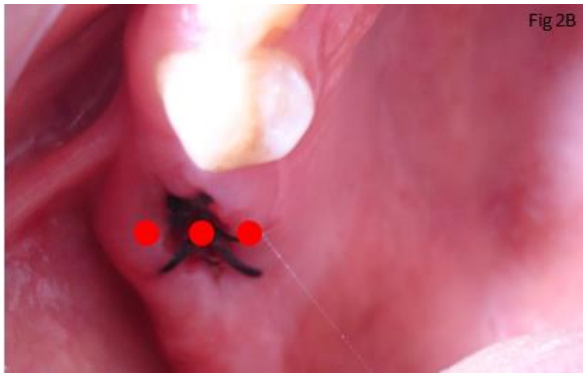
Fig 2B

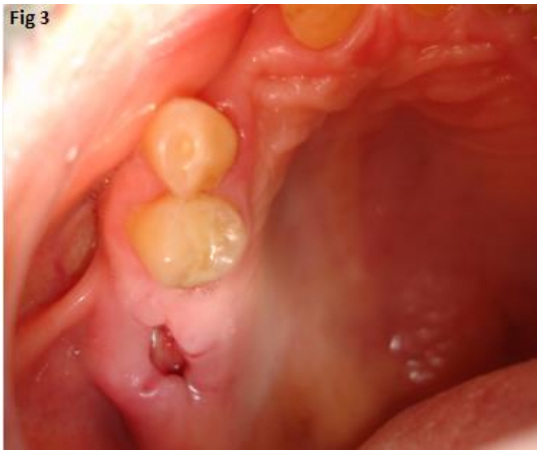
Fig 3

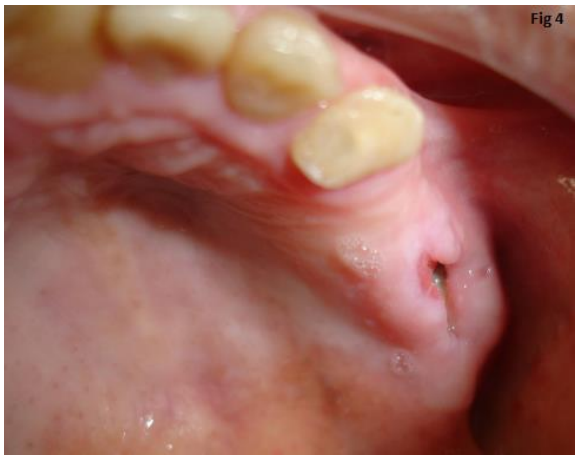
Fig 4

ARTIGO CIENTÍFICO 3

Incorporation of Rosemary (*Rosmarinus officinalis* Linn.) extract in an endodontic sealer: Antimicrobial evaluation

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ABSTRACT

The aim of this study is to evaluate the microbial activity of an endodontic sealer containing rosemary extract in comparison to Sealer 26®.Agar well diffusion assays were used to evaluate the antimicrobial activity of the materials in the presence of the following bacterial strains: *S. mutans* (ATCC 25175), *S. aureus* (ATCC 9811) and *E. faecalis* (ATCC 51299). 100 µl of suspension with each inoculum were placed on glass dishes containing 10 ml of culture medium and homogenized with the aid of swabs. Wells were made in each dish and filled with the endodontic sealers. The dishes were incubated in a bacteriological chamber at 37 ºC for 48 and 24 hours, respectively. Bacterial growth inhibition halos were measured. The Mann-Whitney test was used to

determine the statistical significance of differences between groups. Mean inhibition halos were 27.1, 26.4 and 38.6 mm for the strains of *S. aureus*, *E. faecalis* and *S. mutans* when the rosemary-based sealer was used and 16.3, 15.8 and 25.5 mm, respectively, when Sealer 26® was used. Significant differences between sealers were found for each bacterial strain ($p < 0.05$). The rosemary-based endodontic sealer demonstrated greater antimicrobial efficacy in comparison to Sealer 26®.

Descriptors: *Rosmarinus officinalis;* Root canal obturation; *Staphylococcus aureus*; *Streptococcus mutans*; *Enterococcus faecalis*

INTRODUCTION

Microorganisms and their byproducts are considered the main agents of endodontic disease. Thus, the major goal of endodontic treatment is the destruction of these microorganisms in the root canal system [1]. The success of endodontic treatment depends on effective performance in all phases. While no particular phase is considered the most important, special care must be given to obturation, which involves the use of a solid material (gutta-percha) and a sealer [2].

