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CURSO DE ODONTOLOGIA

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THEREZA CRISTINA FARIAS BOTELHO DANTAS

Terapia Fotodinâmica Antimicrobiana e Plasma de Baixa Temperatura e Pressão como Tratamentos Alternativos Contra Biofilmes Endodônticos Patogênicos

Fortaleza

2015

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Tese apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará, como requisito parcial para obtenção do título de doutor em Odontologia.

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Orientadora: Profa. Dra. Nádia Accioly Pinto Nogueira
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Resumo

A terapia fotodinâmica antimicrobiana (TFDA) e o plasma de baixa temperatura e pressão (PBTP) surgem como tratamentos coadjuvantes à terapia endodôntica convencional, diante do surgimento de cepas resistentes e complexidades anatômicas do canal radicular. Esse estudo foi dividido em 4 capítulos, cujos objetivos foram: Capítulo 1) avaliar o efeito da TFDA mediada pelo TBO em diferentes concentrações e tempos de exposição à luz na produção de EROS e a viabilidade celular em culturas planctônicas de *E. faecalis*. Capítulo 2) avaliar os efeitos da TFDA realizada com AOT e fonte de luz vermelha, em três diferentes tempos de exposição na viabilidade de biofilmes maduros de *E. faecalis* e *C. albicans.* Capítulo 3) avaliar os efeitos do PBTP, em três diferentes tempos de exposição, na viabilidade de biofilmes maduros de *E. faecalis.* Capítulo 4) comparar o efeito da TFDA e do PBTP em canais radiculares infectados com biofilmes maduros de *E. Faecalis,* nos mesmos tempos de exposição para as duas terapias. Nos capítulos 2 e 3, os biofilmes foram crescidos em discos de hidroxiapatita, imersos em Brain-heart infusion broth (*E. faecalis* por 15 dias) ou RPMI 1640 medium (*C. albicans* por 72 horas). Os biofilmes maduros foram submetidos à TFDA mediada pelo fotossentitizador AOT (100 µg/mL) e fonte de luz vermelha (LumaCare®, 630 nm, 2 mm de distância), com densidades de energia de 118,9 J/cm^2, (1 min) 237,8 J/cm^2 (2 min) e 594,5 J/cm^2 (5 min) (capítulo 2). Nas mesmas condições, biofilmes maduros de *E. faecalis* foram também submetidos à ação do PBTP (capítulo 3). Tanto a TFDA como PBTP apresentaram-se eficazes como agentes antimicrobianos em um efeito dose-dependente. No capítulo 4, 96 dentes unirradiculares foram preparados com instrumentação mecanizada. Os espécimes foram divididos em 8 grupos: 1-3 – dentes submetidos à TFDA; 4-6 dentes submetidos ao PBTP e dois grupos-controle – hipoclorito de sódio a 2,5% (positivo) e dentes sem tratamento (negativo). Os dados foram tabulados e tratados estatisticamente pelo método ANOVA ($p<0,05$). Houve uma redução da microbiota em todos os grupos de tratamento, com diferença estatisticamente significante entre os grupos tratados pelo PBTP e TFDA, no tempo de exposição de 5 minutos. Conclui-se então que a TFDA e o PBTP, nos parâmetros testados, apresentaram atividade antimicrobiana eficaz contra os biofilmes estudados, nos dois modelos *in vitro* apresentados.

Palavras-Chave: Fotoquimioterapia. Desinfecção. Endodontia. *E. faecalis*. *C.albicans*. Espécies reativas de oxigênio. Pressão atmosférica.

Abstract

Photodynamic Antimicrobial Therapy (PACT) and Low Pressure Cold Plasma emerged as an effective adjunctive procedure to conventional endodontic treatment, especially in case of persistent infection. This study was divided into four chapters, which objectives were: Chapter 1) investigate the antibacterial effects of photodynamic antimicrobial chemotherapy (PACT), with different concentrations of toluidine blue-O (TBO), at three different exposure times, over suspensions of *Enterococcus faecalis*, using a fluorescence probe – Dihydrorhodamine 1, 2,3 for detecting the release of ROS. Chapter 2) study the antimicrobial effect of PACT mediated by Toluidine blue-O activated by red light (LumaCare® LC122) on *Enterococcus faecalis* and *Candida albicans* biofilms. Chapter 3) a tissue-tolerable-plasma (TTP) was tested for its antimicrobial activity against mature biofilm of a key endodontic bacterium *Enterococcus faecalis.* Chapter 4) evaluate the anti-biofilm efficacy of PACT and TTP applied on saliva-coated-teeth with 2-week *E. faecalis* (ATCC 29212) biofilm, treated with PACT and TTP for 3 different exposure times (1, 2 and 5 minutes) and compared with 2.5% NaOCl irrigation for 5 minutes. In chapters 2 and 3 biofilms were formed on saliva-coated hydroxyapatite discs using batch culture method at 37°C, 5% CO_2. BHI broth was changed daily. In chapter 2, mature *E. faecalis* and *C. albicans* biofilms were subjected to PACT using TBO (100 µg/mL), using a non-coherent red light source (LumaCare®, 630 nm, 2 mm distance) and energy density of 118.9 J/cm^2, 237.8 J/cm^2 and 594.5 J/cm^2 (chapter 2). Using the same growth conditions, mature *E. faecalis* biofilms were subject to TTP on chapter 3. The results were expressed by counting colony forming units (cfu) and group means were compared using 1-way ANOVA. The anti-biofilm effect of PACT and TTP improved as exposure time was increased, reaching the maximum effect after 5 minutes of treatment for both therapies. After 5 min of exposure, there is a significant reduction in cfu numbers in PACT and TTP treatments ($p<0.05$), but neither treatment was as effective as 2.5% NaOCl irrigation. Using root canals *in vitro* model (chapter 4) TTP was better than PACT, at 5 minutes of exposure. Bacterial killing was confirmed by CLSM/COMSTAT and SEM analysis.

Key-words: Photochemotherapy. Sterilization. Endodontics. Reactive oxygen

species. Microorganisms. Atmospheric pressure.

Sumário

1. INTRODUÇÃO GERAL

O sucesso da terapia endodôntica está alicerçado na eficaz descontaminação do sistema de canais radiculares, com a redução significativa dos micro-organismos e seus subprodutos e a manutenção dessa condição, por meio da obturação e do efeito protetor da restauração coronária. Complexidades anatômicas, em especial da porção apical, resistência microbiana e execução inadequada do preparo químico-mecânico são as principais causas para que a infecção persista, não só no interior do canal principal, bem como na intimidade dos túbulos dentinários e canais secundários (SIQUEIRA JR *et al*., 2007).

A caracterização da microbiota endodôntica tem se modificado graças aos métodos moleculares, onde se pode detectar microrganismos de difícil cultivo ou em quantidade insuficiente para detecção por cultura (SIQUEIRA JR *et al*., 2007). Infecções endodônticas, em sua maioria, são mediadas por microrganismos organizados em biofilmes aderidos a superfícies como dentina, cemento radicular e materiais obturadores.

Biofilmes se caracterizam por agregações celulares de bactérias aderidas a uma superfície, embebidas em uma matriz extracelular, produzidas por esses microrganismos (COSTERTON;STEWART;GREENBERG,1999). Nos últimos anos tem sido observado que bactérias organizadas em biofilmes exibem características fisiológicas distintas que resultam no aumento da resistência aos agentes antimicrobianos, quando comparadas aos seus homólogos planctônicos (KOLENBRANDER, 2000). Além disso, outras características são atribuídas aos microrganismos organizados em biofilmes, tais como: a formação de uma barreira física constituída especialmente por polissacarídeos, dificultando a difusão de agentes antimicrobianos às camadas celulares mais profundas, crescimento mais lento decorrente das limitações nutricionais no interior dos biofilmes, ativação de mecanismos gerais de resposta ao estresse, e ainda a expressão de fenótipos específicos (MAH e TOOLE, 2011). Dessa forma, pode-se afirmar que as bactérias no biofilme chegam a ser 10 a 1.000 vezes mais resistente à erradicação quando comparadas aos seus homólogos planctônicos (FUX *et al*., 2005).

A capacidade de organização em biofilmes e de adesão dentinária é

considerada um importante fator de virulência do *Enterococcus faecalis,* um dos micro-organismos mais presentes em casos de infecções secundárias e lesões persistentes, nesses casos havendo a necessidade de reintervenção endodôntica em uma incidência de até 67% (RÔÇAS *et al*., 2004). *E. faecalis* é capaz de crescer em ambientes com extremas diferenças de temperatura (15° - 60°C), pode sobreviver por longos períodos sem nutrição adicional, apresenta resistência ao corante azul de metileno 0,1% e ao ambiente alcalino promovido pelo hidróxido de cálcio (HUBBLE *et al*., 2003; SEDLEY *et al*., 2005).

Diversos autores constataram a presença de *E. faecalis* após o preparo químico-mecânico, independente da técnica utilizada, e que concentrações mais altas de hipoclorito de sódio, usado como solução irrigadora durante o preparo químico-mecânico, foram mais eficientes na desinfecção em profundidade da dentina (WU M. K.. *et al*., 2003; BERBER *et al*., 2006; SIQUEIRA JR. *et al*., 2007).

Apesar de muitos estudos apontarem para o *E. faecalis* como o principal microrganismo responsável pela manutenção das periodontites apicais, fungos também podem estar presentes devido a sua capacidade de invadir túbulos dentinários e sobreviver em ambientes com baixa quantidade de substrato. Dentre as espécies fúngicas mais encontradas, a *Candida albicans* é a mais prevalente nos casos de dentes obturados com lesões refratárias ao tratamento. Fatores de virulência representados pela capacidade de formação de biofilmes também são associados à *C. albicans*, que podem apresentar diferentes padrões de crescimento, variando desde células únicas às complexas arquiteturas em hifas, migrando para dentro dos túbulos dentinários e ramificações do sistema de canais radiculares, resistindo assim aos mecanismos de desinfecção da terapia endodôntica (TURNER *et al*., 2004; LIMA *et a*l., 2015).

Assim, o surgimento de terapias antimicrobianas coadjuvantes à terapia endodôntica convencional configura uma ferramenta interessante para a obtenção de um elevado grau de antissepsia do sistema de canais radiculares, quando associadas ao preparo químico-mecânico, sobretudo nos casos de necessidade de reintervenção e lesões refratárias.

O termo Terapia Fotodinâmica aparece na literatura médica, pela primeira vez em 1941, sendo definida como “*sensibilizador (corante) no tecido*

alvo e presença de oxigênio + luz = destruição celular por oxigênio singleto". Por definição, a TFD é uma reação entre fotossensitizadores e luz, gerando um efeito citotóxico, normalmente por reações oxidativas, sendo reconhecidamente eficaz na inativação de vírus, bactérias resistentes e leveduras. Nessa terapia, um agente químico (corante) é ativado pela luz (sentitização), causando a morte celular, principalmente por apoptose em células eucarióticas (MACHADO, 2000; ACKROYD *et al*., 2001; LEE *et al*., 2004; KONOPLA; DOSLINSKI, 2007).

Dessa forma, o processo fotoquímico promovido pela TFDA provoca dois mecanismos: tipo I – transferência de elétrons - e tipo II – transferência de energia. O mecanismo tipo I induz à formação de produtos oxidados, sendo responsável por 5% da reação. Nos outros 95%, ocorrem o mecanismo tipo II, mais desejável e responsável pela apoptose da célula bacteriana, que posteriormente fagocitada, diferentemente da necrose, não provoca injúria aos tecidos adjacentes. Um vez que não ocorre lise celular, não há extravasamento do conteúdo citoplasmático e, consequentemente, não há lesão tecidual, tornando a TFDA uma terapia eficaz e segura (DOUGHERTY *et al*., 1998; KÜBLER *et al*., 1998; GAD *et a*l., 2004; PASCOAL *et al*., 2015).

As espécies reativas de oxigênio, dentre elas o oxigênio singleto, produzidas nos tecidos após o processo fotoquímico, são altamente citotóxicas. O oxigênio singleto apresenta papel importante no mecanismo de apoptose de células nucleadas, uma vez que esse processo, nas células procariotas, ocorre em sítios ricos em oxigênio como parede celular, lisossomos, DNA etc (PASCOAL *et al*., 2015).

Como a maioria dos microrganismos não possui agentes fotossensíveis, a utilização de um agente fotossensitizador que, uma vez em contato com a célula possa atrair a luz para si, permitindo a formação de radicais livres, é de fundamental importância para a efetividade da TFDA. Esses compostos devem ser não-tóxicos, não-mutagênicos, não-carcinogênicos, capazes de absorver a luz dentro do espectro da fonte utilizada, solúveis em água e serem facilmente eliminados pelo organismo. Dentre os principais grupos utilizados em odontologia estão os fenotiazínicos, representados pelo azul de metileno e azul de toluidina, capazes de sofrer ativação por luz vermelha na faixa entre 600 – 660 nm. A natureza

catiônica do azul de toluidina, permite que esse composto possa interagir tanto com bactérias Gram-positivo como com Gram-negativo, estendendo a ação da terapia fotodinâmica a um maior número de microrganismos (ALLISON *et al*., 2004; DETTY *et al*., 2004; ZANIN *et al*., 2005; WOOD *et al*.,2006; BOUILLAGUET *et al*., 2008; SOUZA *et al*., 2008 VAHABI *et al*., 2011; NAGATA *et al*., 2012).

A literatura apresenta três principais categorias de fontes de luz utilizadas em TFDA: lasers, diodos emissores de luz (LEDs) e lâmpadas halogênicas. Embora tanto lasers como LEDs produzam luzes monocromáticas, em termos de comprimento de onda, LEDs e lâmpadas halogênicas por não apresentarem a mesma especificidade dos lasers, podem ser utilizados com diferentes fotossensitizantes, apresentando o mesmo potencial de inativação (WILSON, PATTERSON, 2008).

Mais recentemente, os efeitos do plasma de baixa temperatura e pressão (PBTP) têm sido estudados na endodontia por suas propriedades antissépticas comparadas aos agentes antibacterianos convencionais. O plasma é considerado o quarto estado da matéria, sendo produzido submetendo-se um ou mais gases a um campo elétrico. Ao receber energia, ocorre uma aceleração no movimento das moléculas do gás, que passam a colidir umas com as outras, favorecendo a transferência de elétrons, partículas carregadas positivamente e negativamente, fótons e radicais livres, tornando o gás ionizado. Todo o processo de produção do plasma ocorre sem gerar significantes aumentos de temperatura, podendo ser tocado livremente com as mãos, sem provocar nenhum dano térmico aos tecidos expostos (KUNHARDT, 2000; MOISAN *et al*., 2001; KOGELSCHATZ, 2002; KIM *et al*., 2014).

Em odontologia têm-se estudado os efeitos do PBTP na modificação das propriedades de biomaterias (SILVA *et al*., 2011; DANTAS *et al*., 2012), clareamento dentário (PAN *et al*., 2010; SUN *et al*., 2010), adesão dentinária (COKELILER *et al*., 2007) e, mais recentemente e especialmente importante para a endodontia (KIM *et al.*, 2014; KOBAN *et al*., 2011; GOREE *et al.*, 2006), como um potente agente anti-biofilme (KOBAN *et al*., 2011; DU *et al*., 2012), por apresentar propriedades antissépticas contra diversos microrganismos, incluindo fungos, vírus, bactérias, parasitas e esporos (SLADEK *et al*., 2003; SLADEK *et al*., 2004; STOFFELS *et al*., 2004;

GOREE *et al* 2006; KUO *et al*., 2006; SLADEK *et al*., 2007; TANG *et al*., 2008; DELBEN *et al*., 2014). Seu mecanismo de ação promove danos à parede celular bacteriana, ocorrendo a lise celular, possivelmente pela produção de radicais livres de oxigênio e nitrogênio (O_2^-, OH, NO), com elevado potencial de oxidação, emissão de radiação ultravioleta (UVC e UVB), sem promover danos por elevação de temperatura ou pressão (LAROUSSE *et al*., 1996; MOREAU *et al*., 2008).

As vantagens do uso da TFDA e do PBTP como auxiliares na terapia antimicrobiana no combate aos biofilme orais, em relação ao uso de agentes antimicrobianos tradicionais, se dá pela rapidez com que ocorre a morte da célula microbiana, evitando dessa forma a manutenção de doses elevadas de agentes antissépticos e antibióticos, tornando o desenvolvimento de resistência bacteriana ou a seleção de cepas resistentes improvável (GURSOY *et al*., 2013). Além disso são terapias de efeito confinado à área da lesão, no caso específico da TFDA, o corante ou a luz sozinhos não apresentam efeitos significativos e a maior concentração de energia produzida pelo plasma ocorre entre 6 – 7 mm além da ponta do dispositivo de aplicação (KIM *et al*., 2010).

Assim faz-se necessária a análise do efeito da Terapia Fotodinâmica e do Plasma de Baixa Temperatura e Pressão na viabilidade celular em biofilmes maduros de *E.faecalis* e *C. albicans*, considerados frequentes em lesões endodônticas persistentes, para verificação de seu potencial antimicrobiano em canais endodônticos infectados.

2. PROPOSIÇÃO:

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

Capítulo 1: Comparar o efeito da Terapia Fotodinâmica realizada com quatro diferentes concentrações do TBO, em três diferentes tempos de exposição em culturas planctônicas de *E. faecalis*, verificando a viabilidade celular e o estresse oxidativo pela quantidade de espécies reativas de oxigênio produzidas.

Capítulo 2: Avaliar a efetividade da Terapia Fotodinâmica realizada com TBO e a fonte de luz vermelha LumaCare® na viabilidade celular, em biofilmes maduros de *E. faecalis* e *C. albicans,* em modelos *in vitro* em discos de hidroxiapatita.

Capítulo 3: Avaliar a efetividade do Plasma de Baixa Temperatura e Pressão na viabilidade celular em biofilmes maduros de *E. faecalis,* em modelos *in vitro* em discos de hidroxiapatita.

Capítulo 4: Comparar o efeito da Terapia Fotodinâmica realizada com TBO e a fonte de luz vermelha LumaCare® com o Plasma de Baixa Temperatura e Pressão na viabilidade celular, em biofilmes maduros de *E. faecalis,* em modelos *in vitro* em dentes humanos extraídos.

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, essa tese é composta por quatro capítulos, contendo artigos a serem submetidos para publicação em revistas científicas, conforme descrito abaixo:

Capítulo 1

"The Use of Dihydrorhodamine 123 for Detecting Reactive Oxygen Species Associated with *Enterococcus faecalis* and Photodynamic Chemotherapy in Different Concentrations of Photosensitizer". Thereza Cristina Farias Botelho Dantas, Mônica Sampaio do Vale, Nádia Accioly Pinto Nogueira, Simone Duarte. Este artigo será submetido à publicação no periódico "Journal of Endodontics".

Capítulo 2

"Photodynamic Antimicrobial Chemotherapy on *Enterococcus faecalis and Candida albicans* Biofilms". Thereza Cristina Farias Botelho Dantas, Mônica Sampaio do Vale, Nádia Accioly Pinto Nogueira, Simone Duarte. Este artigo será submetido à publicação no periódico "Journal of Endodontics".

Capítulo 3

"Antimicrobial Activity of Tissue-Tolerable Plasma on *Enterococcus faecalis* Biofilm". Thereza Cristina Farias Botelho Dantas, Mônica Sampaio do Vale, Nádia Accioly Pinto Nogueira, Simone Duarte. Este artigo será submetido à publicação no periódico "Journal of Endodontics".

Capítulo 4

"Photodynamic Chemotherapy and Tissue Tolerable Plasma: An Effective Approach Against *Enterococcus faecalis* Biofilm". Thereza Cristina

Farias Botelho Dantas, Mônica Sampaio do Vale, Nádia Accioly Pinto Nogueira, Simone Duarte. Este artigo será submetido à publicação no periódico "Journal of Dental Research".

3.1. Capítulo 1

The Use of Dihydrorhodamine 123 for Detecting Reactive Oxygen Species Associated with *Enterococcus faecalis* and Photodynamic Chemotherapy in Different Concentrations of Photosensitizer

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Key-words: *Enterococcus faecalis*, Fungal infection, Photochemotherapy, Biofilms, Root Canal Therapy, Microscopy, Confocal

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Abstract

Introduction: Photodynamic antimicrobial chemotherapy (PACT) uses light of a specific wavelength to activate a nontoxic photoactive dye in the presence of oxygen, inactivating microorganisms essentially by the formation of highly reactive oxygen species (ROS). This study aimed to investigate the antibacterial effects of PACT, testing different concentrations of toluidine blue-O (TBO) and exposure times, over suspensions of *Enterococcus faecalis* planktonic culture, using a fluorescence probe – dihydrorhodamine 123 for detecting the release of ROS.

Methods: Suspensions of *E. faecalis* were standardized and submitted to PACT at different concentrations of TBO (12.5 μg/mL, 25 μg/mL, 50 μg/mL and 100 μg/mL), and exposed to a non-coherent red light (LumaCare®; 630 nm), in different exposure times (0.5 min, 1 min and 2 min). Positive (2% chlorhexidine and 2.5% NaOCl), and negative (light alone and TBO at all concentrations) controls were also included. Antimicrobial efficacy was assessed comparing the mean number of colony-forming units in each group, with a linear mixed model ($p<0.05$). ROS levels were quantified by Confocal/COMSTAT analysis of dihydrorhodamine 123 fluorescence.

Results: PACT produced dose-dependent reductions in CFUs relative to negative controls. No bacteria were counted when the culture was exposed for at least 30 s to the 100 μg/mL TBO concentration, similar to the positive control. TBO concentration had a substantial influence on ROS release, as measured by dihydrorhodamine 123.

Conclusion: PACT, using TBO 100 μg/mL at a short illumination times with LumaCare® induces ROS formation and eliminates *E. faecalis* in planktonic cultures.

Key-words: Photodynamic therapy; fluorescence probes; reactive oxygen species.

Introduction

Enterococcus faecalis is a facultative anaerobic Gram-positive bacterium mostly frequently associated with secondary and persistent endodontic infections (1,2). Although *E. faecalis* is a commensal bacterium that inhabits the gastrointestinal tracts and oral cavity, its resistance to multiple antibiotics can result in nosocomial infections (3-6). *E. faecalis* plays a major role in failure after endodontic treatment and is highly resistant to various disinfectants used during the endodontic treatment, including chlorhexidine, sodium hypochlorite and calcium hydroxide dressings, underscoring the need for better methods of disinfection (7, 8, 9).

Numerous studies have verified that the completely desbridment and bacterial elimination with any of the conventional used techniques and combinations cannot be consistently achieved, due to the complexity of the root canal system (9,10-12). Furthermore, the establishment of sterilization methods with selective bactericidal properties, considering antibiotic resistance showed by an increasing number of bacteria, is highly desired. Thus, the use of PACT arises as a possible alternative in endodontic therapy (13).

PACT is an antimicrobial strategy that uses a nontoxic photosensitizer that is selectively absorbed in a target cell, and a harmless visible light source. Upon photo induced activation of the sensitizer, in the presence of oxygen, a series of reactions produce free radicals and singlet oxygen molecules, which can kill microorganisms by damaging essential cellular molecules (14). The photodynamic effect and the extent of cell damage depends on numerous variables as the photosensitizer, the availability of oxygen, light parameters and light delivery techniques (15-18). However, while disclosing and confirming the excellent antibacterial potential of PACT, none of the studies have consistently examined in which concentration of dye or exposure times, occurs the higher ROS release, which is the greatest potential use of this technology.

Fluorescent probes are frequently employed for the detection of different reactive oxygen species formed during the irradiation of

photosensitized cells (19). Dihydrorhodamine (DHR) 123 is an uncharged and nonfluorescent indicator of reactive oxygen species (ROS) that can passively diffuse across membranes, where it is oxidized to cationic rhodamine 123, which localizes in the mitochondria and exhibits green fluorescence.

This study determined the antimicrobial effect of all combinations of four concentrations of toluidine blue-O and 3 exposure times to a broad–visible light device (LumaCare® LC122) on *E. faecalis* suspensions.

Methods

Bacterial Strains and Media

A standard strain of *E. faecalis* (ATCC 29212) was used for planktonic suspensions. After confirmation of the strain purity by Gram staining and colony morphology, cells were grown in 10 mL sterile brain-heart infusion (BHI) broth (Difco, Detroid, MI), overnight at 37°C and 5% CO_2. The culture in a stationary phase was centrifuged (4500 rpm, 10 min), washed twice in sterile phosphate-buffered saline (PBS1x), re-suspended in fresh nutrient broth, and adjusted spectrophotometrically to a cell density of approximately 3.2×10^7 cfu/mL, an optical density (OD) of 450 nm.

Photosensitizing agent and light sources

A 100 µg/mL toluidine blue-O (TBO, Sigma-Aldrich, St. Luis, MO) stock solution was made and filtered by employing a sterile 0.22 µm membrane and stored in the dark before use. The TBO solutions used in the study were prepared by diluting the stock solution in ultra-pure water.

A single source of a non-coherent light that produces the full spectrum of a visible light was used (LumaCare® LC122A, Medical Group, Newport Beach, CA). This device is capable of delivering red light into a fiber-optic probe (beam diameter = 12 mm), with an irradiance of 3.37 W/cm^2 and centered emission at 630 nm, resulting in the uniform illumination of an area 10 mm^2.

Photodynamic Antimicrobial Chemotherapy

In order to determinate the optimal photosensitizer (TBO) concentration for killing planktonic bacteria, PACT tests were conducted as 10-fold replicates. Fresh cell suspensions of *E. faecalis* in nutrient both were prepared as described above. Aliquots (100 µL) of the bacterial suspensions (approximately 3.2 x 10^7 cfu/mL) were used. Equal volumes of TBO were added, resulting in final concentrations of 12.5 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL. The samples were divided into 12 experimental groups, regarding differences in TBO/concentration and irradiation time used. Three replicates were performed. After 5 min in the dark (pre-irradiation time), the samples were exposed to a red light for three different times, corresponding to the delivery of 59 Jcm^{-2} (30s), 118 Jcm^{-2} (1min) and 237 Jcm^{-2} (2 min) of total energy. The distance between the light and the sample was 2 mm.

The following negatives and positives controls were used for the experiment: (NCG 1) *E. faecalis* incubated with ultra-pure water, with no TBO and exposed to 2 min irradiation, (NCG 2 – NCG 5) *E. faecalis* incubated with TBO at four different concentrations, but no irradiation, (PCG 1) 2% chlorhexidine and (PCG 2) 2.5% sodium hypochlorite. All parameters investigated for each experimental group are shown in **table 1**.

For each group, a ten-fold serial dilution was carried out and plated aerobically at 37°C for 48 hs. The total of the bacterial colony forming units (cfu) by mL was obtained and transformed to logarithm (log_{10}). The treatment response was evaluated by the number of cfu recovered after light exposure.

ROS measurements by Dihydrorhodamine 123 (DHR)

Accumulations of ROS were analyzed using DHR 123 fluorescent probe, using different concentrations of TBO, at 2 min of illumination time. Three controls groups were also investigated to determine the individual fluorescence of only TBO 100µg/ml, only light (237 Jcm^{-2} /2 min) and water with no light.

Tests were carried out adding 500 µL of 15 µM concentration of DHR (Invitrogen/Molecular Probes, Eugene, Oregon) to 10 mL of inoculum, 30 minutes before PACT treatments, and incubating in the dark at 37°C, 5% CO_2. After PACT, aliquots of 200 µL of each sample were transferred to a separate well of a 300 µL flat-botton 96-well microplate (PS, F-botton - Greiner bio one, Germany). The fluorescence signal was monitored using a microplate fluorescence reader (SpectraMax M5 – Molecular Devices), with excitation at 515 nm and emission at 529 nm. The fluorescence intensity at the emission optimum was recorded. The experiment was repeated at least in three different days. Results are summarized in table 2.

Confocal Laser Scanning Microscopy/COMSTAT

Aliquots of 5 µL of each treated suspensions were transferred to a slide, and fluorescent pictures were captured in the stereoscopic microscope Nikon SMZ1500 (Nikon Corporation, Japan), at 1.6X lens and 5.0 X zoom, using the microscope imaging software NIS-Elements Br (Nikon Corporation, Japan). The areas of observations were randomly chosen (5 views per sample). All the images were then analyzed and quantified by the software COMSTAT (20,21), considering the percentage of coverage by green fluorescence on each image captured by CLSM (figure 2). Two replicates were performed by each group exposed to 2 min of irradiation.

Parameters		**TBO**	**TBO**	**TBO**	**TBO**		
59 Jcm^{-2} (30s)		12.5µg/ml	25µg/ml	50µg/ml	100 µg/ml		
118 Jcm^{-2} (1min)		12.5µg/ml	25µg/ml	50µg/ml	100 µg/ml		
237 Jcm^{-2} (2 min)	water	12.5µg/ml	25µg/ml	50µg/ml	100 µg/ml		
Absence of light (2min)		12.5µg/ml	25µg/ml	50µg/ml	100 µg/ml	**2% Chlorhexidine**	**2.5% Sodium Hypochlorite**

Table 1: Distribution of the experimental groups, negative and positive control groups.

Parameters							Mean Value
TBO 100 µg/ml + L	20.029	21.597	23.027	26.155	22.773	22.196	**22.630 (SD 1.854)**
TBO 50 µg/ml + L	20.684	22.847	23.211	21.987	22.661	21.830	**22.203 (SD 0.830)**
TBO 25 µg/ml + L	15.479	17.850	16.440	18.914	18.397	20.209	**17.882 (SD 1.559)**
TBO 12.5 µg/ml + L	12.217	13.262	15.581	13.360	12.292	21.090	**14.634 (SD 3.093)**
TBO 100 µg/ml - L	9.433	10.212	10.250	10.277	7.745	9.821	**9.623 (SD 0.891)**
237 Jcm^{-2} (2 min) + Water	8.172	9.301	10.538	9.624	8.973	12.672	**9.880 (SD 1.435)**
Water - L	8.610	8.826	8.908	8.727	8.916	9.436	**8.904 (SD 0.260)**

Table 2: Fluorogenic interactions between experimental PACT groups, incubated with DHR 123 (15µM), at four different concentrations of TBO and control groups, indicating the amount of ROS released after photo activation. The numbers represent the fluorescent emission intensity on excitation at 515 nm, during 2 min of illumination time in six replication determinations.

Statistical analysis

Counts of colony forming unit were logarithm transformed (base-10) prior to analysis. Treatment groups were then compared by evaluating whether the mean for one group fell within the 95% confidence bounds of any other group. These limits were computed on the basis of the pooled variance estimate from a linear mixed model with fixed factors of group and random factors of sample, and replications within a sample (IBM SPSS; version 22; IBM Corp, Armonk, NY). These models excluded data from groups showing no bacteria (TBO 100 µg/m and the positive controls), which were constants, in order to satisfy the model assumption of homogeneous variances. Comparisons with the excluded groups were then achieved by evaluating whether the 95% confidence limits of each other experimental group included zero.

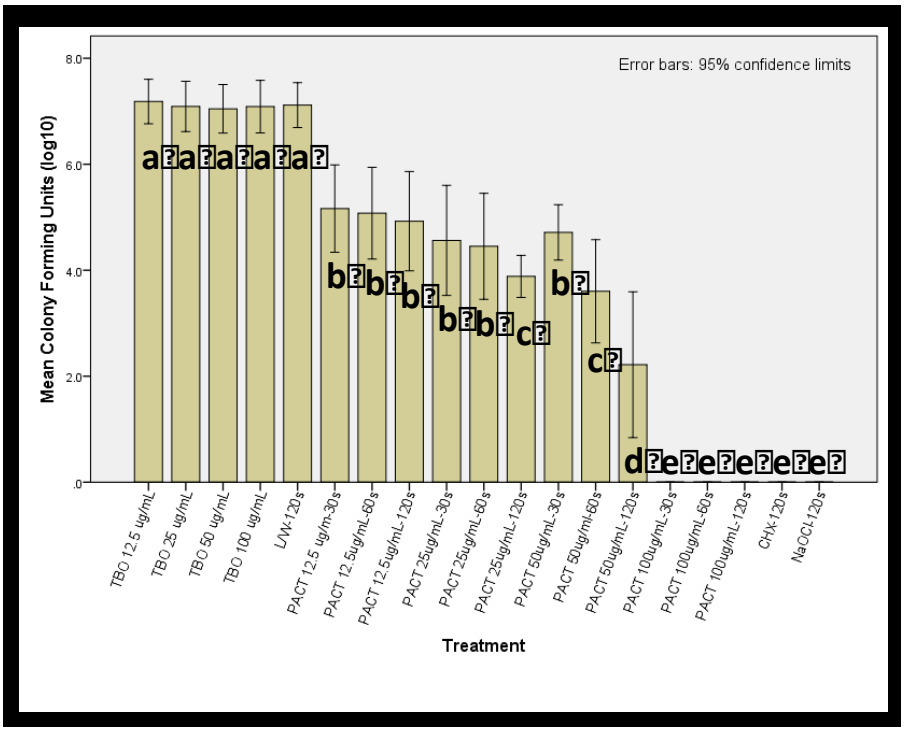

Figure 1. Treatments effects using TBO at 12,5 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL, in three different exposure times, compared with negative and positive controls, on the viability of *E. faecalis* suspensions (Log cfu) after PACT. The letters a, b, c, and d show statistically significant differences.

Results

Effectiveness of PACT disinfection

Figure 1 shows mean counts (after log10 transformation) of approximately 10^7 cfu mL^{-1} in negative controls groups. PACT groups treated with increasing levels of TBO and/or increasing illumination times showed dose-related reductions in bacterial counts. Bacterial counts decreased from 10^5 cfu mL^{-1} to less than 10 cfu mL^{-1} as the TBO dose increased from 12.5 to 100 µg/ml. No bacteria were seen in the positive control groups.

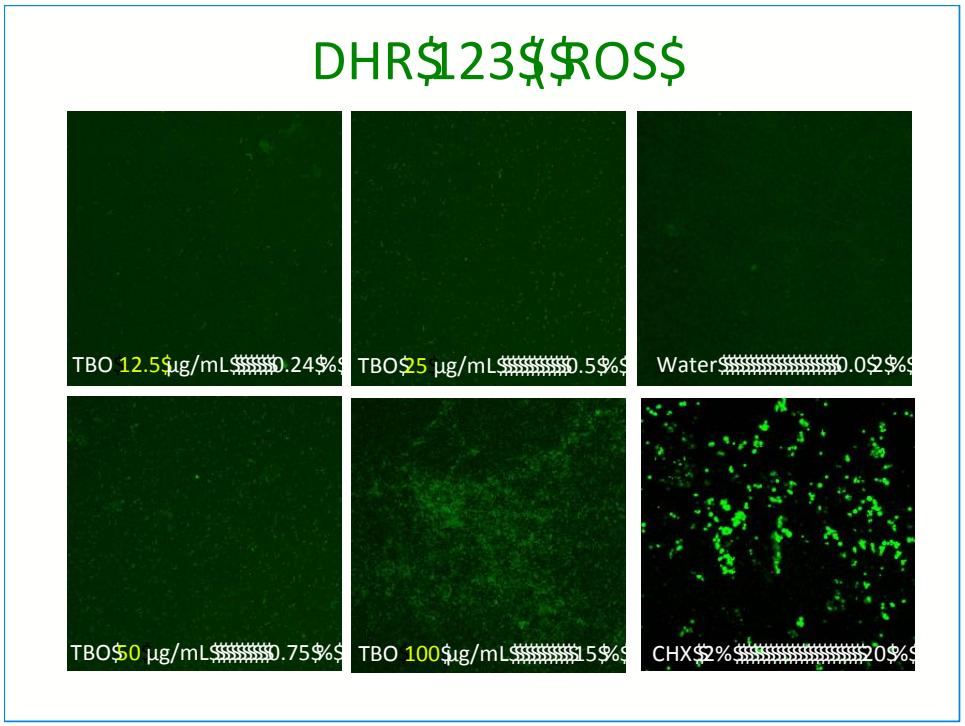

Figure 2. : Effect of PACT on *E. faecalis* suspensions. CLSM Photographs of ROS stain after 2 min of red light exposure. Bright green color is fluorescence intensity of ROS stain. ROS stain is extremely strong as the TBO concentration is increased. The values represent mean of % covered by stained cells of 10 points after light exposure, analyzed by COMSTAT.

The red light exposure induces ROS generation inside cells - relative levels of ROS determined by a fluorescent dye, dihydrorhodamine 123.

One mechanism by which PACT/TBO may reduce the count of cfu mL^{-1} in *E. faecalis* suspensions is through production of ROS. Fluorescence microscopy images were analyzed as an indicator of ROS production in the medium during PACT. Upon light activation, Figure 2 shows, as expected, little or no fluorescence in the control groups. Compared with the control group, the images demonstrate a dose dependent increase in fluorescence as the concentration of TBO increased. Cells treated with chlorhexidine for 2 minutes showed the highest level of fluorescence.

Discussion

PACT is a process that produces singlet oxygen and other ROS through a variety of photochemical mechanisms, resulting in cell death, and it has been presented as a potential antimicrobial therapy (22). Although it is known that photosensitizer accumulates in the target tissues and causes oxidative damage to the bacterial cell when exposed to light of a specific wavelength, the ideal photosensitizer, and its ideal concentration and exposure time remain controversial.

Numerous studies reported different photosensitizer concentrations and irradiation times, focusing on the antibacterial effect of PACT on endodontic pathogens. Schlafer *et al*. (23) demonstrated that photo activated disinfection using laser 628nm of wavelength, in 30s of irradiation time with TBO 100 µg/ml strongly reduce the number of endodontic pathogens in planktonic suspensions, including *E. faecalis*. Bago *et al*. (24) reported a significant reduction in the *E. faecalis* population in root canals infected, using PACT with TBO 155 µg/ml, in a irradiation time of 1min. Vaziri *et al.* (25) also investigated the bactericidal efficacy of PACT against *E. faecalis*, using TBO 15 µg/ml, during 30 s with a diode laser 625 nm of wavelength. The results showed a significant reduction in cfu count in groups which the PACT was used alone in root canals contaminated. The discrepancy of all those studies can be related to the differences in pattern of photosensitizer concentration, light source and exposure time used. This discrepancy corroborating the idea that metabolites produced during PACT, such as ROS, requires ideal photosensitizer concentration and a specific exposure time to produce the same effect on the cells.

The results presented here demonstrated the potential of PACT, using TBO, as an antibacterial therapy, and a positive correlation between the concentration of TBO, the reduction in cfu count after PACT and ROS release. This effect was increased after more prolonged irradiation time, indicating that ROS produced in the medium after PACT, using TBO, promote more effective damage in the cells in a time and dose-dependent manner. We suggest that the specific metabolites produced by PACT, using TBO in an increasing rate of concentration and exposure time, can induce harmful effect on cells, which lead to cellular injury. The maximum concentration used in

this study achieved high bacterial reduced in all exposure times, and orto-toluidine blue at 100 µg/mL has maximum efficacy on *E. faecalis* suspensions in a short exposure time. The associations observed between increasing concentrations of TBO, increased ROS production and reduced bacterial load suggests that PACT acts through a photokiling mechanism.

Different photochemical mechanisms involved in PACT have been studied for many authors, as the bacteria cells are particularly sensitive to membrane damage from ROS generated in solution (26-28). This means that the intracellular uptake of photosensitizer is not as strictly necessary to kill bacteria as previously thought. The ROS generated outside the cell may be able to cause lethal damage to the cell membrane by diffusion (28). It has been demonstrated that ROS can also promote damage to DNA and proteins, leading to cell death (29). In PACT, the light interaction with the photosensitizer drug increases its energy state, which, in the presence of molecular oxygen, forms reactive oxygen species, primarily singlet oxygen, which can react with regions of many biomolecules, producing an increase in the oxidized species (30,31,32). These ROS can diffuse to a distance of approximately 100 nm with a half-life < 0,04 microseconds (15).

The positive correlation observed between ROS production, showed by employing a fluorescence probe, and the concentration of TBO and the reduction of bacterial load after PACT, indicates that the mechanisms by which PACT acts, as a photokiling therapy, involve both ROS production and the direct contact of cells membranes with the selectively photosensitizer, in the presence of low-intensity light source.

Conclusion

Under the conditions of this study, TBO 100 µg/ml, in all illumination times used, showed the best results as a photokilling bactericidal agent and ROS producer. Additional investigations, including biofilms, *in situ* and *in vivo* studies, are needed to further strengthen the currently level of evidence of this study.

References

1. Narayanan LL, Vaishnavi C. Endodontic microbiology. J Conserv Dent 2010;13(4):233-239.

2. Tennert C, Fuhrmann M, Wittmer A, Karygianni L, Altenburger MJ, Pelz K, et al. New bacterial composition in primary and persistent/secondary endodontic infections with respect to clinical and radiographic findings. J Endod 2014;40(5):670-677.

3. Arias CA, Murray BE. The rise of the *Enterococcus:* beyond vancomycin resistance. Nat Rev Microbiol 2012;10(4):266-278.

4. Burley KM, Sedgley CM. CRISPR-Cas, a prokaryotic adaptive immune system, in endodontic, oral, and multidrug-resistant hospital-acquired Enterococcus faecalis. J Endod 2012;38(11):1511-1515.

5. Matos RC, Lapaque N, Rigottier-Gois L, Debarbieux L, Meylheuc T, Gonzalez-Zorn B, et al. Enterococcus faecalis prophage dynamics and contributions to pathogenic traits. PLoS genet 2013;9(6):e1003539.

6. Rocas IN, Siqueira JF, Jr., Santos KR. Association of Enterococcus faecalis with different forms of periradicular diseases. J Endod 2004;30(5):315-320.

7. Beyth N, Kesler Shvero D, Zaltsman N, Houri-Haddad Y, Abramovitz I, Davidi MP, et al. Rapid kill-novel endodontic sealer and Enterococcus faecalis. PloS one 2013;8(11):e78586.

8. Radcliffe CE, Potouridou L, Qureshi R, Habahbeh N, Qualtrough A, Worthington H, et al. Antimicrobial activity of varying concentrations of sodium hypochlorite on the endodontic microorganisms Actinomyces israelii, A. naeslundii, Candida albicans and Enterococcus faecalis. Int Endod J 2004;37(7):438-446.

9. Pileggi G, Wataha JC, Girard M, Grad I, Schrenzel J, Lange N, et al. Blue light-mediated inactivation of Enterococcus faecalis in vitro. Photodiagnosis Photodyn Ther 2013;10(2):134-140.

10. Paiva SS, Siqueira JF, Jr., Rocas IN, Carmo FL, Leite DC, Ferreira DC, et al. Clinical antimicrobial efficacy of NiTi rotary instrumentation with NaOCl irrigation, final rinse with chlorhexidine and interappointment medication: a molecular study. Int Endod J 2013;46(3):225-233.

11. Mickel AK, Chogle S, Liddle J, Huffaker K, Jones JJ. The role of apical size determination and enlargement in the reduction of intracanal bacteria. J Endod 2007;33(1):21-23.

12. Card SJ, Sigurdsson A, Orstavik D, Trope M. The effectiveness of increased apical enlargement in reducing intracanal bacteria. J Endod 2002;28(11):779-783.

13. Souza LC, Brito PR, de Oliveira JC, Alves FR, Moreira EJ, Sampaio-Filho HR, et al. Photodynamic therapy with two different photosensitizers as a supplement to

instrumentation/irrigation procedures in promoting intracanal reduction of Enterococcus faecalis. J Endod 2010;36(2):292-296.

14. Konopka K, Goslinski T. Photodynamic therapy in dentistry. J Dent Res 2007;86(8):694-707.

15. Chrepa V, Kotsakis GA, Pagonis TC, Hargreaves KM. The effect of photodynamic therapy in root canal disinfection: a systematic review. J Endod 2014;40(7):891-898.

16. Foschi F, Fontana CR, Ruggiero K, et al. Photodynamic inactivation of Enterococcus faecalis in dental root canals in vitro. Lasers Surg Med 2007;39:782–7.

17. Soukos NS, Chen PS, Morris JT, et al. Photodynamic therapy for endodontic disinfection. J Endod 2006;32:979–84.

18. Fimple JL, Fontana CR, Foschi F, et al. Photodynamic treatment of endodontic polymicrobial infection in vitro. J Endod 2008;34:728–34

19. Price M, Kessel D. On the use of fluorescence probes for detecting reactive oxygen and nitrogen species associated with photodynamic therapy. J Biomed Opt 2010;15(5):051605.

20. Klein MI, Duarte S, Xiao J, Mitra S, Foster TH, Koo H. Structural and molecular basis of the role of starch and sucrose in Streptococcus mutans biofilm development. Appl Environ Microbiol 2009;75(3):837-841.

21. Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersboll BK, et al. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology 2000;146 (Pt 10):2395-2407.

22. Vera DM, Haynes MH, Ball AR, Dai T, Astrakas C, Kelso MJ, et al. Strategies to potentiate antimicrobial photoinactivation by overcoming resistant phenotypes. Photochem Photobiol 2012;88(3):499-511.