Microorganisms can cause persistent and secondary infections of root canal systems. *Enterococcus faecalis* is an anaerobic, facultative bacterium related to the failure of root canal treatment as well as pulp necrosis and periapical lesions. Although the genus *Enterococcus* is not present in considerable amounts in the initial flora of untreated root canals, once stabilized, it becomes viable and resistant to antimicrobial treatment and medicinal interactions, persisting after the obturation [3]. *Staphylococcus aureus* is a spherical (coccus), Gram-positive bacterium that appears in pairs in short chains or bunches upon microscopic

examination [4]. It is one of the main agents of hospital-acquired infection and the main cause of surgical infection throughout the world [5]. This bacterium is considered one of the most resistant species and a possible cause of the failure of root canal treatment [6]. The mutans streptococci are a group of heterogeneous cocci that make up part of the resident microbiota in the oral cavity. Many are infectious and considered the main etiologic agent of dental caries as well as important contributors to infectious endocarditis [7].

Endodontic sealers perform a special function in filling root canals. These products have inherent antimicrobial activity, which contributes to the control of the microbial population. In typical clinical situations, the complete elimination of bacteria from root canal system appears to be impossible. However, the use of sealers with antibacterial properties helps reduce the number of microorganisms and contributes to the avoidance of infection [8]. Thus, the incorporation of antimicrobial agents in endodontic sealers can enhance these desirable properties [9].

The current trend is the development of endodontic sealers that maintain and/or enhance the properties of traditional fillers and are more biocompatible [10]. In principle, it is possible to improve the properties of these materials through the addition of antimicrobial agents. A number of such substances have been employed in dentistry in the form of mouthwashes, restorative materials and toothpastes [11].

There is an ongoing search for the development of an endodontic sealer that meets all the requirements to be considered ideal. Many researchers have investigated the use of plant extracts and phytochemicals with antimicrobial properties. In recent years, studies have demonstrated the effectiveness of these extracts, which mainly stems from the antimicrobial action of their secondary metabolites [12]. One such plant, rosemary (*Rosmarinus officinalis* Linn.), has been described to have a number of medicinal properties, which justifies its

traditional use in folk remedies. The plants contain essential oil, flavonoids, phenols and terpenoids, which have antioxidant and antimicrobial properties [13].

Considering the need for a biocompatible endodontic sealer that forms a physical barrier and demonstrates therapeutic efficacy against microorganisms associated with endodontic infection, the aim of the present study was to evaluate the microbial activity of an endodontic sealer containing rosemary extract in comparison to Sealer 26® (Dentsply Maillefer, USA), which is widely used in dentistry.

MATERIALS AND METHODS

Production of rosemary-based endodontic sealer

Rosmarinus officinallis Linn. was acquired and identified through comparisons with material deposited in the herbarium of the Pernambuco Institute of Agricultural Research (Brazil). The material was washed and dried at room temperature. Leaves and stems were weighed (320 g) and macerated in 1 L of ethanol for 30 days under refrigeration. The extract was filtered and placed in a rotary evaporator at a temperature of 50^{o}C. The dried extract was divided into portions for the microbiological, toxicological and phytochemical tests and kept under refrigeration between tests. A sealer similar to Sealer 26® (Dentsply Maillefer, USA) was mixed at a compounding pharmacy (Sensoriale® Manipulação Farmacêutica e Cosmética, Recife, Brazil). The blended sealer did not contain calcium hydroxide, which was replaced with the rosemary extract at the minimum inhibitory concentration (MIC) for the bacteria tested. The MIC of the extract for each bacterium was tested in a previous experiment [14] (Chart 1). As three different microorganisms were tested, the largest MIC was used while respecting the LD_{50} for this phytotherapeutic substance. The sealer produced exhibited similar

physical and organoleptic characteristics to Sealer 26® and was compounded in the same way, following the manufacturer's instructions.

Antimicrobial evaluation of rosemary-based endodontic sealer

Agar well diffusion assays [15] were performed for the evaluation of the antimicrobial activity of the rosemary-based endodontic sealer in the presence of *S. mutans* (ATCC 25175), *S. aureus* (ATCC 9811) and *E. faecalis* (ATCC 51299). The bacterial strains were acquired from the Microbial Collection Laboratory of the Department of Antibiotics of the Federal University of Pernambuco (Brazil) and reactivated in test tubes containing Brain Heart Infusion (BHI®, DIFCO, USA). The strains were inoculated on glass dishes containing 10 ml of BHI® agar medium. The growth conditions of each bacterium were respected: aerobiotic conditions for 24 h for *S. aureus* and *E. faecalis* and anaerobiotic conditions for 48 h for *S. mutans* in a bacteriological chamber at 37 ºC. Colonies of each inoculum were diluted in test tubes with sterile water and placed in a vortex mixer for one minute to achieve turbidity similar to tube n^0 2 of the McFarland Scale®.