23. Schlafer S, Vaeth M, Horsted-Bindslev P, Frandsen EV. Endodontic photoactivated disinfection using a conventional light source: an in vitro and ex vivo study. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010;109(4):634-641.

24. Bago I, Plecko V, Gabric Panduric D, Schauperl Z, Baraba A, Anic I. Antimicrobial efficacy of a high-power diode laser, photo-activated disinfection, conventional and sonic activated irrigation during root canal treatment. Int Endod J 2013;46(4):339-347.

25. Vaziri S, Kangarlou A, Shahbazi R, Nazari Nasab A, Naseri M. Comparison of the bactericidal efficacy of photodynamic therapy, 2.5% sodium hypochlorite, and 2% chlorhexidine against Enterococcous faecalis in root canals; an in vitro study. Dent Res J 2012;9(5):613-618.

26. Huang L, Xuan Y, Koide Y, Zhiyentayev T, Tanaka M, Hamblin MR. Type I and Type II mechanisms of antimicrobial photodynamic therapy: an in vitro study on gram-negative and gram-positive bacteria. Lasers Surg Med 2012;44(6):490-499.

27. Sanchez-Cruz P, Dejesus-Andino F, Alegria AE. Roles of hydrophilicities and

hydrophobicities of dye and sacrificial electron donor on the photochemical pathway. J Photochem Photobiol A Chem 2012;236:54-60.

28. Kasimova KR, Sadasivam M, Landi G, Sarna T, Hamblin MR. Potentiation of photoinactivation of Gram-positive and Gram-negative bacteria mediated by six phenothiazinium dyes by addition of azide ion. Photochem Photobiol Sci: Official journal of the European Photochemistry Association and the European Society for Photobiology 2014;13(11):1541-1548.

29. Imlay JA. Pathways of oxidative damage. Annu Rev Microbiol 2003;57:395-418.

30. Rosenthal I, Ben-Hur E. Role of oxygen in the phototoxicity of phthalocyanines. International journal of radiation biology 1995;67(1):85-91.

31. Dougherty TJ. Photodynamic therapy: Part II. Seminars in surgical oncology. Int J Radiat Biol 1995;67(1):85-91.

32. Gursoy H, Ozcakir-Tomruk C, Tanalp J, Yilmaz S. Photodynamic therapy in dentistry: a literature review. Clin Oral investig 2013;17(4):1113-1125.

3.2. Capítulo 2

Photodynamic Antimicrobial Chemotherapy on *Enterococcus faecalis and Candida albicans* Biofilms

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Key-words: *Enterococcus faecalis*, Fungal infection, Photochemotherapy, Biofilms, Root Canal Therapy, Microscopy, Confocal

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Declaration of interests:

There are no potential conflicts of interests relating to this study.

Abstract

Introduction: Photodynamic antimicrobial chemotherapy (PACT) uses a resonant source of light to activate a nontoxic photosensitizer being able to form highly reactive oxygen species, that are harmful to microorganisms. This study aimed to investigate the antimicrobial effect of PACT on *Enterococcus faecalis* and *Candida albicans* biofilms.

Methodology: *Enterococcus faecalis* and *Candida albicans* biofilms were formed on saliva-coated-hydroxyapatite discs and treated with PACT (LumaCare®; 630 nm; 2 mm distance). The light irradiation time periods were 1, 2 and 5 minutes. Control groups were 2.5% NaOCl (positive), photosensitizer without light irradiation, ultra-pure water, and light irradiation without photosensitizer (negatives). The antimicrobial effect of PACT was determined on the basis of the number of colony-forming-units per milligram of biofilm dry weight in addition to visualization by confocal laser and scanning electron microscopies (CLSM/SEM). The CLSM images were quantified by COMSTAT.

Results: Relative to the negative controls, PACT reduced *E. faecalis* and *Candida albicans* biofilms in a dose-dependent manner. Treatment for 5 minutes had higher antimicrobial efficacy and was able to kill *Candida albicans* biofilms cells completely ($p<0.05$). The SEM analysis visually showed a change of the structure of the biofilm after PACT treatment for both biofilms. Confocal laser microscopic images and COMSTAT analysis indicated that PACT treatment induced death and destruction in all different layers of biofilms studied, in 5 minutes exposure groups.

Conclusion: PACT with toluidine blue-O exhibited remarkable lethal effects on this *E. faecalis and C. albicans* biofilms *in vitro* model.

Key-words: *Enterococcus faecalis*, fungal infection, photodynamic therapy.

Introduction

Enterococcus faecalis and *Candida albicans* are microorganisms capable of resisting to endodontic disinfection procedures, being associated with secondary or persistent endodontic infection (1, 2). Their ability to adhere, colonize and form biofilms on the surface of dentin, cementum and substrates such as gutta-percha or endodontic sealers has been demonstrated in several studies (3-6). Considering that biofilms characteristically exhibit increased resistance to a wide range of a antimicrobial agents (7, 1), develop a sterilization method that cause damage to biofilm in different depths is highly desired.

In recent years photodynamic chemotherapy (PACT) has been used to target microorganisms in bacterial, fungal and viral infections, based on the concept that a nontoxic photosensitizer (PS) agents, activated by a light source of an appropriate wavelength, in the presence of oxygen, induces cell death due the production of singlet oxygen, hydroxyl radicals and others reactive oxygen species (ROS). These radicals are cytotoxic to cells of the target tissue by damaging so important components such as plasma membrane and DNA (8-11). As the PS are used topically and these reactions occur in a limited space, this modality seems to be effective against localized infections with considerably low systemic adverse reactions (9,12).

Considering the increase in the occurrence of resistance of *Candida ssp*. to conventional antifungals in the last decades (13, 14), in addition to the increasing ability of the *E. faecalis* to withstand endodontics procedures, as a alkaline environment tolerable species (15), the searching for more effective treatment options seems absolutely necessary. In Endodontics, PACT has shown to be effective (16), being a remarkable tool to obtain better antisepsis in endodontic treatment (17, 18).

The aim of this study was therefore use PACT disinfection method against an *in vitro* biofilm model, that closely mimics the *in vivo* biofilm, to assay the eradication of *E. faecalis* and *C. albicans* biofilms, focusing on the anti-biofilm property.

Methods

E. faecalis culture and biofilm (ATCC 29212):

The inoculum was obtained from single colonies isolated on agar plates, inoculated in brain-heart infusion broth (BHI) and incubated overnight at 37°C, under microaerophilic conditions (5% CO_2). Cell density was set at 3.2 X 10^7 colony forming units by milliliter (cfu mL^{-1}) for the stock solution. Biofilms of *E. faecalis* were formed on saliva-coated hydroxyapatite discs (C-HA) (0.635 cm^2) placed into 24-well culture plates with 2 mL of sterile BHI broth, containing 3.2 X 10^7 CFU mL^{-1}, at 37° C in 5% CO_2 for 15 days (19). Stimulated saliva from a health volunteer who did not use any antibiotic for 6 months before the start of the experiments was used for the adhesion studies. To prevent nutrient deficiency, the BHI broth was completely replaced every 24 hours.

C. albicans culture and biofilm (ATCC14053):

Strains were grown overnight at 37°C in yeast nitrogen base medium supplemented with dextrose. The culture was adjusted to a concentration of approximately 1.0 x 10^7 cells ml^{-1} in RPMI 1640 medium buffered to pH 7.0 with 3-(N-morpholino)-propanesulfonic acid (MOPS; Sigma-Aldrich, St. Luis, MO).

Biofilms of *C. albicans* were formed on C-HA as described above. 1 mL of cell suspension was transferred to each well of a 24-well culture plates containing 1 mL of sterile RPMI medium, and the samples were incubated for 90 min at 37°C in a 3-D rotator (Lab Line – Thermo scientific, USA). Following, unattached microorganisms were removed by washing the discs with 1 X PBS. 2 mL of fresh RPMI medium was added into the wells in order to completely cover the discs, and then incubated at 37°C for 72 h to allow the biofilm to grow and maturate. Fresh medium was changed every 24 hours (20).

Photosensitizing agent and light sources

Toluidine blue-O (TBO) at 100 µg/mL in ultrapure water (Sigma-Aldrich, St. Luis, MO) was used as a PS in this study. The solution was filtered in 0.22 µm PES membrane and immediately used.

A non-coherent light that produces the full spectrum of a visible red light was used as the irradiation source (LumaCare® LC122A, Medical Group, Newport Beach, CA) with 630 nm wavelength, spot size113.1 mm^2 and output power of 95.5 mW cm^{-2}.

Exposure of the biofilms to red light

After biofilms formation, four samples of C-HA discs containing each biofilm studied, were submitted to PACT and control groups according to the experimental conditions of each group. After 5 min in the dark (pre-irradiation time), the samples were exposed to red light. The distance from the tip of the fiber to the biofilms surface was 2 mm, the irradiation time periods were set at 1, 2 and 5 minutes and power density of 118.9 Jcm^{-2}, 237.8 Jcm^{-2} and 594.5 Jcm^{-2}, respectively.

To determine whether photosensitizer alone induced any toxic effects on microorganisms viability, the biofilm samples were exposed to PS, under identical conditions, but not exposed to light. Biofilms were also exposed to irradiation of red light, with no previous exposure to PS. Samples with no exposure to either PS or red light (ultra-pure water groups) were also used as a negative control group. All control groups were treated for 5 minutes.

Microbiological analysis

At the end of the experimental period, the biofilms were gently rinsed in phosphate buffered saline. The opposite surface of the disc, with adherent biofilm that was not irradiated by the light, was removed with a sterile cotton swab. The same procedure was performed in control groups.

At the end of the experimental period, the discs were placed in 5 mL of sterile saline solution and subject to a ultrasound bath (Ultrasonic Cleaner, FS140, Fisher Scientific, Pittsburgh, PA, USA) for 10 minutes. Three

intervals of 15-s sonication pulses were used to homogenize the removed biofilms (Fisher Scientific, Sonic Dismembrator model 100; USA). The homogenized suspension was used for bacterial viability (cfu mg^{-1} of biofilm dry weight) (21).

A ten-fold serial dilution was carried out and 50 µL was placed onto blood agar. The plates were incubated at 37°C in 5% CO_2 for 48 h, and then the number of CFU mg-1 of biofilm dry weight were obtained. The experiment was repeated at least in three different days.

Dry weight

For the dry weight determination, three volumes of cold ethanol (-20°C) were added to 1 mL biofilm suspension, and the resulting precipitate was centrifuged (10,000 g for 10 min at 4°C). The supernatant was discarded, and the pellet was washed with cold ethanol, and then lyophilized and weighed (21, 28).

Confocal Laser Scanning Microscopy/COMSTAT

The presence of dead and live bacteria on the biofilm surface was visualized by confocal laser scanning microscope and analyzed by COMSTAT. Two biofilm samples of each group were stained with Live/Dead Baclight Bacterial Viability kit (Molecular probes. Invitrogen, Eugene, Oregon. USA) in accordance with the manufacturer

Each sample was processed and analyzed individually and 5 images of each biofilm were taken from randomly chosen areas in each disc. All the samples were examined under a CLSM (Leica Lasertechnik GmbH, Heidelberg, Germany), with a HCX APOL U-V-I 40X/0.8-numerical-aperture water immersion objective. The stained samples were incubated at room temperature in the dark for 30 min and examined under CLSM. The bacterial biomass ($\mu m^3/\mu m^2$) were quantified by COMSTAT (22, 23).

Biofilm Verification by Scanning Electron Microscopy (SEM)

The biofilm morphology changes were observed by SEM after the treatment with PACT and visually compared with the controls groups. Two specimens of each group were immersed in a fixative solution 4%

paraformaldehyde at room temperature for 1 hour. The specimens were then submitted to increasing concentrations of ethanol for serial dehydration (ethanol 70%, 85% and 100%). The dehydrated specimens were dried using a desiccator, overnight, sputter-coated with gold-palladium, mounted on a stub and examined by SEM (Hitachi S3500N Variable Pressure Scanning Electron Microscopy, Boston, MA, USA) at 1500x or 4000x magnifications at 6-12Kv .

Statistical analysis

Colony forming units and dry weight were logarithmic transformed (base-10) prior to analysis. Treatment groups were compared, for each biofilm, by evaluating whether one mean fell within the 95% confidence intervals of any other mean. These limits were computed on the basis of the pooled variance estimate from a linear mixed model with fixed factors of group and random factors of subject and replications within a subject (IBM SPSS; version 22; IBM Corp, Armonk, NY). These models excluded data from the positive control, which was a constant, in order to satisfy the model assumption of homogeneous variances. Comparisons with the positive control were then achieved by evaluating whether the 95% confidence limits of each other experimental group included zero.

Results

Figure 01 shows the number of CFUs and dry weight (CFU mg^{-1}) determined for each microorganisms on each treatments and controls. The results revealed that there was a tendency for a dose-dependent response for *E. faecalis* and *C. albicans* biofilms, compared to control groups, showing the highest results in 5 minutes of irradiation. In comparison of two biofilms investigated, 5 minutes of exposure to a non-coherent red light were able to disrupt nearly completely *C. albicans* biofilm statistically indistinguishable from the positive control group ($p<0.05$).

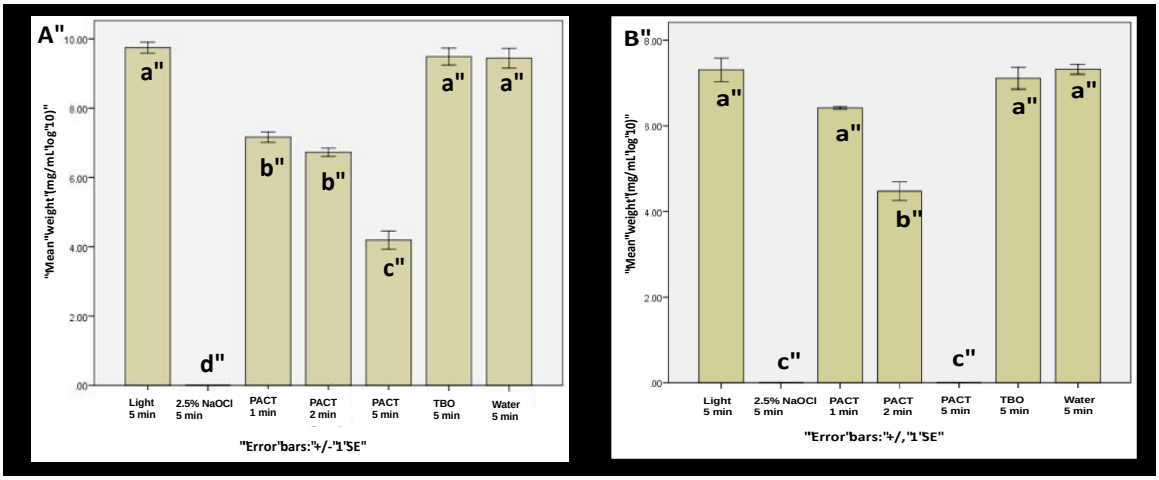

Figure 01: CFU count and dry weight in *E. faecalis* and *C. albicans* biofilms. CFU count and dry weight after PACT treatment for 1, 2 and 5 minutes compared with 2.5% sodium hypochlorite (positive control), ultrapure water, light without TBO and TBO without irradiation (negative controls). Data represent the mean values and error bars represent standard deviation. Values marked with different letters are significantly different from each other ($p<0,05$). (A) represent values for *E. faecalis* and (B) for *C. albicans* biofilms.

SEM

Scanning electron microscopy images showed highly thick and organized *E. faecalis* biofilm structure. However, after 5 minutes of PACT, the biofilm 3-dimensional architecture and morphology were destroyed (Fig. 02). *C. albicans* biofilm colonization with filamentous hyphae were observed on negative control groups. PACT were able to disrupt the architectural structure of the yeasts cells, showing the shrunken, rough and fractured appearance (Fig. 03). There was a visual difference between red light group and the control groups.

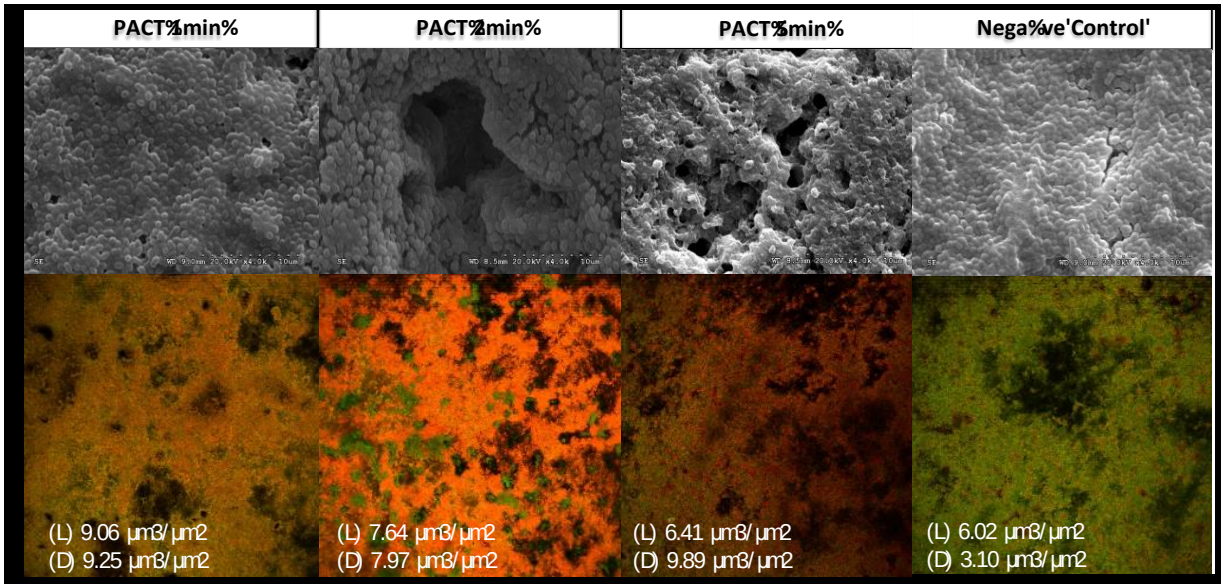

Figure 02: Scanning electron micrographs of C-HA discs colonized with *E. faecalis* biofilm at magnification of 4000x. Comparative analysis of each group, in three different exposure times of PACT, showing the morphological structure of the biofilm severely modified. Confocal Scanning Laser Microscopy images obtained from the mid-area of C-HA disc showing overlap of live (L) and dead (D) cells. The average of total bacterial biomass calculated by COMSTAT in all of 5 points are described.

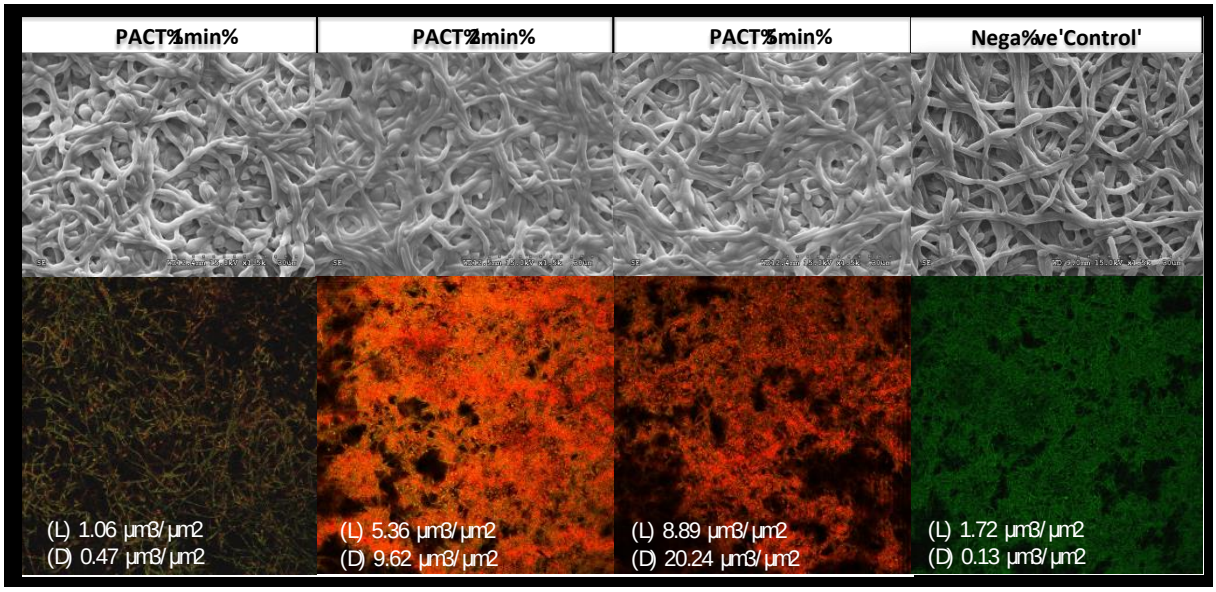

Figure 3: Scanning electron micrographs of C-HA discs colonized with *C. albicans* biofilm at magnification of 1,500x. Comparative analysis of each group, in three different exposure times of PACT, showing the morphological structure of the biofilm severely modified. Confocal Scanning Laser Microscopy images obtained from the mid-area of C-HA disc showing the overlap of live (L) and dead (D) cells. The average of total fungus biomass calculated by COMSTAT in all of 5 points are described.

Confocal Lasers Scanning Microscopy/COMSTAT

There is a clear inversion of the proportion of live (blue line) and dead cells (red line) within the biofilms when compared to the treatment and control groups. However, the results presented by *C. albicans* biofilm, indicates that prolonged PACT exposure were able to remarkable increase the presence of dead cells in the deeper and outer layer of the biofilm (Fig. 04). The total biomass calculated by COMSTAT analysis confirm that PACT with red light is effective as an anti-biofilm therapy (Fig. 03).

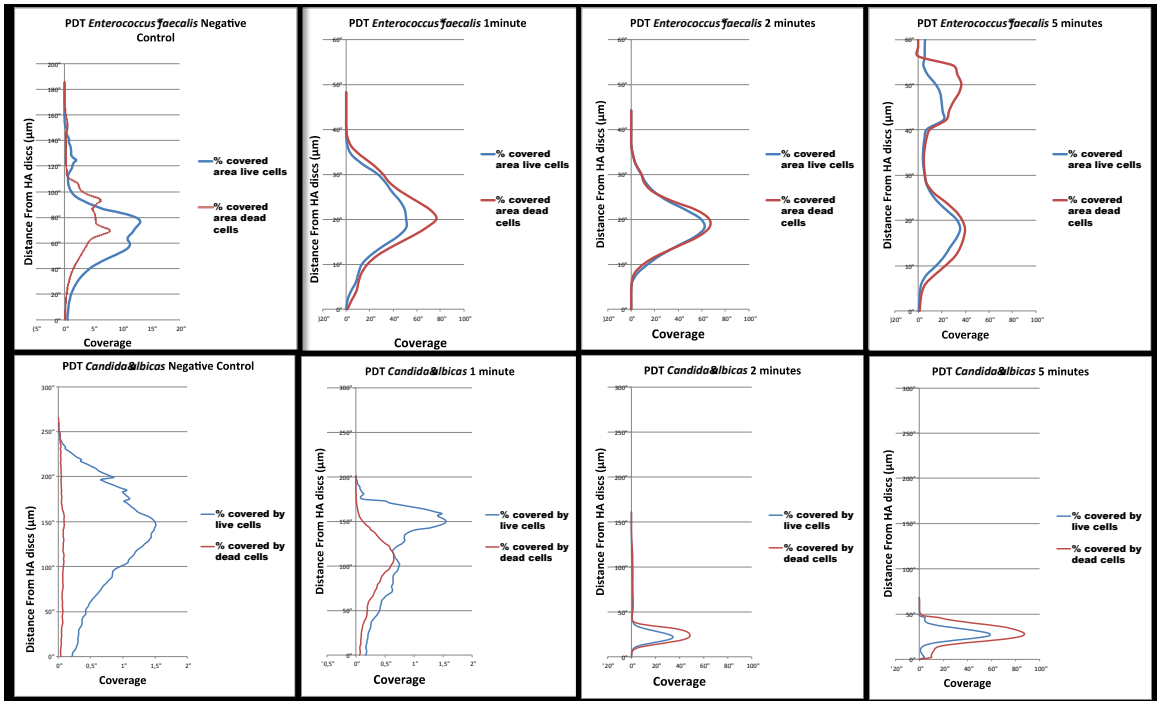

Figure 04. Representative images of the *E. faecalis* and *C. albicans* biofilms viability after different exposure times of PACT rendered images of the outer layers of biofilm. Graphics show the area occupied (%) by green (live cells) and red (dead cells), from the substratum surface to the top layer of the biofilm analyzed by COMSTAT .

Discussion

The current study evaluated the anti-biofilm properties of PACT in eradication of *E. faecalis* and *C. albicans,* using biofilms grown on saliva-coated HA discs. Results have supported the promising and surpass effect of photokilling activity in a dose-dependent manner effect, in terms of final colony-forming unit per milligram of biofilm dry weight and important cell viability and morphological modifications observed under CLSM and SEM.

There are many variables to be taken into account when developing a reliable and safe PACT protocol. One of the most controversial factors is the photosensitizer concentration. For photodynamic disinfection, TBO has been tested at concentrations ranging from 10 μg/mL to 100 μg/mL (24-26), showing different results related to the use of planktonic or biofilm form of growth bacteria. In this study, TBO was used at a concentration of 100 μg/mL as photosensitizer, since its efficacy has been determinate in our preliminaries studies (data not shown), showing antibacterial and antifungal effects at this concentration (27, 28).

In the present study, a significant CFU count reduction was observed after 5 min of irradiation for *E. faecalis* groups treated by PACT. Light illumination alone did not produce bacterial reduction ($p<0.05$), which indicates there is no significant harmful thermal effects. This results corroborate with previous reports that achieved good outcomes when PACT was tested over *E. faecalis* biofilm *(*16, 29*)*. Bergmans *et al*. (24), showed a significant reduction in CFU counts of *E. faecalis* (88.4%), using TBO at 12.7 μg/mL irradiated by diode laser for 2.5 minutes. In contrast, Souza *et al*. (30), using similar combination of TBO at 15 μg/mL, irradiated by diode laser for 4 minutes, found results with no statistical significance in terms of reduction of the *E. faecalis* load.

Although eukaryotic species, such as *C. albicans,* are less susceptible to killing with PACT than prokaryotic bacteria (6), mean values of *C. albicans* CFU mg^{-1} of biofilm dry weight, showed in our results, confirm the known fungicidal activity of PACT (31, 32). After 5 min of irradiation there was no cell growth at all, similarly to the effect 2.5% sodium hypochlorite long used as “gold standard” endodontic irrigant solution. While was more effective than PACT in eliminating *E. faecalis* biofilm, 2.5% sodium hypochlorite and PACT were similarly effective in eliminating *C. albicans* biofilm without the

disadvantage of cytotoxic effects to cells of periapical region caused by accidentally leakage. Furthermore, it is reasonable to agree with previous studies that have shown the relationship between ROS production in PACT and the changes in the expression of virulence determinants of yeasts (6, 33, 34), contributing to antimicrobial activity by damaging lipids, DNA, and proteins of microbial cells (35). Plasma membrane is another site of cellular damage that has been demonstrated in yeasts following PACT (36).

The images of confocal laser scanning microscopy exhibited a large amount of death cells compared with controls groups, reaching the entire thickness of biofilms. Similar analyze by scanning electronic microscopy images also showed an intense modification on biofilm morphological structure. This results suggest either effects on cells viability and disorganization and desegregation of the microorganisms in the biofilm (28).

In conclusion, our findings suggest the use of this *in vitro* biofilm model, that closely mimics the *in vivo* biofilm, provides an excellent test of the potential of PACT in achieving biofilm inactivation against keystone pathogens present in persistent endodontic infection. However this therapy presents different challenges regarding its susceptibility to different microorganisms, there is no doubt that further refinement and enhancement of PACT procedure might be necessary to extrapolations these results to clinical approaches.

References

1. Wilson CE, Cathro PC, Rogers AH, Briggs N, Zilm PS. Clonal diversity in biofilm formation by Enterococcus faecalis in response to environmental stress associated with endodontic irrigants and medicaments. I Endod J 2015;48(3):210-219.

2. Turner SR, Love RM, Lyons KM. An in-vitro investigation of the antibacterial effect of nisin in root canals and canal wall radicular dentine. Int Endod J 2004;37(10):664-671.

3. Nair PN. Pathogenesis of apical periodontitis and the causes of endodontic failures. Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists. Crit Rev Oral Biol Med 2004;15(6):348-381.

4. Leonardo MR, Rossi MA, Silva LA, Ito IY, Bonifacio KC. EM evaluation of bacterial biofilm and microorganisms on the apical external root surface of human teeth. J Endod 2002;28(12):815-818.

5. George S, Basrani B, Kishen A. Possibilities of gutta-percha-centered infection in endodontically treated teeth: an in vitro study. J Endod 2010;36(7):1241-1244.

6. Senges C, Wrbas KT, Altenburger M, Follo M, Spitzmuller B, Wittmer A, et al. Bacterial and Candida albicans adhesion on different root canal filling materials and sealers. J Endod 2011;37(9):1247-1252.

7. Andrade FB, Midena RZ, Koga-Ito CY, Duarte MAH. Conventional and Natural products against oral infections. In: Méndez-Vilas A. Microbial pathogens and strategies for combating them: science, technologies and education 2013;3:1574-1583.

8. Ng R, Singh F, Papamanou DA, Song X, Patel C, Holewa C, et al. Endodontic photodynamic therapy ex vivo. J Endod 2011;37(2):217-222.

9. Fekrazad R, Ghasemi Barghi V, Poorsattar Bejeh Mir A, Shams-Ghahfarokhi M. In vitro photodynamic inactivation of Candida albicans by phenothiazine dye (new methylene blue) and Indocyanine green (EmunDo(R)). Photodiagnosis Photodyn Ther 2015;12(1):52-57.

10. Pinheiro SL, Donega JM, Seabra LM, Adabo MD, Lopes T, do Carmo TH, et al. Capacity of photodynamic therapy for microbial reduction in periodontal pockets. Lasers Med Sci 2010;25(1):87-91.

11. Novaes AB, Jr., Schwartz-Filho HO, de Oliveira RR, Feres M, Sato S, Figueiredo LC. Antimicrobial photodynamic therapy in the non-surgical treatment of aggressive periodontitis: microbiological profile. Lasers Med Sci 2012;27(2):389-395.

12. Fekrazad R, Poorsattar Bejeh Mir A, Ghasemi Barghi V, Shams-Ghahfarokhi M. Eradication of C. albicans and T. rubrum with photoactivated indocyanine green, Citrus aurantifolia essential oil and fluconazole. Photodiagnosis Photodyn Ther 2015;12(2):289-297

13. Bremenkamp RM, Caris AR, Jorge AO, Back-Brito GN, Mota AJ, Balducci I, et al. Prevalence and antifungal resistance profile of Candida spp. oral isolates from patients with type 1 and 2 diabetes mellitus. Arch Oral Biol 2011;56(6):549-555.

14. Koga-Ito CY, Lyon JP, Vidotto V, de Resende MA. Virulence factors and antifungal susceptibility of Candida albicans isolates from oral candidosis patients and control individuals. Mycopathologia 2006;161(4):219-223.

15. Lew HP, Quah SY, Lui JN, Bergenholtz G, Hoon Yu VS, Tan KS. Isolation of alkaline-tolerant bacteria from primary infected root canals. J Endod 2015;41(4):451-456.

16. Nunes MR, Mello I, Franco GC, de Medeiros JM, Dos Santos SS, Habitante SM, et al. Effectiveness of photodynamic therapy against Enterococcus faecalis, with and without the use of an intracanal optical fiber: an in vitro study. Photomed Laser Surg 2011;29(12):803-808.

17. Silva LA, Novaes AB, Jr., de Oliveira RR, Nelson-Filho P, Santamaria M, Jr., Silva RA. Antimicrobial photodynamic therapy for the treatment of teeth with apical periodontitis: a histopathological evaluation. J Endod 2012;38(3):360-366.

18. Silva Garcez A, Nunez SC, Lage-Marques JL, Jorge AO, Ribeiro MS. Efficiency of NaOCl and laser-assisted photosensitization on the reduction of Enterococcus faecalis in vitro. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;102(4):e93-98.

19. Guerreiro-Tanomaru JM, de Faria-Junior NB, Duarte MA, Ordinola-Zapata R, Graeff MS, Tanomaru-Filho M. Comparative analysis of Enterococcus faecalis biofilm formation on different substrates. J Endod 2013;39(3):346-350.

20. Delben JAM, R. M.; Wei, X.; Castro, M. L.; Assunção, W. G.; Da Silva, R. N. F.; Duarte, S. Low-temperature plasma: an effective approuch against Candida albicans biofilm. Plasma Med 2014;4(1-4):231-244.

21. Duarte S, Gregoire S, Singh AP, Vorsa N, Schaich K, Bowen WH, et al. Inhibitory effects of cranberry polyphenols on formation and acidogenicity of Streptococcus mutans biofilms. FEMS Microbiol Lett 2006;257(1):50-56.

22. Klein MI, Duarte S, Xiao J, Mitra S, Foster TH, Koo H. Structural and molecular basis of the role of starch and sucrose in Streptococcus mutans biofilm development. Appl Environ Microbiol 2009;75(3):837-841.

23. Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersboll BK, et al. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology 2000;146 (Pt 10):2395-2407.

24. Bergmans L, Moisiadis P, Huybrechts B, Van Meerbeek B, Quirynen M, Lambrechts P. Effect of photo-activated disinfection on endodontic pathogens ex vivo. Int Endod J 2008;41(3):227-239.

25. Paschoal MA, Santos-Pinto L, Lin M, Duarte S. Streptococcus mutans photoinactivation by combination of short exposure of a broad-spectrum visible light and low concentrations of photosensitizers. Photomed Laser Surg 2014;32(3):175-180.

26. Garcia VG, Longo M, Gualberto Junior EC, Bosco AF, Nagata MJ, Ervolino E, et al. Effect of the concentration of phenothiazine photosensitizers in antimicrobial photodynamic therapy on bone loss and the immune inflammatory response of induced periodontitis in rats. J Period Res 2014;49(5):584-594.

27. Souza RC, Junqueira JC, Rossoni RD, Pereira CA, Munin E, Jorge AO. Comparison of the photodynamic fungicidal efficacy of methylene blue, toluidine blue, malachite green and low-power laser irradiation alone against Candida albicans. Lasers Med Sci 2010;25(3):385-389.

28. de Souza DL, Lima RA, Zanin IC, Klein MI, Janal MN, Duarte S. Effect of twice-daily blue light treatment on matrix-rich biofilm development. PloS One 2015;10(7):e0131941.

29. Fimple JL, Fontana CR, Foschi F, Ruggiero K, Song X, Pagonis TC, et al. Photodynamic treatment of endodontic polymicrobial infection in vitro. J Endod 2008;34(6):728-734.

30. Souza LC, Brito PR, de Oliveira JC, Alves FR, Moreira EJ, Sampaio-Filho HR, et al. Photodynamic therapy with two different photosensitizers as a supplement to instrumentation/irrigation procedures in promoting intracanal reduction of Enterococcus faecalis. J Endod 2010;36(2):292-296.

31. Soares BM, da Silva DL, Sousa GR, Amorim JC, de Resende MA, Pinotti M, et al. In vitro photodynamic inactivation of Candida spp. growth and adhesion to buccal epithelial cells. J Photochem Photobiol B 2009;94(1):65-70.

32. Prates RADS, E. G.; Yamada, A. M.; Suzuki, L. C.; Paula, C. R.; Ribeiro, M. S. Light parameters influence cell viability in antifungal photodynamic therapy in a fluence and rate fluence-dependent manner. Laser Phys Lett 2009;19:1038-1044.

33. Munin E, Giroldo LM, Alves LP, Costa MS. Study of germ tube formation by Candida albicans after photodynamic antimicrobial chemotherapy (PACT). J Photochem Photobiol B 2007;88(1):16-20.

34. Kato IT, Prates RA, Sabino CP, Fuchs BB, Tegos GP, Mylonakis E, et al. Antimicrobial photodynamic inactivation inhibits Candida albicans virulence factors and reduces in vivo pathogenicity. Antimicrob Agents Chemother 2013;57(1):445-451.

35. Seider K, Heyken A, Luttich A, Miramon P, Hube B. Interaction of pathogenic yeasts with phagocytes: survival, persistence and escape. Curr Opin Microbiol 2010;13(4):392-400.

36. Giroldo LM, Felipe MP, de Oliveira MA, Munin E, Alves LP, Costa MS. Photodynamic antimicrobial chemotherapy (PACT) with methylene blue increases membrane permeability in Candida albicans. Lasers Med Sci 2009;24(1):109-112.

3.3. Capítulo 3

Antimicrobial activity of Tissue-Tolerable Plasma on *Enterococcus faecalis* Biofilm

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Key-words: *Enterococcus faecalis*, Plasma gases, Disinfection, Biofilms, Root Canal Therapy, Microscopy, Confocal

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Declaration of interests:

There are no potential conflicts of interests relating to this study

Abstract

Introduction: Plasma-based dental applications have attracted much attention in antimicrobial disinfection procedures due to ability to operate at room temperature and to generate high concentrations of highly reactive free radicals. This study investigated the antimicrobial activity of tissue-tolerable-plasma (TTP) against 2-week *Enterococcus faecalis* biofilm.

Methods: Saliva-coated-hydroxyapatite-discs with 2-week *E. faecalis* (ATCC 29212) biofilms were treated with TTP and divided to undergo (1-3) TTP treatment for 3 different exposure times (1, 2 and 5 min), (4) 2.5% NaOCl for 5 min (positive control group), (5) argon gas alone and (6) buffered-saline-solution for 5 min (negative control groups). Biofilms were processed and the number of colony forming units (cfu/mL) was recorded. Three independent replicates were performed. The antimicrobial efficacy was assessed by CFU method, dry weight determination, in addition to visualization by confocal laser and scanning electron microscopies (CLSM/SEM). The CLSM images were quantified by COMSTAT.

Results: TTP produced dose-depedent reduction in CFUs relative to negative controls. Treatment for 5 minutes had higher antimicrobial efficacy than TTP 1 or 2 min ($p<0.05$). The SEM analysis showed morphology changes of the *E. faecalis* biofilm by plasma. CLSM images indicated that TTP treatment induced a destruction in deep layers of *E. faecalis* biofilm. COMSTAT analysis showed the prevalence of dead cells, in 5 min exposure group, in all different layers of biofilm studied.

Conclusion: Although the current results are limited to an *in vitro* model, TTP had a high efficacy in disinfecting the *E. faecalis* biofilm.

Key-words: *Enterococcus faecalis* biofilms, cold plasma, disinfection.

Introduction

Microorganisms and their products are etiologic factors of pulp and periapical disease. Although the occurrence of *Enterococcus faecalis* in primary endodontic infections is low, they become important species amongst the microbial survivors of root canal treatment (1). The ability to form a biofilm is an important factor contributing to the persistence of *E. faecalis* at the root canal, following optimal endodontic treatment, considering the biofilms are orders of magnitude more resistant to phagocytosis, antimicrobials and antibodies than planktonic bacteria, making biofilms extremely difficult to eradicate from living host tissues (2, 3).

The presence of a biofilm is related to the majority of the infections (4). Biofilm can form on virtually any type of surface, although hydroxyapatite and bovine dentin are more commonly used for analyses of oral biofilm characteristics (5-9). The ability of microorganism to establish and form a biofilm on a specific surface depends on their capacity of attachment to a substratum, microcolony formation and build up of the biofilm, in addition to the nature of surface and environmental conditions (4). Furthermore, learning about how the architecture of the biofilm can interfere with its resistance is also important in order to propose an effective method to eradicate it.

Plasma is an electrical discharge produced by subjecting one or more gases to electrical field, either as constant or alternating amplitude (10). The ability to reach gas phase without the need to elevate the temperature allows the use of tissue tolerable plasma (TTP) for the treatment of temperature sensitive materials, including biological matter such as cells and tissues, without causing any thermal damage to the host (11, 12). Among the potential active plasma agents, it is widely accepted that chemically reactive oxygen and nitrogen species (RONS) such as O, O_3, NO e NO_2, play a crucial role in the biofilm inactivation process (13). According to all these characteristics, recently plasma-based dental application has attracted much attention as an effective emerging anti-biofilm agent (14, 15).

The aim of this study was therefore, to use a promising disinfection method to treat a new *in vitro* biofilm model, targeting the eradication of *E. faecalis* mature biofilm, focusing on the anti-biofilm property of tissue tolerable plasma.

Methods

Inoculum and Biofilm Model

E. faecalis (ATCC 29212) was obtained from singles colonies isolated on agar plates, inoculated in brain-heart infusion broth (BHI) and incubated overnight at 37° C and 5% CO_2. Saliva-coated discs were prepared after 1 hour incubation, in a 3-D rotator (Lab Line – Thermo scientific, USA), at 37°C in 24-well culture plates, containing 2 mL of a sterile clarified human whole saliva diluted in adsorption buffer (50 mM KCl, 1.0 mM KPO_4, 1.0 MM CaCl, 1.0 mM $MgCl_2$ – pH=6.5–1:1), containing 1mM phenylmethylsulphonyl fluoride (PMSF- 1:1000) (25). The solution was clarified by centrifugation at 8,500 rpm for 10 min at 4°C, filtered with a 0.22 µm pore size filter (Stericup, Millipore, USA).

Biofilms of *E. faecalis* were formed on saliva-coated hydroxyapatite discs (C-HA) (0.635 cm^2) placed into 24-well culture plates with 2 mL of sterile BHI broth, containing 3.2 X 10^7 CFU mL^{-1}, at 37° C in 5% CO_2 for 15 days (16). To prevent nutrient deficiency, the BHI broth was completely replaced every 24 hours.

Plasma System

The experiments were conducted using a TTP sterilization apparatus, developed by the Leibniz Institute for Plasma Science and Technology (Neoplas Tools – Kinpen, Greifswald, Germany). The device consists of a hand-held unit (length = 170 mm, diameter = 20 mm, weight = 170 g), a gas flow system, pulse generator and a plasma exposure stage. The main features are as follow: compressed gas: argon, gas flow: 5 L/min, inlet pressure: 1.5 bar, power input: 8 W at 220 V, 50/60 Hz. The plasma is generated from the top of the electrode and expands to the surrounding air outside the nozzle.

The distance between the jet nozzle and the biofilm surface was approximately 3 mm, and the temperature was measured with a thermometer for 5 min and resulted in 31.56 ± 0.48°C (Fig. 2).

Sterilization Test

The C-HA discs were randomly divided into 6 groups of 4 discs. The discs in group 1 (negative control group) were immersed in phosphate-buffered saline (PBS 1x) for 5 minutes. The groups 2-4 were treated by TTP for 1, 2 and 5 minutes, respectively. The discs in group 5 (positive control group) were treated for 5 minutes with 2.5% sodium hypochlorite. The C-HA discs (groups 2-4) were similarly scanned by the plasma tip, with a frequency of approximately 30 movements per minute, performed by the same operator, in order to treat the entire surface of the disc. After the treatment, the C-HA discs were gently rinsed in phosphate buffered saline. The opposite surface, with adherent biofilm that was not touched by the plasma, was cleaned with a sterile cotton swab. The same procedure was performed with the discs in the control groups.

At the end of the experimental period, the discs were placed in 5 mL of sterile saline solution and subject to a ultrasound bath (Ultrasonic Cleaner, FS140, Fisher Scientific, Pittsburgh, PA, USA) for 10 minutes. Three intervals of 15-s sonication pulses were used to homogenize the removed biofilms (Fisher Scientific, Sonic Dismembrator model 100; USA). The homogenized suspension was used for bacterial viability (cfu mg^{-1} of biofilm dry weight) (17).

A ten-fold serial dilution was carried out and 50 µL was placed onto blood agar. The plates were incubated at 37°C in 5% CO_2 for 48 h, and then the number of CFU mg-1 of biofilm dry weight were obtained. Each assay was carry out in triplicate.

Effect of Blowing and Heating on Cell Reduction – Negative control (group 6)

In order to distinguish the TTP effect from the possible gas blowing effect and heating, the survival rates of the *E. faecalis* biofilm were also assessed by using the same argon flow rate, in a exposure time of 5 min, but with the plasma switched off. The CFU assay follows the same protocol described previously (18).

Dry weight

For the dry weight determination, three volumes of cold ethanol (-20°C) were added to 1 mL of biofilm suspension, and the resulting precipitate was centrifuged (10,000 g for 10 min at 4° C). The supernatant was discarded, and the pellet was washed with cold ethanol, and then lyophilized and weighed (17).