100µl of homogenized suspension were inoculated on sterile glass dishes containing 10 ml of BHI® medium and homogenized with the aid of sterile swabs so that the entire medium was inoculated. Wells measuring 10 mm in diameter were made with a perforator in the center of each plate and filled with the test sealer and Sealer 26® as the positive control – both blended following the manufacturer's instructions. An inoculated dish with a well filled with sterile gelose of the medium was used as the negative control. The dishes were incubated in accordance with the requirements of each microorganism. All assays were conducted in triplicate. Following incubation, the bacterial growth inhibition halos were measured with the aid of halo meter and the results were expressed as mean values in millimeters.

The normality test demonstrated the need for a nonparametric test, as no variables exhibited normal distribution. The Mann-Whitney test was used to determine the statistical significance of differences between the materials ($p < 0.05$).

RESULTS

Mean inhibition halos were 27.1, 26.4 and 38.6 mm for the strains of *S. aureus*, *E. faecalis* and *S. mutans* when the rosemary-based sealer was used and 16.3, 15.8 and 25.5 mm, respectively, when Sealer 26® was used. Significant differences between sealers were found for each bacterial strain ($p < 0.05$) (Table 1).

DISCUSSION

There has been growing interest in research into the anaerobic microorganisms that infect root canal systems, especially with regard to long-term infections [1,9,16]. Anaerobic bacteria are well adapted to survive in necrotic pulp tissue, where the blood supply is either limited or nonexistent. Facultative anaerobic microorganisms can interact with strict anaerobic microorganisms, leading to changes in the nutritional relationship and oxygen tension-reduction, which favor microbial bonding and survival [9]. Thus, the antimicrobial action of endodontic sealers may participate in the control of infection.

The ideal endodontic sealer should exhibit adequate antimicrobial activity, provide a hermetic seal and offer a low degree of toxicity to biological tissues. Antimicrobial substances can be added to enhance the properties of endodontic sealers [9,17]. Gjorgievska *et al* [11] evaluated the effect of the addition of benzalkonium chloride and cetylperydinium chloride to endodontic sealers on strains of *S. mutans*, *L. casei* and *A. viscosus* and found an increase in the antimicrobial activity of all sealers analyzed, demonstrating that these substances have potential clinical applications in root canal treatment. However, further studies are needed to

evaluate whether the addition of such substances can compromise the physicochemical characteristics of endodontic sealers.

In the present study, Sealer 26® was used for the purposes of comparison to the rosemary-based sealer. Sealer 26® is composed of bismuth oxide, calcium hydroxide and epoxy resin and is known for its excellent sealing property and satisfactory biocompatibility [18]. A number of studies have evaluated the antimicrobial activity of Sealer 26® in the presence of bacterial strains or microorganisms of the oral cavity [2,3,18].

In the present study, the rosemary-based endodontic sealer demonstrated greater inhibition halos in comparison to Sealer 26®. Gomes *et al* [2] also found lesser antimicrobial activity with Sealer 26® in comparison to Endo-fill®, Endomethasone®, Endomethasone N® and AH-Plus®, but the differences did not achieve statistical significance. Cruz *et al* [19] and Kooper *et al* [20] found smaller inhibition halos with Sealer 26® for all microorganisms tested in comparison to Rickert®/N-Rickert® and Endo-Fill®. However, Kooper *et al* [20] found a larger inhibition halo for *S. aureus* using Sealer 26®, which is in agreement with findings described by Leonardi *et al* [21], who compared this substance to Endo-fill®, AH-Plus® and Acroseal® with regard to the same bacterium. Tanomaru-Filho *et al* [18] also report the adequate performance of Sealer 26®. Maia *et al.* [3] report the antimicrobial action of this product in the presence *E. faecalis*. However, this bacterium is known to be resistant to calcium hydroxide [22]. Thus, the action of Sealer 26® on *E. faecalis* may be due to the release of another substance, such as formaldehyde, which occurs after the mixture of the other components of the product [23].

Aal-Saraj *et al* [16] evaluated the antimicrobial activity of a novel endodontic sealer containing nano-hydroxyapatite epoxy resin (nanoseal) in comparison to the commercial products AH 26®, Tubliseal®, Sealapex® and Roekoseal® in the presence of the facultative

anaerobic bacteria *E. faecalis, P. aeruginosa*, *S. mutans, S. sobrinus* and *E. coli*. The authors found that the nanoseal had better antimicrobial action in comparison to Roekoseal®, similar action to AH 26® and lesser action in comparison to Tubliseal® and Sealapex® in the presence of *E. faecalis, P. aeruginosa* and *E. coli* as well as greater action in comparison to Sealapex® and Roekoseal®, similar action to AH 26® and lesser action in comparison to Tubliseal® in the presence of *S. mutans* and *S. sobrinus*.

In the present study, agar well diffusion was used to test antimicrobial activity. This is one of the most widely used methods for this purpose [2,24,25]. However, the size of the inhibition zone does not indicate the absolute antimicrobial effect of an endodontic sealer. According to Bodrumlu & Semiz [24], the inhibition zone can be affected by the diffusibility of the sealer through the agar, the interaction between the sealer and components of the medium and the *in vivo* micro-environmental conditions.

The mean inhibition halos for *S. aureus, E. faecalis* and *S. mutans* were significantly larger with the rosemary-based endodontic sealer in comparison to Sealer 26®. This finding may be explained by the fact that rosemary has anti-inflammatory, antioxidant and antimicrobial properties [13]. This plant is made up of essential oil, flavonoids, phenols and terpenoids. Klancnik *et al* [26] report greater sensitivity to rosemary extract among Gram-positive bacteria in comparison to Gram-negative bacteria.

The essential oil of rosemary is considered to have the greatest antimicrobial action. Bernardes *et al* [27] analyzed the effect of rosemary extract on *E. faecalis, S. salivarius, S. sanguinis, S. mitis, S. mutans* and *S. sobrinus*. The authors also performed both biomonitored fractioning and a chromatographic analysis of the extract to identify the main components, attributing the antimicrobial activity mainly to carnosic acid and carnosol. Moghtader &

Afzali [28] report that the essential oil of rosemary has antibacterial, antinociceptive and antifungal properties.

Hofling *et al* [29] found strong antifungal activity of rosemary extract on some species of the genus Candida. Fungal adherence to biomaterials used in medicine, such as catheters, valves and implants, was the object of a study by Chifiriuc *et al* [30], who combined the unique properties of nanoparticles with the antimicrobial activity of the essential oil from rosemary to create a nanobiological system that could be used to cover the surface of these materials and impede both microbial colonization and the development of biofilm. The authors found that the essential oil fortified with nanoparticles was able to inhibit and control fungal adherence (*C. tropicalis* and *C. albicans*). Del-Campo *et al* [31] evaluated the effect of rosemary extract on different microorganisms and found activity against Gram-positive bacteria, such as *S. aureus*, *B. cereus* and *S. mutans*. According to the authors, the phenols in the plant are responsible for this activity.

CONCLUSION

In the present study, the rosemary-based endodontic sealer demonstrated greater antimicrobial efficacy in comparison to Sealer 26® with regard to *S. aureus*, *E. faecalis* and *S. mutans.* Further studies are needed to evaluate the effectiveness of rosemary-based endodontic sealers in daily clinical use.

TABLES and CHARTS

Table 1: Mean inhibition halos (in mm) using Sealer 26 and the rosemary-based endodontic sealer in the presence of *S. aureus*, *E. faecalis* and *S. mutans*

Bacterial strain	Sealer	Mean	Standard deviation	p-value[1]

S. aureus	Sealer 26	16.3	1.5	0.030*
	Rosemary	27.1	1.0	
E. faecalis	Sealer 26	15.8	1.0	0.013*
	Rosemary	26.4	1.3	
S. mutans	Sealer 26	25.5	1.0	0.030*
	Rosemary	38.6	2.5	

[1]Nonparametric Mann-Whitney test

* Statistically significant difference

Chart 1: MIC of rosemary extract for bacterial strains studied[14]

Bacterial strain	MIC
S. aureus (ATCC 9811)	6.25 mg/ml
E. faecalis (ATCC 51299)	6.25 mg/ml
S. mutans (ATCC 25175)	14.9 mg/ml

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Anexos

Parecer do Comitê de Ética

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DO RIO GRANDE DO NORTE
COMITÊ DE ÉTICA EM PESQUISA DO HOSPITAL UNIVERSITÁRIO ONOFRE LOPES (CEP-HUOL)

CERTIFICADO

O Comitê de Ética em Pesquisa do Hospital Universitário Onofre Lopes (CEP-HUOL), divisão – Comitê de Ética em *Pesquisa Envolvendo Animais*, *analisou o projeto:*

Título: Manifestações Orais em Pacientes Transplantados Renais do Hospital Universitário Onofre Lopes.

Protocolo – 152/07.

Pesquisador Responsável: Maria Ângela Fernandes Ferreira.

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