Biofilm analysis by Confocal Laser Scanning Microscopy/COMSTAT

The presence of dead and live bacteria on the biofilm surface was visualized by confocal laser scanning microscope and analyzed by COMSTAT. Two biofilm samples of each group were stained with Live/Dead Baclight Bacterial Viability kit (Molecular probes. Invitrogen, Eugene, Oregon. USA) in accordance with the manufacturer

Each sample was processed and analyzed individually and 5 images of each biofilm were taken from randomly chosen areas in each biofilm. All the samples were examined under a CLSM (Leica Lasertechnik GmbH, Heidelberg, Germany), with a HCX APOL U-V-I 40X/0.8-numerical-aperture water immersion objective. The stained samples were incubated at room temperature in the dark for 30 min and examined under CLSM. The bacterial biomass ($\mu m^3/\mu m^2$) were quantified by COMSTAT (19,20).

Biofilm Visualization by Scanning Electron Microscopy (SEM)

The biofilm morphology changes were observed by SEM after the treatment with TTP and visually compared with the controls groups. Two specimens of each group were immersed in a fixative solution 4% paraformaldehyde at room temperature for 1 hour. The specimens were then submitted to increasing concentrations of ethanol for serial dehydration (ethanol 70%, 85% and 100%). The dehydrated specimens were dried using a desiccator, overnight, sputter-coated with gold-palladium, mounted on a stub and examined by SEM (Hitachi S3500N Variable Pressure Scanning Electron Microscopy, Boston, MA, USA) at 2,500x or 4,000x magnifications at 6-12Kv .

Statistical analysis

Colony forming units and dry weight were logarithmic transformed (base-10) prior to analysis. Treatment groups were compared, for each biofilm, by evaluating whether one mean fell within the 95% confidence intervals of any other mean. These limits were computed on the basis of the pooled variance estimate from a linear mixed model with fixed factors of group and random factors of subject and replications within a subject (IBM SPSS; version 22; IBM Corp, Armonk, NY). These models excluded data from the positive control, which was a constant, in order to satisfy the model assumption of homogeneous variances. Comparisons with the positive control were then achieved by evaluating whether the 95% confidence limits of each other experimental group included zero.

Results

Effectiveness of PACT and TTP Disinfection.

Fig. 1 shows that the initial total CFU mg^{-1} count reached 10^9. All TTP treatment groups showed reduced CFUs of *E. faecalis* compared with negative control groups ($p < 0.05$). Maximum exposure time of 5 minutes showed significantly better results, approximately 10^5, than 1 and 2 minutes groups, approximately 10^8. None of the exposure times tested were as good as the positive control group, where there were no detectable residual CFUs in the sample.

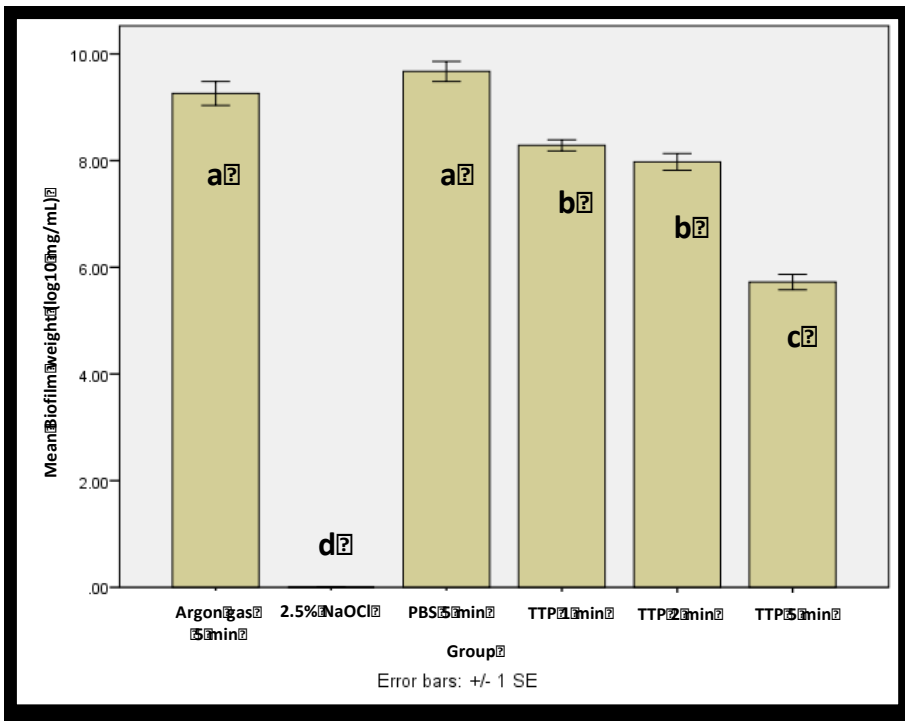


Figure 1: CFU count and dry weight in the C-HA discs with *E . faecalis* biofilm after TTP treatment for 1, 2 and 5 minutes. The positive control group shows the *E. faecalis* biofilm treated with 2.5% sodium hypochlorite. Negative controls groups were PBS and argon gas. Values marked with different letters are significantly different from each other (p<0,05).

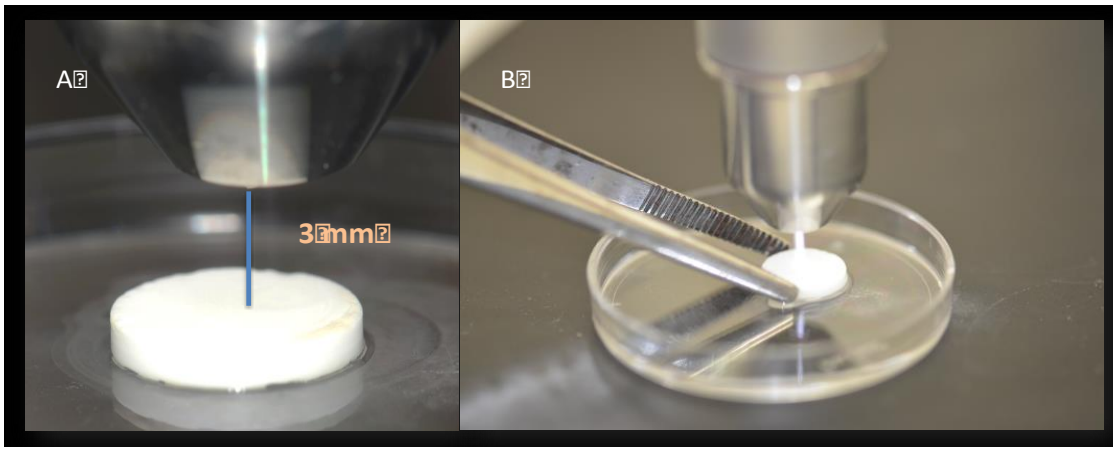


Figure 2: (A) Photographs of argon/plasma hand-held unit showing the distance between the jet nozzle and biofilm surface. (B) The C-HA disc contaminated with 2-week *E. faecalis* biofilm being scanned by the plasma tip.

SEM investigation

The SEM images illustrate the effects of the plasma treatment of *E. faecalis* biofilm resulting in a significant biofilm morphology and structure changes. There was a visual difference between the all plasma exposure times. After 5 min exposure, the images shows mostly the C-HA disc surface with rare biofilm structure attached.

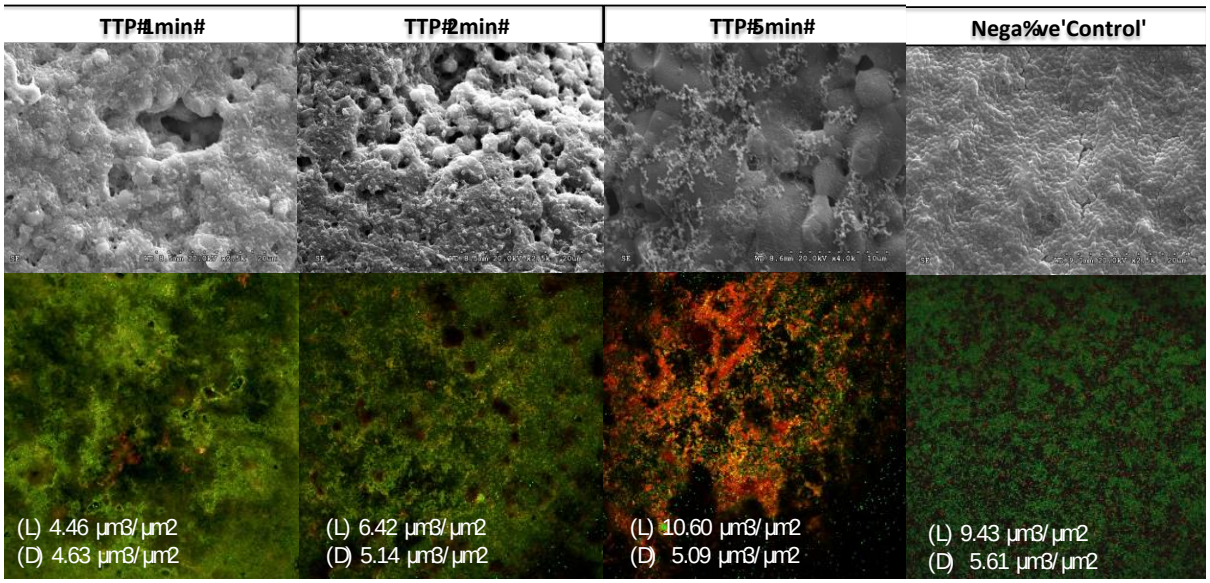


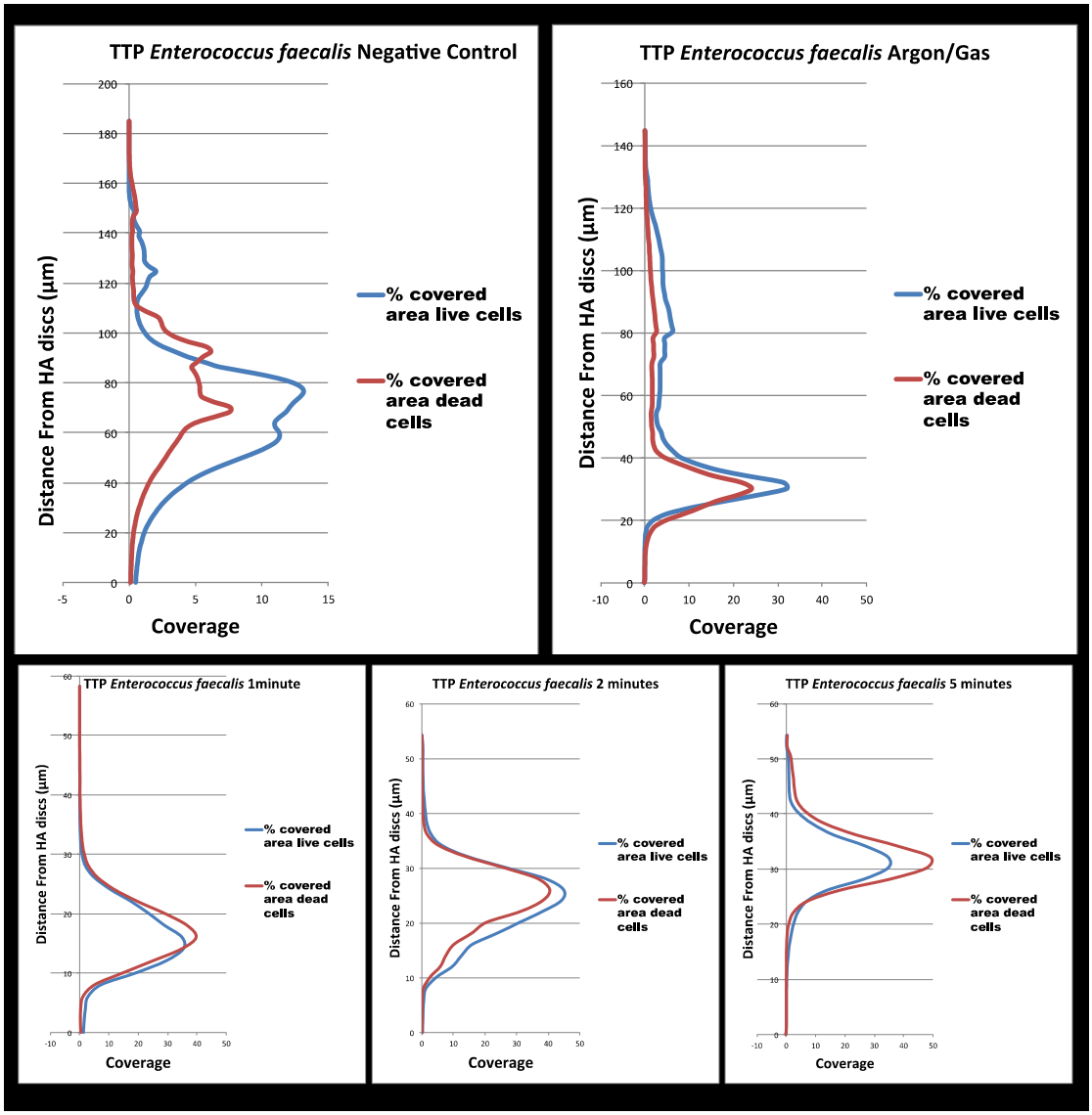
Figure 3: SEM Comparative analysis of *E. faecalis* biofilm groups at 2.500x, in three different exposure times of TTP and PBS group, with tip-to-sample distance of 3mm, showing the morphological structure of the biofilm severely modified. In TTP 5 min group a large surface of HA discs free of biofilm is visualized at 4.000x. Confocal Scanning Laser Microscopy images obtained from the mid-area of HA disc showing the overlap of live (L) and dead (D) cells. The average of total bacterial biomass calculated by COMSTAT in all of 5 points are shown.

Confocal Laser Scanning Microscopy/COMSTAT

The CLSM 3D and 2-dimensional images show the efficacy of TTP therapy. After 5 minutes of TTP exposure, HA discs infected with 2-week *E. faecalis* biofilm, near the whole layers exhibited red fluorescence in different percentages and thickness, which indicated that bacteria was dead by the effect of therapy. Lower antibacterial effect exhibited 1 and 2 minutes groups, alternating percentage of green (live cells) and red (dead cells) coverage throughout the layers of biofilm. Negatives control and argon gas groups, showed the whole biofilm covered by green fluorescence, which suggest that bacteria are alive in all layers of biofilm. COMSTAT software analyzed the fraction of the area occupied by biomass in each layer of the biofilm, in means of the average of percentage of coverage, in each image of a stack, among the 5 points randomly chosen. Different peaks in different areas of

biofilm show how deeply the applied therapy affect the biofilm. TTP therapy shows highly peaks of dead cells in deep layers of biofilm, which suggest that, TTP show high rate of killing *E. faecalis* biofilm in different layers of biofilm (Fig.4).

Figure 04. Representative three-dimensional images of the *E. faecalis* biofilm viability after different exposure times of TTP rendered images of the outer layers of biofilm. The graphics show the percentage area occupied by green (live cells) and red (dead cells), from the substratum surface to the top layer of the biofilm analyzed by COMSTAT .



Discussion

Eliminating residual microorganisms within the biofilm is a challenging task. Bacteria organized in biofilms have a low metabolic rate and seems to be more resistant to conventional antimicrobial therapies (8). Scientific evidence has shown that *E. faecalis* is often isolated in persistent endodontic infection and it is, therefore, present in many *in vitro* studies regarding the effectiveness of different antimicrobial application for endodontic therapy. The potential antibacterial activity of the sodium hypochlorite, as a widely used irrigant agent during the traditional endodontic treatment, is well documented as a “gold standard” (21).

Hydroxyapatite (HA) substrates were chosen because it mimics the tooth mineral and it is easily available (17), and therefore previous *in vitro* microbiological studies show a suitable layer of biofilm growth on HA disc surface in different cultures ages (8, 9, 16). Guerreiro-Tanomaro *et al* (16) comparing *E. faecalis* biofilm formation on different substrates, also concluded that hydroxyapatite was the substrate with the best conditions for biofilm development. Shen *et al* (22) and Dufour *et al* (23) recently reported the pattern of the effect of biofilm age (maturation) on the resistance of bacteria, showing that two-weeks or more-old biofilms became more resistant than newly grown biofilm, but the differences were rather small. Although there is no earlier literature supporting the use of saliva coated HA discs with *E. faecalis* biofilm, this *in vitro* model showed to be suitable for TTP anti-biofilm efficacy assay (17).

We used a single-electrode of a tissue-tolerable atmospheric pressure plasma, with argon gas as the working gas, against *E. faecalis* biofilms formed on saliva coated HA discs. In accordance with a previous study (3), the proportion of live cells decrease significantly with the longer exposure to plasma, reaching the maximum level of deactivation within 5 minutes of treatment, in a directly time-dependent manner. Similarly, Du *et al.* (14) reported that, for the same exposure times, the atmospheric pressure plasma and 2% chlorhexidine were equally effective at reducing bacteria CFU numbers. Different results are also been reported by Pan *et al.* (3) and Li *et al*. (24), showing a significant decrease in the number of CFUs after prolonged cold plasma treatment of 8 to 10 minutes and 12 minutes of exposure,

respectively. In our experiments, the temperature on the plasma tip, during 5 min of treatment, was slightly above the room temperature, which excludes harmful thermal effects on the bacterial cells.

To determine changes in *E. faecalis* biofilms with respect of morphology and topography we used SEM images to accurately the biofilm matrix morphology possibly altered by the effect of plasma. In addition, the CLSM images showed larges areas of the biofilm stained in red, corresponding to the damage on the bacterial cells caused by TTP treatment. The COMSTAT analysis also demonstrate that TTP was able to penetrate the biofilms and treat the deepest parts of it, which is presented in figure 4.

We hypothesize the inactivation of *E. faecalis* biofilm by TTP can be attributed to the production of RONS which play a main role in the inactivation process, as it has been suggested in several previous studies (3, 13, 14, 25). Ultraviolet radiation is also other possible mechanism contributing to the inactivation, leading to destruction of the matrix of the extra-cellular polymeric substance in the biofilm (10), which is confirmed by the CLSM image analysis.

Considering all the challenges related to root canal disinfection during endodontic treatment, the development of new methods and alternative therapies are highly desirable, mostly aiming to reach the treatment goals without toxicity to the patient. More studies need to be conducted to be able to translate the *in vitro* data to the clinical approaches. However the test settings in the present study indicate that use of a non-thermal atmospheric plasma may be remarkably useful as a disinfecting alternative treatment against *Enterococcus faecalis* biofilms. This study is novel and generates new hypothesis regarding the use of alternative therapies to complement the endodontic treatment.

Conclusion

Under the operating conditions used in this *in vitro* study, the use of tissue tolerable plasma has a distinct advantage of achieving high rates of *Enterococcus faecalis* biofilm inactivation in a short period of time.

References

1. Stuart CH, Schwartz SA, Beeson TJ, Owatz CB. Enterococcus faecalis: its role in root canal treatment failure and current concepts in retreatment. J Endod 2006;32(2):93-98.

2. Distel JW, Hatton JF, Gillespie MJ. Biofilm formation in medicated root canals. J Endod 2002;28(10):689-693.

3. Pan J, Sun K, Liang Y, Sun P, Yang X, Wang J, et al. Cold plasma therapy of a tooth root canal infected with enterococcus faecalis biofilms in vitro. J Endod 2013;39(1):105-110.

4. Hancock V, Witso IL, Klemm P. Biofilm formation as a function of adhesin, growth medium, substratum and strain type. Int J Med Microbiol 2011;301(7):570-576.

5. Brandle N, Zehnder M, Weiger R, Waltimo T. Impact of growth conditions on susceptibility of five microbial species to alkaline stress. J Endod 2008;34(5):579-582.

6. Del Carpio-Perochena AE, Bramante CM, Duarte MA, Cavenago BC, Villas-Boas MH, Graeff MS, et al. Biofilm dissolution and cleaning ability of different irrigant solutions on intraorally infected dentin. J Endod 2011;37(8):1134-1138.

7. Ordinola-Zapata R, Bramante CM, Cavenago B, Graeff MS, Gomes de Moraes I, Marciano M, et al. Antimicrobial effect of endodontic solutions used as final irrigants on a dentine biofilm model. Int Endod Journal 2012;45(2):162-168.

8. Pappen FG, Shen Y, Qian W, Leonardo MR, Giardino L, Haapasalo M. In vitro antibacterial action of Tetraclean, MTAD and five experimental irrigation solutions. Int Endod Journal 2010;43(6):528-535.

9. Shen Y, Stojicic S, Qian W, Olsen I, Haapasalo M. The synergistic antimicrobial effect by mechanical agitation and two chlorhexidine preparations on biofilm bacteria. J Endod 2010;36(1):100-104.

10. Moisan M, Barbeau J, Moreau S, Pelletier J, Tabrizian M, Yahia LH. Low-temperature sterilization using gas plasmas: a review of the experiments and an analysis of the inactivation mechanisms. Inter J Pharm 2001;226(1-2):1-21.

11. Kunhardt EE. Generation of large-volume, atmospheric-pressure, nonequilibrium plasmas. LEEE Transitions on Plasma Sci 2000;28(1):189-200.

12. Kogelschatz U. Filamentary, patterned, and difuse barrier discharges. LEEE Transitions on Plasma Sci 2000;30(4):1400-1408.

13. Cao YY, P.; Lu, X. . Efficacy of atmospheric pressure plasma as an antibacterial agent against Enterococcus faecalis in vitro. Plasma Sci Technol 2011;13:93-98.

14. Du T, Ma J, Yang P, Xiong Z, Lu X, Cao Y. Evaluation of antibacterial effects by atmospheric pressure nonequilibrium plasmas against Enterococcus faecalis biofilms in vitro. J Endod 2012;38(4):545-549.

15. Koban I, Holtfreter B, Hubner NO, Matthes R, Sietmann R, Kindel E, et al. Antimicrobial efficacy of non-thermal plasma in comparison to chlorhexidine against dental biofilms on titanium discs in vitro - proof of principle experiment. J Clin Periodontol 2011;38(10):956-965.

16. Guerreiro-Tanomaru JM, de Faria-Junior NB, Duarte MA, Ordinola-Zapata R, Graeff MS, Tanomaru-Filho M. Comparative analysis of Enterococcus faecalis biofilm formation on different substrates. J Endod 2013;39(3):346-350.

17. Duarte S, Gregoire S, Singh AP, Vorsa N, Schaich K, Bowen WH, et al. Inhibitory effects of cranberry polyphenols on formation and acidogenicity of Streptococcus mutans biofilms. FEMS Microbiol Lett 2006;257(1):50-56.

18. Delben JAM, R. M.; Wei, X.; Castro, M. L.; Assunção, W. G.; Da Silva, R. N. F.; Duarte, S. Low-temperature plasma: an effective approuch against Candida albicans biofilm. Plasma Med 2014;4(1-4):231-244.

19. Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersboll BK, et al. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology 2000;146 (Pt 10):2395-2407.

20. Klein MI, Duarte S, Xiao J, Mitra S, Foster TH, Koo H. Structural and molecular basis of the role of starch and sucrose in Streptococcus mutans biofilm development. Appl Environ Microbiol 2009;75(3):837-841.

21. Siqueira JF, Jr., Rocas IN, Favieri A, Lima KC. Chemomechanical reduction of the bacterial population in the root canal after instrumentation and irrigation with 1%, 2.5%, and 5.25% sodium hypochlorite. J Endod 2000;26(6):331-334.

22. Shen Y, Stojicic S, Haapasalo M. Antimicrobial efficacy of chlorhexidine against bacteria in biofilms at different stages of development. J Endo 2011;37(5):657-661.

23. Dufour DL, V. Bacterial bioflm: structure, function, and antimicrobial resistance. Endod Topics 2012;22:2-16.

24. Li Y, Sun K, Ye G, Liang Y, Pan H, Wang G, et al. Evaluation of Cold Plasma Treatment and Safety in Disinfecting 3-week Root Canal Enterococcus faecalis Biofilm In Vitro. J Endod 2015;41(8): 1325-1330.

25. Lu XJ, Z.; Xiong, Q. A single electrode roo-temperature plasma jet device for biomedical applications. Appl Phys Lett 2008;92:151504.

3.4. Capítulo 4

Photodynamic Chemotherapy and Tissue Tolerable Plasma: An Effective Approach Against *Enterococcus faecalis* Biofilm

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Key-words: Photochemotherapy. Photodynamic antimicrobial chemotherapy. Tissue-tolerable plasma. Microorganisms. Biofilms

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Declaration of interests:

There are no potential conflicts of interests relating to this study.

Abstract

Introduction: Although multispecies biofilm are involved in almost all infections of the oral cavity, *Enterococcus faecalis* is a key microorganism involved in chronic apical periodontitis. The aim of this study was to evaluate the activity of Photodynamic Chemotherapy (PACT) and Tissue Tolerable Plasma (TTP) against 2-week *E. faecalis* biofilms.

Methods: Saliva-coated**-**teeth with 2-week *E. faecalis* (ATCC 29212) biofilm were treated with PACT and TTP for 3 different exposure times (1, 2 and 5 minutes) and compared with 2.5% NaOCl irrigation for 5 minutes. Biofilms were processed, serially diluted and cultured on agar plates and the number of colony-forming units (cfu/mL) was recorded. Anti-biofilm efficacy was assessed by counting colony-forming units and group means were compared using 1-way ANOVA. Confocal laser scanning microscopy analyzed by COMSTAT software was used to confirm the viability of the biofilm in outer layers of the biofilm. Scanning electron microscopy was used to asses the morphological changes of *E. faecalis* biofilm by PACT and TTP.

Results: The anti-biofilm effect of PACT and TTP improved as exposure time was increased, reaching the maximum effect after 5 minutes for both therapies. At 5 min exposure, there is a significant reduction in CFUs in PACT and TTP treatments ($p<0.05$), but neither treatment was as effective as 2.5% NaOCl irrigation. Bacterial killing was confirmed by CLSM/COMSTAT and SEM analysis.

Conclusion: TTP and PACT are effective adjunctive therapies, contributing for sterilization effect on fully matured biofilm within 5 minutes.

Keywords: photosensitizer, antimicrobial, oral biofilms, reactive oxygen species (ROS).

Introduction

Enterococcus faecalis often exist in the form of biofilm and are responsible for persistent pulpo-periapical infections processes (Siqueira and Roças. 2008; Murad et al. 2014). Effective root canal disinfection is undoubtedly a fundamental component of successful root canal treatment, nevertheless numerous studies have shown that complete elimination of bacteria from the root canal system cannot be consistently achieved with any of the currently used techniques, mostly because of the intricate canal anatomy and the innate resistance capacity of biofilms (Bonsor et al. 2006; Mickel et al. 2007; Roças et al. 2013, Li et al. 2015). In this regard, the use of Photodynamic chemotherapy (PACT) and, a novel antimicrobial intervention, Tissue Tolerable Plasma (TTP) have emerged as a technological complementary to endodontic treatment, focusing on an effective antimicrobial therapy (de Oliveira BP et al. 2014; Chrepa et al. 2014).

In PACT the light activates a specific photosensitizer forming short-lived and highly reactive oxygen species, including singlet oxygen, causing serious damage to microorganisms through irreversible oxidation of cell compounds (Garcez et al. 2013). Plasma is essentially a ionized gas generated by electromagnetic field. TTP has been increasingly highlighted in recent years in the biomedical field for its outstanding sterilization effects (Du et al. 2012; Li et al. 2015), tooth whitening (Pan et al. 2010), wound healing (Isbary et al. 2010), cell detachment and migration (Volotskova and Steep 2011), apoptosis of tumor cells (Lee et al. 2009) and dentin bond strength (Hirata et al. 2015).

Considering preparation of the root canal leaves part of the canal walls untouched, regardless of the instrumentation technique used (Wu et al. 2003), resulting in inconsistent removal of the contaminated innermost layer of dentin (Siqueira et al. 2007), novel approaches for disinfecting root canals may include the use of TTP and PACT. Both therapies can act as adjuvants to conventional endodontic treatment, potentially leading to a significant further reduction of bacterial load in the root canal system.

Within this background, the current study compared the antimicrobial activity of PACT and TTP on extracted human teeth infected with *Enterococcus faecalis* biofilm, focusing on the anti-biofilm property of each treatment.

Methods

Sample Preparation

Ninety six freshly intact human straight single-rooted pre-molar teeth with fully formed apices were used for cultivation of *E. faecalis* biofilm in the study. The teeth were selected, root surfaces cleaned and stored in 0.1% thymol solution at 4°C. The crown was removed at 2-3 mm bellow the cement-enamel junction to obtain a standard length of 12 mm. The working length (WL) was established by deducing 0.5 mm from the canal length. The canals were enlarged by a conventional multifile rotary system (BioRaCe [FKG, La-Chaux-de-Fonds, Switezerland]) driven with the VDW Silver motor (VDW GmbH), used in a crown-down manner, according to the manufacturer's instruction (500-600 rpm/ 1N/cm^2), using the following sequence: BR0 (25/0.08), BR1 (15/0.05), BR2 (25/0.04), BR3 (25/0.06), BR4 (35/0.04), and BR5 (40/0.04). After the glide path at each nickel-titanium file, root canals were irrigated with 1 mL 2.5% NaOCl for 1 minute. A final ultrasound bath (Ultrasonic Cleaner, FS140, Fisher Scientific, Pittsburgh, PA) with 17% EDTA for 3 minutes, followed by ultrapure water for 10 minutes (Klein et al. 2009) were performed for all groups. All root apical foramina were sealed with temporally filling paste Coltosol F (Colténe AG, Altstätten, Switzerland). Finally, the root canals were autoclaved for 20 minutes at 121°C to ensure no bacterial contamination and stored at 4°C (22). As no identifying information about the donor was collected, this research does not meet the definition of human subjects 45 CFR_46.102(f) and does not require Internal Review Board oversight.

Inoculum and Biofilm Model

Enterococcus faecalis (ATCC 29212) was obtained from singles colonies isolated on agar plates, inoculated in brain-heart infusion broth (BHI) and incubated overnight at 37°C under microaerophilic conditions (5% CO_2). Cell density was set at 3.2 X 10^7 colony forming units (CFU) by milliliter for the stock solution. Saliva-coated roots were prepared through 1 hour incubation with clarified human whole saliva added to adsorption buffer (50 mM KCl, 1.0 mM KPO^4, 1.0 mM CaCl, 0.1 mM $MgCl_2$ – pH=6.5 - 1:1) and phenylmethylsulphonyl

fluoride PMSF (1:1000), at 37° C in a 3D rotator (Lab-Line- Thermo scientific, USA) (Duarte et al. 2006). Saliva was collected from donors in a good health, maintained into ice-chilled tube and then centrifuged and filtered in 0.22 µm PES membrane (Stericup, Milipore, USA). The roots were placed in 24-well culture plates containing 1.8 mL of sterile BHI and 0.2 mL inoculum, at 37°C in 5% CO_2 for 14 days (Guerreiro-Tanomaru et al. 2013). To prevent nutrient deficiency, the BHI culture medium was completely replaced every 24 hours, without addition of new microorganisms. Random samples were examined microscopically to ensure the presence of *E. faecalis*.

Experimental Groups

The root canals were removed from 24 well-plate and randomly divided into six experimental groups and two controls groups, with 4 specimens each, according to the protocol of disinfection used. Saliva-coated-teeth with 2-week *E. faecalis* biofilm were treated with PACT or TTP for 3 different exposure times (1, 2 and 5 min). The root canals in the positive control group were used to evaluate the effectiveness of irrigation with 2.5% NaOCl for 5 min and roots in negative control group did not received any treatment. Three independent replicates were performed (n=12).

Plasma System

The experiments were conducted using an argon/gas plasma sterilization apparatus (Neoplas Tools – Kinpen, Greifswald, Germany). The device consists of a hand-held unit (length = 170 mm, diameter = 20 mm, weight = 170 g) connected to a high-frequency power supply. The main features are as follow: operating gas: argon (5 L/min), inlet pressure: 1.5 bar, power input: 8 W at 220 V, 50/60 Hz. A pin-type electrode (1 mm diameter) is mounted at the center of a quartz capillary (inner diameter 1.6 mm). The plasma plume emerge from the top of the electrode (1.5 mm diameter) and expands to the surrounding air outside the nozzle for a distance of up to 15 mm.

Photosensitizing agent and light sources

The photosensitizer used in this study consisted of Toluidine blue- O (TBO) at 100 µg/mL in ultrapure water (Sigma-Aldrich, St. Luis, MO). The solution was filtered in 0.22 µm PES membrane and immediately used.

A single source of a non-coherent light that produces the full spectrum of a visible red light was used (LumaCare® LC122A, Medical Group, Newport Beach, CA). This device is capable of delivering red light into a fiber-optic probe (spot size113.1 mm^2), with 630 nm wavelength, and output power of 95.5 mW cm^{-2}.

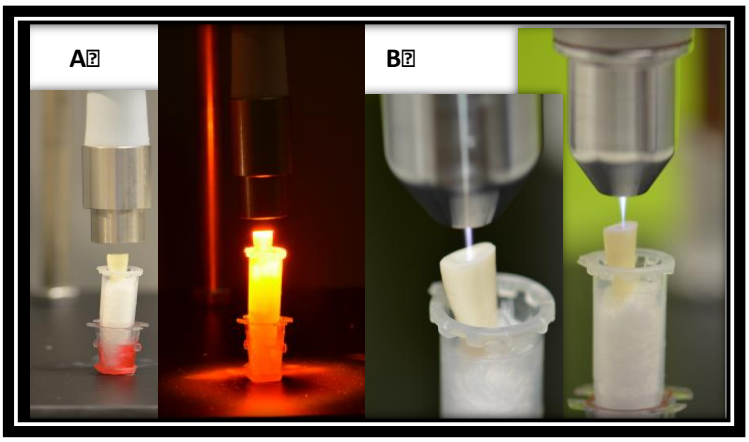

Figure 1. Representative image of root canals being treated with LumaCare® (A) and argon plasma (B).

In vitro PACT experiments

The root canals were completely filled with 100 µL of TBO/100 µg/mL. After 5 min in the dark (pre-irradiation time), the samples were exposed to 118.9 Jcm^{-2} (1 min), 237.8 Jcm^{-2} (2 min) and 594.5 Jcm^{-2} (5 min) of total energy. The work distance to activate de photosensitizer was set at 3 mm, avoiding thermal effects, which has been previously tested (data not shown) (Fig. 1).

In vitro TTP experiments and Controls groups

The specimens in TTP groups were used to evaluate the effectiveness of 3 different treatment exposure times (1, 2 and 5 minutes) on disinfection the of

E. faecalis biofilms in root canals. The distance between the jet nozzle and the biofilm surface was approximately 3 mm. The temperature was measured with a thermometer for 5 minutes and resulted in 31.56 ± 0.48°C (Fig. 1).

The root canals in positive control group were used to evaluate the effectiveness of irrigation with 2.5% NaOCl for 5 minutes with a sterile plastic 27 gauge syringe (Becton Dickinson & Co, New Jersey, USA). The needle was placed 1 mm short of the WL. The NaOCl was agitated with an ISO size 40 hand K-file (Dentsply Maillefer) and left undisturbed for 5 minutes. The roots in the negative control group did not received any treatment.

Assessment of Anti-biofilm Activity of PACT and TTP

The root canals were sampled to verify the presence of microbial growth and microorganisms viability, before and after the experimental period. An initial microbial sampling of each root canal in experimental and positive control groups was performed with a #40 sterile paper point inserted into the canal to collect the bacteria for 1 minute. The paper points were transferred to centrifuge tubes containing sterile saline solution, shaken vigorously for 1 minute to break up the clumps, and the baseline *E. faecalis* CFU was determined.

The antimicrobial efficacy was determined by the amount of recovered CFU and dry weight determination. After treatments, 15 µL of sterile saline solution was injected into the root canal. The biofilm suspension was collected with spiral utility brush (Versa Brush - Vista Dental, USA) driven with the VDW Silver motor at 500 rpm for 1 minute. The same procedure was performed in negative and positive control groups.

The spiral brush was then transferred to sterilized glass culture tubes containing 5 mL of sterile saline solution and the tubes were placed in a ultrasound bath (Ultrasonic Cleaner, FS140, Fisher Scientific, Pittsburgh, PA) for 10 minutes. The removed biofilms were subjected to sonication using 3 15-s pulses at an output of 7W (Fisher Scientific, Sonic Dismembrator, model 100; USA). The homogenized suspension was used either for bacterial viability (CFU mL^{-1}) and dry weight (CFU mg^{-1} of biofilm dry weight).

A ten-fold serial dilution was carried out and plated on blood agar, incubated in 5% CO_2 at 37°C for 48 h, and then the total number CFU mL^{-1} was

obtained. Three independent replicates were performed.

Dry weight

For the dry weight determination, three volumes of cold ethanol (-20°C) were added to 1 mL of biofilm suspension, and the resulting precipitate was centrifuged (10,000 g for 10 min at 4° C). The supernatant was discarded, and the pellet was washed with cold ethanol, and then lyophilized and weighed (Duarte et al. 2006).

Biofilm analysis by Confocal Laser Scanning Microscopy/COMSTAT

The viability of *E. faecalis* cells after TTP and PACT treatments were visualized by CLSM and quantified by COMSTAT (Heydorn et al. 2000; Klein et al. 2009). Two specimens for each group condition were cleaned and instrumented as described, split into two halves, autoclaved for 20 minutes at 121°C, and infected with *E. faecalis* biofilm for 2 weeks. The different treatments were conducted, the biofilms were stained with Live/Dead® Bacterial Viability kit (Baclight Bacterial Viability kit L7012; Molecular probes, Inc). The stains were prepared according to the manufacturer's instruction. The samples were incubated at room temperature for 30 min before the images were taken.

Each sample was processed and analyzed individually and 5 images of each biofilm were taken from random areas in each semi-root. All the samples were examined under a CLSM (Leica Lasertechnik GmbH, Heidelberg, Germany), with a HCX APOL U-V-I 40X/0.8-numerical- aperture water immersion objective. The bacterial biomass ($\mu m^3/\mu m^2$) was quantified by COMSTAT.

Biofilm Visualization by Scanning Electron Microscopy (SEM)

The cell morphology of *E. faecalis* biofilms were observed by SEM after the treatment with PACT and TTP and visually compared with the controls groups. Two specimens of each group were immersed in a fixative solution 4% paraformaldehyde at room temperature for 1 hour, followed by dehydration in ascending ethanol concentrations (70%, 85% and 100%). After drying overnight

at 37°C, the dehydrated specimens were mounted in aluminum stubs, sputter-coated with gold-palladium and examined by SEM (Hitachi S3500N Variable Pressure Scanning Electron Microscopy, Boston, MA, USA).

Results

Statistical Analysis.

Counts of CFU and dry weight were logarithmic transformed (base-10) and evaluated with a 1-way ANOVA for independent samples. Pairs of treatment means were then compared using the Tukey HSD method with a type 1 error rate of 5%. IBM SPSS version 21 (IBM, Inc., Armonk, NY) was used to perform the analysis. The confidence interval was set at 95%.

Effectiveness of PACT and TTP disinfection.

Figure 02 shows in (A) the CFU mL^{-1} baseline values, (B) the values after treatment in means of CFU mL^{-1} and in (C) CFU mg mL^{-1} of biofilm dry weight. After PACT and TTP therapies, there was a dose-dependent reduction in CFU relative to the control group (NC), showing that when exposure times reached 5 minutes, there was a significant reduction in CFU in both PACT and TTP compared with the negative control, about 5 and 4 logs, respectively (both $p<0.05$).

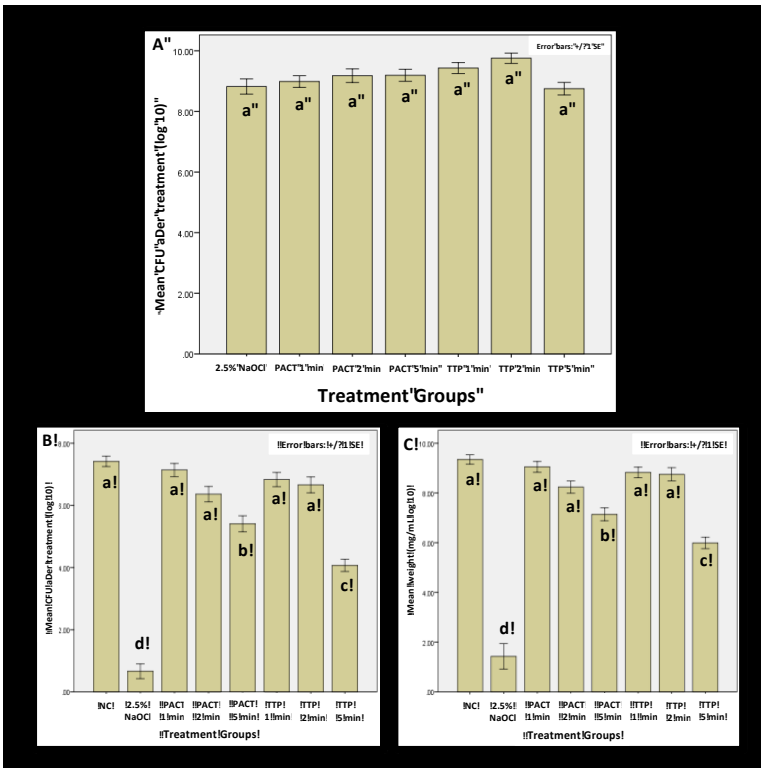

Figure 2. Disinfection results of TTP and PACT. (A) CFU mL^{-1} baseline values before treatments. *E. faecalis* biofilm was treated in three different exposure times and controls groups. In 5 min treatment groups, there is a significant decrease in means of CFU mL^{-1} (B) and CFU mg/mL^{-1} of biofilm dry weight (C). Values marked with different letters are significantly different from each other ($p<0,05$).

SEM

Scanning electron microscopy images (Figure 03) were taken of the sliced root canals with different magnifications. A highly organized *E. faecalis* biofilm structure was observed. However, after 5 min of both therapies, the 3-dimensional architectures were destroyed.

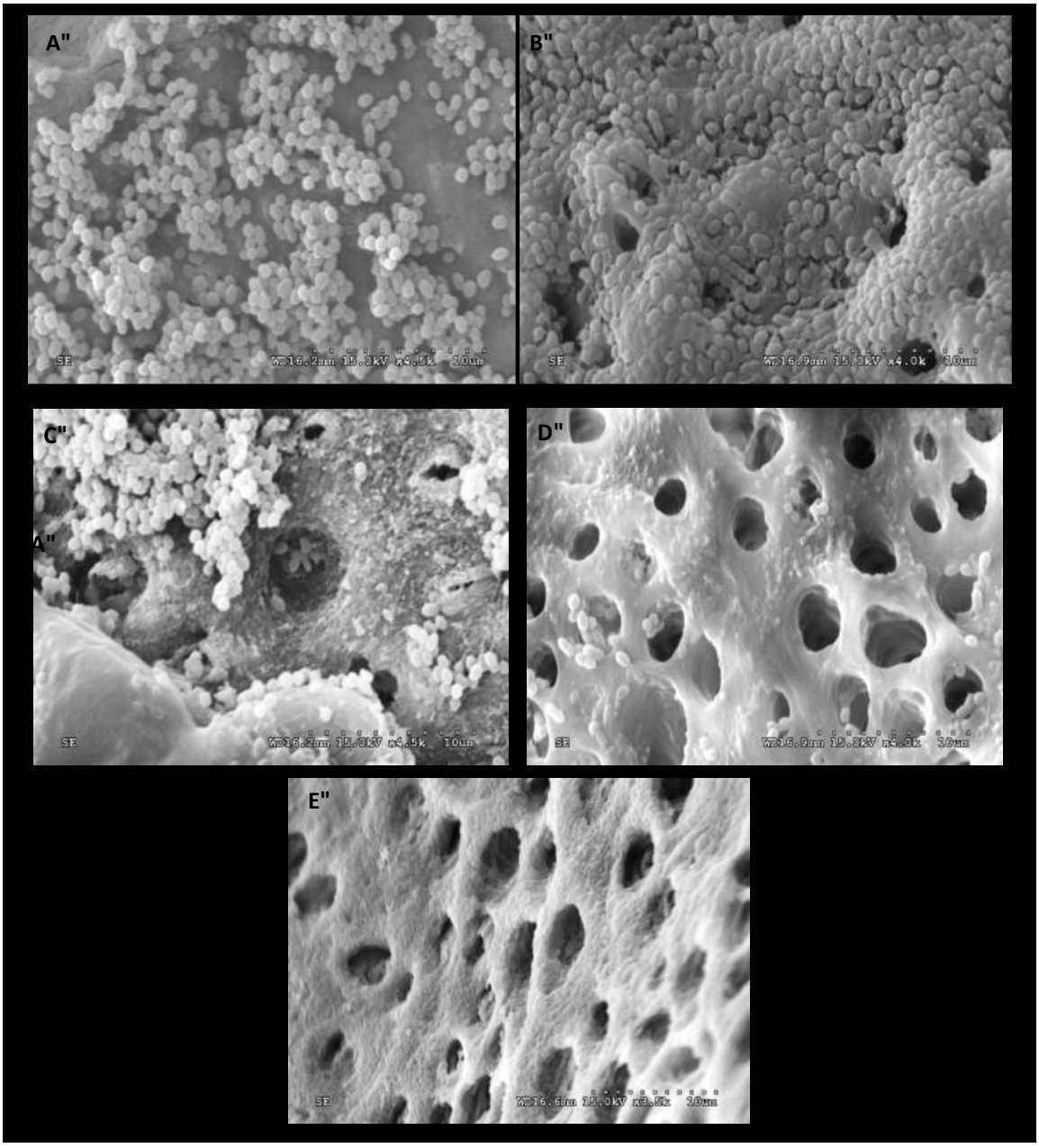

Figure 03. (A,C) *E. faecalis* biofilm adhered to the pulpal wall at 4.5K. (B) Aspect of dentin covered by *E. faecalis* biofilm morphologically changed after 5 minutes of light irradiation at 4.0K. (D) shows the aspect of dentin tubules after 5 minutes of plasma treatment at 4.0K magnification. (E) reveals the modification of the dentin wall after irrigation with 2.5% NaOCl for 5 minutes at 3.5K.

CLSM/COMSTAT

Figure 04 shows the CLSM biofilm images and the COMSTAT analysis after PACT and TTP treatment. In both therapies, the percentage of dead cells (biomass mm^3/mm^2) is always higher than alive cells, which suggest that, either PACT and TTP, show high rate of killing *E. faecalis* biofilm in all layers of biofilm.

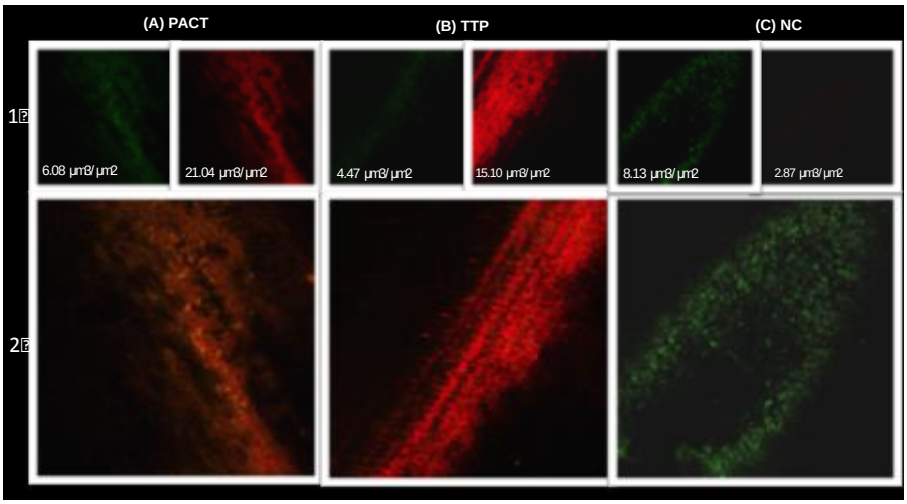

Figure 04. Viability of the two-weeks *E. faecalis* biofilms showed by confocal laser scanning microscopy. The biofilms were treated by PACT (A) and TTP (B) for 5 min and compared to root canals which did not received any treatment (C). (1) Red cells are considered dead and green cells are alive with the average of total biomass (µm3/µm2), in five points chosen in split roots samples, calculated by COMSTAT shown respectively. (2) Overlap of live and dead stacks.

COMSTAT software analyzed the fraction of the area occupied by biomass (green/ live and red/ dead) in each layer of the biofilm, in means of the average of percentage of coverage, in each image of a stack, among the 5 points randomly chosen in each sliced root. Different peaks in different areas of biofilm show how deeply the applied therapy affected the biofilm. TTP therapy shows highly peaks of dead cells in deep layers of biofilm, more than presented by PACT graphics, although in both therapies, the percentage of dead cells is always high than alive cells (Fig.5).

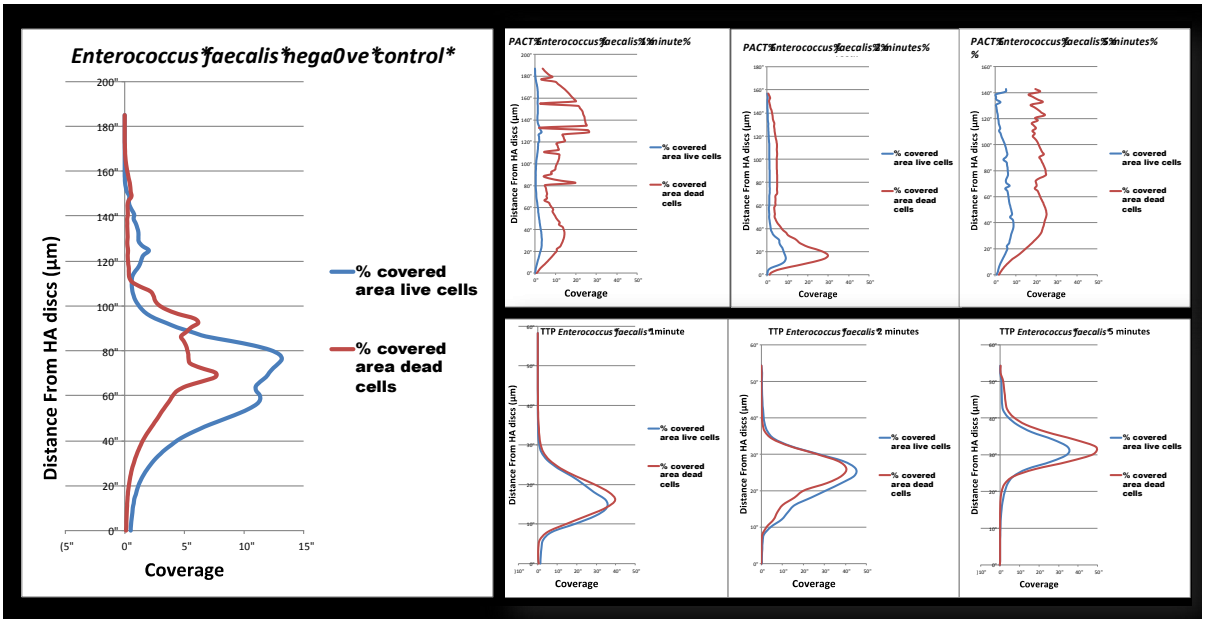

Figure 05. Representative images of the *E. faecalis* biofilm viability after different exposure times to TTP and PACT rendered images of the outer layers of biofilm. The graphics show the area occupied (%) by green (live cells) and red (dead cells) analyzed by COMSTAT, from the substratum surface to the top layer of the biofilm.

Discussion

It is well established that endodontic infections are caused by microorganisms, usually bacteria, in the biofilm mode of growth, residing in root canal system, including dentin tubules of the affected teeth (Zehnder. 2006). Organized in biofilms, microorganisms are firmly attached to a surface and are enclosed in a polymeric matrix formed by polysaccharides, nucleic acid, proteins, water and cell debris (Duarte et al. 2011; Li et al. 2015). Thus, biofilms can be up to 1.000-folder more resistant than their planktonic counterparts (Costerton et al. 1999), since the matrix offers protection against host defenses and often restricts the penetration of antimicrobial agents (Ordinola-Zapata et al. 2013; Lopez-Jimenes et al. 2015; Li et al. 2015). *E. faecalis* biofilm is commonly adopted model to investigate different antimicrobial methods (Stuart et al. 2006), moreover 2-weeks old biofilms become more resistant and hard to eliminate with traditional treatment (Stojicic et al. 2013; Li et al. 2015).

In an attempts to promote effective root canal disinfection, many studies search for a new methods to provide additional antibacterial therapies for

root canal system (Costerton et al. 1999; Lee et al. 2009; Isbary et al. 2010; Pan et al. 2010; Volotskova and Steep. 2011; Vaziri et al. 2012; Du et al. 1012; Garcez et al. 2013; Pan et al. 2013; Muhammad et al 2014; Li et al. 2015; Paschoal et al. 2015). Recently, novel techniques including PACT and TTP have emerged as alternative in the removal of oral biofilms, and thus in prevention of oral cavity infection. As per our knowledge, this is the first time the anti-biofilm effect of PACT and TTP are compared in mature *E. faecalis* biofilms, using a root canal model. The hypothesis of this study is that near-complete elimination of *E. faecalis* biofilm may be achieved by using PACT or TTP in infected extracted human teeth.

Most studies evaluate root canal disinfection by sampling with paper points. This technique is limited by only sampling bacteria from the fluid in the canal. In the present study, dentin walls were brushed, which allowed collect the biofilm in inner parts of the root canal system. We used three different approaches: CFU counting, CLSM/COMSTAT and SEM analysis to study the efficacy of the treatments in the elimination of *E. faecalis* biofilm formed on dentin walls.

Sodium hypochlorite was used as a positive control due to its well-known effective disinfectant efficacy and a wide antimicrobial spectrum (Du et al. 2015). Historically, NaOCl has been used as a “gold-standard” irrigant in endodontics, as it covers most of the requirements for endodontic irrigant when compared to any other known compound. However, it is also known to be toxic when in contact with vital tissues (Zehnder 2006).

Our CFU counting results showed that, either PACT and TTP therapies, in all tested conditions, induced reduction of mature *E. faecalis* biofilm, in a statistically significant way, reaching the maximum inactivation after 5 minutes of irradiation ($p<0.05$), without generating significant heat (data not shown) (Rios et al. 2011). This results corroborate with previous reports showed by Upadya *et al.* (2010), and Chrepa *et al.* (2014), correlating the PACT and tissue/cells damage in a dose-dependent manner, highlighting the importance of power light density. Li *et al*. (2015), comparing different exposure times of cold plasma, showed completely inactivation of 2-week *E. faecalis* biofilm after 12 minutes of treatment. Analogous results proposed by Pan *et al*. (2013), showed that CFU counting of *E. faecalis* biofilm is dependent on the plasma exposure

time and complete inactivation was achieved in 10 minutes.

Our SEM results confirmed visual morphological alterations and surface injuries observed in treated samples produced by either PACT and TTP, specially after 5 minutes of treatment. Similar results were also reported by Cheng *et al.* (2012) and Li *et al*. (2015), using SEM images to reveal the shrunken, rough and fractured appearance of the biofilm that remained after PACT and TTP, respectively.

With the double-staining method provided by CLSM, we were able to distinguish bacteria with intact (green) and damaged (red) membranes (Silva et al. 2014), as well as, indicate their viability after each treatment condition, whereas the large proportion of areas covered by red-stained bacteria was shown even in the deep portions of treated biofilms.

It must be highlighted that inactivation of *E. faecalis* biofilm can be attributed to reactive oxygen and nitrogen species, which led to oxidative damages to cell membrane, DNA, and proteinaceous enzymes generated by TTP application (Pan et al. 2013; Lunov et al. 2015; Paschoal et al. 2015). PACT is known by the generation of highly reactive singlet oxygen species and free radicals produced by photochemical reactions between the non-coherent light application and TBO (Paschoal et al. 2015).

It was believed that the slightly better bacterial killing results achieved by TTP compared to those reached by PACT, after 5 minutes of irradiation, may be due to incomplete TBO penetration in the dentin tubules (Bonsor et al. 2006) and failure of TBO to penetrate the whole canal biofilms (Li et al. 2015). As a gaseous medium, one of the advantages of TTP is the capability to penetrate irregular cavities/fissures and kill bacteria (Liu et al. 2016).

Our findings indicate that PACT and TTP have the potential to be used as co-adjunctive to the antimicrobial procedure in conventional endodontic treatment, by producing additional reduction in bacterial CFU numbers. Further studies need to be conducted aiming to translate this *in vitro* study to a clinical research.

Conclusion

The results of the study indicate that PACT and TTP are effective adjunctive procedures to conventional endodontic treatment, potentially increasing the success rate of antimicrobial protocols, especially in case of persistent endodontic infections.

References

Bonsor SJ, Nichol R, Reid TM, Pearson GJ .2006. An alternative regimen for root canal disinfection. *Br Dent J* 201(2)*:*101-105; discussion 198; quiz 120.

Cheng X, Guan S, Lu H, Zhao C, Chen X, Li N *et al.* 2012. Evaluation of the bactericidal effect of Nd:YAG, Er:YAG, Er,Cr:YSGG laser radiation, and antimicrobial photodynamic therapy (aPDT) in experimentally infected root canals. *Lasers Surg Med* 44(10)*:*824-831.

Chrepa V, Kotsakis GA, Pagonis TC, Hargreaves KM. 2014. The effect of photodynamic therapy in root canal disinfection: a systematic review. *J Endod* 40(7)*:*891-898.

Costerton JW, Stewart PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* 284(5418)*:*1318-1322.

de Oliveira BP, Aguiar CM, Camara AC .2014. Photodynamic therapy in combating the causative microorganisms from endodontic infections. *Eur J Dent* 8(3)*:*424-430.

Du T, Ma J, Yang P, Xiong Z, Lu X, Cao Y. 2012. Evaluation of antibacterial effects by atmospheric pressure nonequilibrium plasmas against Enterococcus faecalis biofilms in vitro. *J Endod* 38(4)*:*545-549.

Du T, Wang Z, Shen Y, Ma J, Cao Y, Haapasalo M. 2015. Combined Antibacterial Effect of Sodium Hypochlorite and Root Canal Sealers against Enterococcus faecalis Biofilms in Dentin Canals. *J Endod 41(8):1294-1298.*

Duarte S, Gregoire S, Singh AP, Vorsa N, Schaich K, Bowen WH *et al.* 2006. Inhibitory effects of cranberry polyphenols on formation and acidogenicity of Streptococcus mutans biofilms. *FEMS Microbiol Lett* 257(1)*:*50-56.

Duarte S, Kuo SP, Murata RM, Chen CY, Saxena D, Huang KJ, Popovic S. 2011. Air plasma effect on dental disinfection. Phys Plasmas. 18(7):073503

Garcez AS, Fregnani ER, Rodriguez HM, Nunez SC, Sabino CP, Suzuki H *et al.* 2013. The use of optical fiber in endodontic photodynamic therapy. Is it really relevant? *Lasers Med Sci* 28(1)*:*79-85.

Guerreiro-Tanomaru JM, de Faria-Junior NB, Duarte MA, Ordinola-Zapata R, Graeff MS, Tanomaru-Filho M. 2013. Comparative analysis of Enterococcus faecalis biofilm formation on different substrates. *J Endod* 39(3)*:*346-350.

Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersboll BK *et al.* 2000. Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* 146 (Pt 10)(2395-2407.

Hirata R, Teixeira H, Ayres AP, Machado LS, Coelho PG, Thompson VP *et al.* 2015. Long-term Adhesion Study of Self-etching Systems to Plasma-treated Dentin. *J Adhes Dent* 17(3)*:*227-233.

Isbary G, Morfill G, Schmidt HU, Georgi M, Ramrath K, Heinlin J *et al.* 2010. A first prospective randomized controlled trial to decrease bacterial load using cold atmospheric argon plasma on chronic wounds in patients. *Br J Dermatol* 163(1)*:*78-82.

Klein MI, Duarte S, Xiao J, Mitra S, Foster TH, Koo H. 2009. Structural and molecular basis of the role of starch and sucrose in Streptococcus mutans biofilm development. *Appl Environ Microbiol* 75(3)*:*837-841.

Lee HS, C.; Kim, Y. (2009). Degradation of adhesion molecules of G361 melanoma cells by a non-thermal atmospheric pressure microplasma. *New J Phys* 11:115026.

Li Y, Sun K, Ye G, Liang Y, Pan H, Wang G *et al.* 2015. Evaluation of Cold Plasma Treatment and Safety in Disinfecting 3-week Root Canal Enterococcus faecalis Biofilm In Vitro. *J Endod 41(8):1325-1330.*

Liu Y, Liu Q, Yu QS, Wang Y.2016. Nonthermal Atmospheric Plasmas in Dental Restoration. *J Dent Res* pii: 0022034516629425.

Lopez-Jimenez L, Fuste E, Martinez-Garriga B, Arnabat-Dominguez J, Vinuesa T, Vinas M (2015). Effects of photodynamic therapy on Enterococcus faecalis biofilms. *Lasers Med Sci* 30(5)*:*1519-1526.

Lunov O, Churpita O, Zablotskii V, Deynaka IG, Meshkovskii IK, Jager A, Sykova E, Kubinova S, Dejneka A. 2015. Non-thermal plasma mills bacteria: Snanning electron microscopy observations. Appl Phys Lett. 106:053703.

Mickel AK, Chogle S, Liddle J, Huffaker K, Jones JJ. 2007. The role of apical size determination and enlargement in the reduction of intracanal bacteria. *J Endod* 33(1)*:*21-23.

Muhammad OH, Chevalier M, Rocca JP, Brulat-Bouchard N, Medioni E. 2014. Photodynamic therapy versus ultrasonic irrigation: interaction with endodontic microbial biofilm, an ex vivo study. *Photodiagnosis Photodyn Ther* 11(2)*:*171-181.

Murad CF, Sassone LM, Faveri M, Hirata R, Jr., Figueiredo L, Feres M. 2014. Microbial diversity in persistent root canal infections investigated by checkerboard DNA-DNA hybridization. *J Endod* 40(7)*:*899-906.

Ordinola-Zapata R, Bramante CM, Garcia RB, de Andrade FB, Bernardineli N, de Moraes IG *et al.* 2013. The antimicrobial effect of new and conventional endodontic irrigants on intra-orally infected dentin. *Acta Odontol Scand* 71(3-4)*:*424-431.

Pan J, Sun P, Tian Y, Zhou HX, Wu HY, Bai N *et al.* 2010. A Novel Method of Tooth Whitening Using Cold Plasma Microjet Driven by Direct Current in Atmospheric-Pressure Air. *Ieee Transactions on Plasma Sci* 38(11)*:*3143-3151.

Pan J, Sun K, Liang YD, Sun P, Yang XH, Wang J *et al.* 2013. Cold Plasma Therapy of a Tooth Root Canal Infected with Enterococcus faecalis Biofilms In Vitro. *J Endod* 39(1)*:*105-110.

Paschoal MA, Dantas TCB, Duarte S. 2015. Photodynamic and Tissue Tolerable Plasma Therapies as Alternatives to Antimicrobials to Control Pathogenic Biofilms. *Curr Oral Health Rep* 2:57-62.

Rios A, He J, Glickman GN, Spears R, Schneiderman ED, Honeyman AL. 2011. Evaluation of photodynamic therapy using a light-emitting diode lamp against Enterococcus faecalis in extracted human teeth. *J Endod* 37(6)*:*856-859.

Rocas IN, Lima KC, Siqueira JF, Jr. 2013. Reduction in bacterial counts in infected root canals after rotary or hand nickel-titanium instrumentation--a clinical study. *Int Endod J* 46(7)*:*681-687.

Silva TC, Pereira AF, Buzalaf MA, Machado MA, Crielaard W, Deng DM. 2014. Diverse outcomes of Photodynamic Antimicrobial Chemotherapy on five Enterococcus faecalis strains. *Photodiagnosis Photodyn Ther* 11(3)*:*283-289.

Siqueira JF, Jr., Guimaraes-Pinto T, Rocas IN. 2007. Effects of chemomechanical preparation with 2.5% sodium hypochlorite and intracanal medication with calcium hydroxide on cultivable bacteria in infected root canals. *J Endod* 33(7)*:*800-805.

Siqueira JF, Jr., Rocas IN. 2008. Clinical implications and microbiology of bacterial persistence after treatment procedures. *J Endod* 34(11)*:*1291-1301 e1293.

Stojicic S, Shen Y, Haapasalo M. 2013. Effect of the source of biofilm bacteria, level of biofilm maturation, and type of disinfecting agent on the susceptibility of biofilm bacteria to antibacterial agents. *J Endod* 39(4)*:*473-477.

Stuart CH, Schwartz SA, Beeson TJ, Owatz CB. 2006. Enterococcus faecalis: its role in root canal treatment failure and current concepts in retreatment. *J Endod* 32(2)*:*93-98.

Upadya MH, Kishen A. 2010. Influence of bacterial growth modes on the susceptibility to light-activated disinfection. *Int Endod J* 43(11)*:*978-987.

Vaziri S, Kangarlou A, Shahbazi R, Nazari Nasab A, Naseri M. 2012. Comparison of the bactericidal efficacy of photodynamic therapy, 2.5% sodium hypochlorite, and 2% chlorhexidine against Enterococcous faecalis in root canals; an in vitro study. *Dent Res J (Isfahan)* 9(5)*:*613-618.

Volotskova O, Shashurin A, Stepp M A. 2011. Plasma-controlled cells migration: localization of cold plasma-cell interaction region. *Plasma Med* 1:85-92.

Wu MK, van der Sluis LW, Wesselink PR. 2003. The capability of two hand instrumentation techniques to remove the inner layer of dentine in oval canals. *Int Endod J* 36(3)*:*218-224.

Zand V, Milani AS, Amini M, Barhaghi MH, Lotfi M, Rikhtegaran S *et al.* 2014. Antimicrobial efficacy of photodynamic therapy and sodium hypochlorite on monoculture biofilms of Enterococcus faecalis at different stages of development. *Photomed Laser Surg* 32(5)*:*245-251.

Zehnder M. 2006. Root canal irrigants. *J Endod* 32(5)*:*389-398.

4. Discussão Geral

A constante busca por uma melhor desinfecção do sistema de canais radiculares e por métodos eficientes para sua resolução são assuntos bastante atuais na área da especialidade endodôntica, visto que a presença de microrganismos se constitui em fator significativo para a manutenção ou aparecimento da lesão periapical. Conhecer o comportamento dos agentes biológicos envolvidos na patogênese endodôntica é imperioso para definir o protocolo terapêutico, prever o diagnóstico e buscar novas terapias no combate à infecção.

O *E. faecalis* é reportado como microrganismo de alta incidência nos casos de insucessos em endodontia. Sua capacidade de organização em biofilmes, adesão à dentina e penetração nos túbulos dentinários se constituem em importantes fatores de virulência (RÔÇAS *et al*., 2004; RÔÇAS *et al*., 2008; MEIRE *et al*., 2012; WANG *et al*., 2012). Assim como o *E. faecalis, a Candida albicans* também pode ser encontrada em casos de infecções secundárias persistentes do sistema de canais radiculares (WALTIMO *et al*., 2003; SIQUEIRA JR. *et al*., 2004; MIRANDA *et al*., 2009), compartilhando da mesma característica de adesão a diferentes tipos de substratos e organização em biofilmes (SEN, BASKI, 2009). Nesses experimentos, procurou-se estabelecer um modelo de biofilme maduro de *E. faecalis* e *Candida albicans* que pudessem determinar *in vitro* sua susceptibilidade as duas terapias antimicrobianas estudas, por meio dos métodos de cultura e recursos de microscopia confocal de varredura a laser (CLSM) e eletrônica de varredura (SEM).

Nos capítulos 2 e 3 foram escolhidos discos de hidroxiapatita revestidos com saliva esterilizada como substratos para formação dos biofilmes. Gerreiro-Tanomaro *et al*. (2013), comparando a formação de biofilmes maduros de *E. faecalis* em diferentes substratos, encontraram nos discos de hidroxiapatita excelentes resultados, produzindo biofilmes maduros em 15 e 21 dias. Embora estudos mostrem biofilmes maduros de *C. albicans* em discos de hidroxiapatita em apenas 24 horas de incubação, nossos

estudos estão de acordo com os achados de Delben *et al*. (2014), nos quais foram desenvolvidos biofilmes maduros de *C. albicans* em 72 horas.

Apesar das vantagens do modelo de biofilme utilizado no presente estudo, é difícil reproduzir em laboratório as condições clínicas sob as quais os agentes antimicrobianos se difundem através dos biofilmes estudados. Esses modelos *in vitro* não necessariamente simulam as mesmas condições encontradas no sistema de canais radiculares. O uso do presente modelo de biofilme pode mascarar algumas variáveis nas quais as bactérias podem sobreviver à aplicação das terapias antimicrobianas estudadas. Uma dessas variáveis, aparentemente, é a presença de irregularidades anatômicas, como ramificações do canal principal, istmos e achatamentos que podem aumentar a massa vertical do biofilme na estrutura dentinária, como também limitar a ação antimicrobiana. Na tentativa de minimizar os efeitos dessas variáveis nos resultados desse estudo, no capítulo 4 foram utilizados modelos de biofilmes de *E. faecalis* em canais radiculares de dentes humanos extraídos.

A terapia fotodinâmica apresenta ação antimicrobiana com a vantagem da não indução da resistência bacteriana. Diante do surgimento cada vez mais de microrganismos antibiótico-resistentes e de uma complexa anatomia do sistema de canais radiculares, esse tipo de terapia apresenta vantagens com potencialidade para mais estudos. Dentre os sensitizadores mais utilizados em endodontia estão os fenotiazínicos como o azul de toluidina, com grande eficácia comprovada em vários estudos *in vitro* (SCHLAFER *et al*., 2010; VAZIRI *et al*., 2012; BAGO *et al*., 2013).

Há na literatura uma grande variabilidade nos parâmetros utilizados na PACT, dentre eles a escolha da concentração dos sensitizadores e do tempo de exposição aparecem como os mais controversos, apresentando ainda uma grande variação de resultados quando estudados em culturas planctônicas ou bactérias organizadas em biofilmes. Os diferentes resultados encontrados na literatura, corroboram com a ideia de que a produção de metabólitos pela PACT, como as espécies reativas de oxigênio (ERO), dentre elas o oxigênio singleto, requerem uma concentração ideal do sensitizador em tempos de exposição específicos. No capítulo 1, a ação antimicrobiana da PACT, utilizando diferentes concentrações de TBO, foi analisada em culturas planctônicas de *E. faecalis.* Os resultados mostraram

que o efeito antibacteriano da PACT apresentou um comportamento dose-tempo-dependente, sendo a concentração de 100 µg/mL de TBO a mais eficaz em todas as condições de irradiação estudadas. A dihidrorodamina 1 2 3, já utilizada como marcador da presença de ERO intracelular em células eucariontes (PASSOS *et al*., 2013) e em suspensões planctônicas (FARRELLL *et al*., 2011; RISTIC *et al*., 2014), mostrou-se como uma importante ferramenta na visualização dos EROs produzidos nas diferentes condições de concentração e irradiação, corroborando com a escolha do TBO /100 µg/mL para os estudos realizados nos capítulos 2 e 4.

O rápido efeito dos agentes antibacterianos em culturas planctônicas de *E. faecalis* podem não refletir o efeito dessa terapia em bactérias organizadas em biofilmes, uma vez que os fatores de virulência adquiridos aumentam a resistência dos biofilmes em 10 a 1.000 vezes, quando comparados aos seus homólogos planctônicos (FUX *et al*., 2005). Diante disso, no capítulo 2, estudamos a eficácia antibacteriana da PACT em biofilmes de *E. faecalis* e *C. albicans*. Nossos resultados confirmaram a atividade antimicrobiana e fungicida da PACT, quando realizada com TBO a 100 µg/mL e luz vermelha, apresentando o mesmo padrão dose-tempo-dependente que os observados no capítulo 1.

Resultados semelhantes foram observados nos estudos de SOARES *et al*. (2009), onde o efeito dose-dependente, utilizando TBO 25 µM e uma quantidade de energia de 180 J/cm^2, também foi analisado em biofilmes de *C. albicans* aderidos às células do epitélio bucal. Foram observados ainda diferentes resultados estudando a atividade antibacteriana da PACT/TBO em biofilmes de *E. faecalis*, utilizando o laser diodo como fonte emissora de luz e concentrações mais baixas de TBO. Bergmans *et al*. (2008) mostraram uma redução de 88,4% de unidades formadoras de colônias em biofilmes de *E. faecalis*, utilizando TBO a 12,7 µg/mL em um tempo de exposição de 2,5 minutos, em contrapartida SOUZA *et al*. (2010), não encontraram resultados estatisticamente significantes ao utilizar uma concentração de TBO a 15 µg/mL em um tempo de exposição de 4 minutos.

No capítulo 3 foi estudado o efeito antimicrobiano do plasma de baixa temperatura e pressão em biofilmes de *E. faecalis,* em três tempos de exposição diferentes. Recentemente tem crescido bastante o interesse pelo

plasma, como um novo conceito nos tratamentos antimicrobianos, pela comunidade cientifica. Na endodontia, estudos *in vitro* demonstraram o potencial antimicrobiano do plasma, principalmente em relação ao *E. faecalis* (LU *et al*., 2009; JIANG *et al.*, 2009; WANG *et al*., 2011; LI *et al.*, 2015).

Nossos resultados confirmaram a efetividade antibacteriana do plasma na viabilidade de células de *E. faecalis* organizadas em biofilmes, tanto no modelo em discos de hidroxiapatita como no modelo em dentes humanos extraídos. Assim como a PACT, os resultados obtidos com o plasma também acompanham o padrão de dependência do tempo de exposição empregados, adquirindo-se máxima efetividade no tempo de exposição de 5 minutos. Tal fato pôde ser confirmado tanto pelo método de cultura para posterior contagem de unidades formadoras de colônias, como também pela análise da viabilidade celular e do grau de penetrabilidade das duas terapias estudadas nas camadas mais profundas do biofilme, por meio de imagens de microscopia confocal de varredura a laser.

Severas modificações estruturais no biofilme também puderam ser observadas nas imagens de microscopia eletrônica de varredura (SEM), confirmando os achados de Li Y. *et al*. (2015), nos quais também foram observadas destruição do aspecto tridimensional dos biofilmes como também desaparecimento do padrão esférico do *E. faecalis*, o que foi explicado pelo efeito da liberação de espécies reativas de oxigênio, promovendo um estresse oxidativo, gerando a morte do *E. faecalis* principalmente por danos à parede celular.

O capítulo 4 apresentou os resultados dos estudos da PACT e do plasma, porém com os biofilmes de *E. faecalis* crescidos em paredes de dentina de dentes humanos extraídos, contaminados após o preparo químico-mecânico. Nesse estudo, pudemos observar o efeito das duas terapias levando em consideração a complexidade do sistema de canais radiculares e à presença de microrganismos no interior dos túbulos dentinários. Nossos resultados confirmaram a efetividade das duas terapias, PACT e plasma, já observadas em discos de hidroxiapatita, tendo o plasma, após 5 minutos de exposição, melhores resultados antimicrobianos com significância estatística. Nesse sentido, Pan J. *et al*. (2013) e Li Y. *et al*. (2015) encontraram total inativação de biofilmes de *E. faecalis* em

canais radiculares após aplicação do plasma, com tempos de exposição mais elevados, 10 e 12 minutos respectivamente. Resultados dose-dependentes em PACT em biofilmes de *E. faecalis* em canais radiculares também foram reportados pelos trabalhos de Upadia *et al*. (2010) e Chrepa *et al*. (2014).

O uso do equipamento LumaCare® apresentou como maior vantagem a potência, interferindo diretamente na quantidade de energia liberada após os tempos de exposição empregados, porém apresentou como desvantagem, o diâmetro de suas sondas cambiáveis que não permitem a inserção do dispositivo no interior do canal radicular. Tal fato não sugere prejuízos aos resultados desse estudo, o que também foi comprovado por Nunes *et al*. (2011), que, observando os efeitos da PACT em canais radiculares contaminados com *E. faecalis* com e sem o uso de fibra óptica, sugeriram que o uso desta não contribuiu significativamente para o sucesso da terapia, quando comparado com dispositivos de luz colocados na entrada dos canais radiculares.

Em nosso conhecimento, este é o primeiro estudo a comparar os efeitos da PACT com o plasma de baixa temperatura e pressão. Porém mais estudos são necessários para se verificar o potencial do Plasma e da PACT na terapia endodôntica *in vivo*, quanto aos possíveis efeitos citotóxicos em células humanas e um maior número de casos com *E. faecalis* para verificar de forma confiável a sua susceptibilidade às duas terapias.

5. Conclusão Geral

Com base nos resultados desta tese, pode-se concluir que:

I. O efeito da terapia fotodinâmica mediada pelo TBO apresentou um efeito dose-dependente, mostrando um superior efeito antimicrobiano quando se utilizou a concentração de 100 µg/mL em todos os tempos de exposição estudados.

II. A terapia fotodinâmica mediada pelo TBO 100 µg/mL apresentou atividade antimicrobiana e fungicida, na inativação de biofilmes maduros de *E. faecalis* e *C. albicans* no modelo *in vitro* apresentado.

III. O plasma de baixa temperatura e pressão apresentou atividade antimicrobiana, na inativação de biofilmes maduros de *E. faecalis* em curtos períodos de exposição, no modelo *in vitro* apresentado.

IV. O plasma de baixa temperatura e pressão e a terapia fotodinâmica mediada da pelo TBO 100 µg/mL mostraram-se eficientes como terapias antimicrobianas em modelos *in vitro* de canais radiculares, tendo o plasma superior efeito antimicrobiano quando se aumentou os tempos de exposição para 5 minutos. Dessa forma podem representar alternativas viáveis de terapia coadjuvantes ao tratamento endodôntico convencional no combate ao maduro biofilme de *E. faecalis,* frequentemente encontrado em infecções endodônticas persistentes.

Referências Gerais

1. ACKROYD R, KELTY C, BROWN N, REED M. The history of photodetection and photodynamic therapy. **Photochemistry and photobiology** 2001;74(5):656-669.

2. ALLISON RR, DOWNIE GH, CUENCA R, HU XH, CHILDS CJ, SIBATA CH. Photosensitizers in clinical PDT. **Photodiagnosis and photodynamic therapy** 2004;1(1):27-4

3. BAGO I, PLECKO V, GABRIC PANDURIC D, SCHAUPERL Z, BARABA A, ANIC I. Antimicrobial efficacy of a high-power diode laser, photo-activated disinfection, conventional and sonic activated irrigation during root canal treatment. **International endodontic journal** 2013;46(4):339-347.

4. BERBER VB, GOMES BP, SENA NT, VIANNA ME, FERRAZ CC, ZAIA AA, et al. Efficacy of various concentrations of NaOCl and instrumentation techniques in reducing Enterococcus faecalis within root canals and dentinal tubules. **International endodontic journal** 2006;39(1):10-17.

5. BERGMANS L, MOISIADIS P, HUYBRECHTS B, VAN MEERBEEK B, QUIRYNEN M, LAMBRECHTS P. Effect of photo-activated disinfection on endodontic pathogens ex vivo. **International endodontic journal** 2008;41(3):227-239.

6. CHREPA V, KOTSAKIS GA, PAGONIS TC, HARGREAVES KM. The effect of photodynamic therapy in root canal disinfection: a systematic review. **Journal of endodontics** 2014;40(7):891-898.

7. COKELILER D, ERKUT S, ZEMEK J, BIEDERMAN H, MUTLU M. Modification of glass fibers to improve reinforcement: a plasma polymerization technique. **Dental materials : official publication of the Academy of Dental Materials** 2007;23(3):335-342.

8. COSTA DANTAS MC, DO PRADO M, COSTA VS, GAIOTTE MG, SIMAO RA, BASTIAN FL. Comparison between the effect of plasma and chemical treatments on fiber post surface. **Journal of endodontics** 2012;38(2):215-218.

9. COSTERTON JW, STEWART PS, GREENBERG EP. Bacterial biofilms: a common cause of persistent infections. **Science 1999**;284(5418):1318-1322.

10. DELBEN JAM, R. M.; WEI, X.; CASTRO, M. L.; ASSUNÇÃO, W. G.; DA SILVA, R. N. F.; DUARTE, S. Low-temperature plasma: an effective approuch against Candida albicans biofilm. **Plasma Med** 2014;4(1-4):231-244.

11. DETTY MR, GIBSON SL, WAGNER SJ. Current clinical and preclinical photosensitizers for use in photodynamic therapy. **Journal of medicinal chemistry** 2004;47(16):3897-3915.

12. DOUGHERTY TJG, C. J.; HENDERSON, B., W.; JORI, G.; KESSEL, D.; KORBELIK, M.; MOAN, J.; PENG, Q. Photodynamic Therapy. **Journal of the National Cancer Institute** 1998;90:889-890.

13. DU TF, MA JZ, YANG P, XIONG ZL, LU XP, CAO YG. Evaluation of Antibacterial Effects by Atmospheric Pressure Nonequilibrium Plasmas against Enterococcus faecalis Biofilms In Vitro. **Journal of endodontics** 2012;38(4):545-549.

14. FARRELL H, HAYES J, LAFFEY J, ROWAN N. Studies on the relationship between pulsed UV light irradiation and the simultaneous occurrence of molecular and cellular damage in clinically-relevant Candida albicans. **Journal of microbiological methods** 2011;84(2):317-326.

15. FRADE JP, ARTHINGTON-SKAGGS BA. Effect of serum and surface characteristics on Candida albicans biofilm formation. **Mycoses** 2011;54(4):e154-162.

16. FUX CA, COSTERTON JW, STEWART PS, STOODLEY P. Survival strategies of infectious biofilms. **Trends in microbiology** 2005;13(1):34-40.

17. GAD F, ZAHRA T, HASAN T, HAMBLIN MR. Effects of growth phase and extracellular slime on photodynamic inactivation of gram-positive pathogenic bacteria. **Antimicrobial agents and chemotherapy** 2004;48(6):2173-2178.

18. GOREE J, LIU B, DRAKE D, STOFFELS E. Killing of S-mutans bacteria using a plasma needle at atmospheric pressure. **Ieee T Plasma Sci** 2006;34(4):1317-1324.

19. GUERREIRO-TANOMARU JM, DE FARIA-JUNIOR NB, DUARTE MA, ORDINOLA-ZAPATA R, GRAEFF MS, TANOMARU-FILHO M. Comparative analysis of Enterococcus faecalis biofilm formation on different substrates. **Journal of endodontics** 2013;39(3):346-350.

20. HUBBLE TS, HATTON JF, NALLAPAREDDY SR, MURRAY BE, GILLESPIE MJ. Influence of Enterococcus faecalis proteases and the collagen-binding protein, Ace, on adhesion to dentin. **Oral microbiology and immunology** 2003;18(2):121-126.

21. JIANG CC, M. T.; SCHAUDINN, C.; GORUR, A.; VERNIER, P. T.; COSTERTON, W.; JARAMILLO, D. E.; SEDGHIZADEH, P. P.; GUNDERSEN, M. A.. . Pulsed atmospheric-pressure cold plasma for endodontic disinfection. **IEEE Translations of Plasma Sci** 2009;37:1190-1195.

22. KIM JH, LEE MA, HAN GJ, CHO BH. Plasma in dentistry: a review of basic concepts and applications in dentistry. **Acta odontologica Scandinavica** 2014;72(1):1-12.

23. KIM SJ, CHUNG TH, BAE SH. Striation and plasma bullet propagation in an atmospheric pressure plasma jet. **Phys Plasmas** 2010;17(5).

24. KOBAN I, HOLTFRETER B, HUBNER NO, MATTHES R, SIETMANN R, KINDEL E, et al. Antimicrobial efficacy of non-thermal plasma in comparison to chlorhexidine

against dental biofilms on titanium discs in vitro - proof of principle experiment. **Journal of Clinical Periodontology** 2011;38(10):956-965.

25. KOGELSCHATZ U. Filamentary, patterned, and difuse barrier discharges. **LEEE Transitions on Plasma Science** 2000;30(4):1400-1408.

26. KOLENBRANDER PE. Oral microbial communities: biofilms, interactions, and genetic systems. **Annual review of microbiology** 2000;54:413-437.

27. KONOPKA K, GOSLINSKI T. Photodynamic therapy in dentistry. **J Dent Res** 2007;86(8):694-707.

28. KUNHARDT EE. Generation of large-volume, atmospheric-pressure, nonequilibrium plasmas**. Ieee T Plasma Sci** 2000;28(1):189-200.

29. KUO SP, BIVOLARU D, WILLIAMS S, CARTER CD. A microwave-augmented plasma torch module**. Plasma Sources Sci T** 2006;15(2):266-275.

30. LAROUSSE M. Sterilization of contaminated matter with an atmospheric pressure plasma. **IEEE Trans Plasma Sci** 1996;24(3):1188-1191.

31. LEE MT, BIRD PS, WALSH LJ. Photo-activated disinfection of the root canal: a new role for lasers in endodontics. **Australian endodontic journal : the journal of the Australian Society of Endodontology Inc** 2004;30(3):93-98.

32. LI Y, SUN K, YE G, LIANG Y, PAN H, WANG G, et al. Evaluation of Cold Plasma Treatment and Safety in Disinfecting 3-week Root Canal Enterococcus faecalis Biofilm In Vitro. **Journal of endodontics** 2015.

33. LIMA SM, SOUSA MG, FREIRE MDE S, ALMEIDA JA, CANTUARIA AP, SILVA TA, et al. Immune Response Profile against Persistent Endodontic Pathogens Candida albicans and Enterococcus faecalis In Vitro. **Journal of endodontics** 2015;41(7):1061-1065.

34. LU XC, Y.; XIONG, Z.; XIAN, Y.; PAN, Y. . An RC plasma device for sterilization of root canal teeth. **IEEE Trans Plasma Sci** 2009;37:668-673.

35. MACHADO AEATFP, potencial de aplicação e perspectivas. Terapia fotodinâmica: princípios, potencial de aplicação e perspectivas. **Quim Nova** 2000;23(2):237-243.

36. MAH TF, O'TOOLE GA. Mechanisms of biofilm resistance to antimicrobial agents. **Trends in microbiology** 2001;9(1):34-39.

37. MEIRE MA, COENYE T, NELIS HJ, DE MOOR RJ. In vitro inactivation of endodontic pathogens with Nd:YAG and Er:YAG lasers. **Lasers in medical science** 2012;27(4):695-701.

38. MIRANDA TT, VIANNA CR, RODRIGUES L, MONTEIRO AS, ROSA CA, CORREA A, JR. Diversity and frequency of yeasts from the dorsum of the tongue and necrotic

root canals associated with primary apical periodontitis. **International endodontic journal** 2009;42(9):839-844.

39. MOISAN M, BARBEAU J, MOREAU S, PELLETIER J, TABRIZIAN M, YAHIA LH. Low-temperature sterilization using gas plasmas: a review of the experiments and an analysis of the inactivation mechanisms. **International journal of pharmaceutics** 2001;226(1-2):1-21.

40. MOREAU M, ORANGE N, FEUILLOLEY MGJ. Non-thermal plasma technologies: New tools for bio-decontamination. **Biotechnol Adv** 2008;26(6):610-617.

41. NAGATA JY, HIOKA N, KIMURA E, BATISTELA VR, TERADA RS, GRACIANO AX, et al. Antibacterial photodynamic therapy for dental caries: evaluation of the photosensitizers used and light source properties. **Photodiagnosis and photodynamic therapy** 2012;9(2):122-131.

42. NUNES MR, MELLO I, FRANCO GC, DE MEDEIROS JM, DOS SANTOS SS, HABITANTE SM, et al. Effectiveness of photodynamic therapy against Enterococcus faecalis, with and without the use of an intracanal optical fiber: an in vitro study. **Photomedicine and laser surgery** 2011;29(12):803-808.

43. PAN J, SUN K, LIANG Y, SUN P, YANG X, WANG J, et al. Cold plasma therapy of a tooth root canal infected with enterococcus faecalis biofilms in vitro. **Journal of endodontics** 2013;39(1):105-110.

44. PAN J, SUN P, TIAN Y, ZHOU HX, WU HY, BAI N, et al. A Novel Method of Tooth Whitening Using Cold Plasma Microjet Driven by Direct Current in Atmospheric-Pressure Air. **Ieee T Plasma Sci** 2010;38(11):3143-3151.

45. PASCHOAL MAD, T. C, B.; DUARTE, S. Photodynamic and Tissue Tolerable Plasma Therapies as Alternatives to Antimicrobials to Control Pathogenic Biofilms. **Curr Oral Health Rep** 2015;2:57-62.

46. PASSOS JF, MIWA S, VON ZGLINICKI T. Measuring reactive oxygen species in senescent cells. **Methods in molecular biology** 2013;965:253-263.

47. RISTIC BZ, MILENKOVIC MM, DAKIC IR, TODOROVIC-MARKOVIC BM, MILOSAVLJEVIC MS, BUDIMIR MD, et al. Photodynamic antibacterial effect of graphene quantum dots. **Biomaterials** 2014;35(15):4428-4435.

48. ROCAS IN, HULSMANN M, SIQUEIRA JF, JR. Microorganisms in root canal-treated teeth from a German population. **Journal of endodontics** 2008;34(8):926-931.

49. ROCAS IN, SIQUEIRA JF, JR., SANTOS KR. Association of Enterococcus faecalis with different forms of periradicular diseases. **Journal of endodontics** 2004;30(5):315-320.

50. SCHLAFER S, VAETH M, HORSTED-BINDSLEV P, FRANDSEN EV. Endodontic photoactivated disinfection using a conventional light source: an in vitro and ex

vivo study. **Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics** 2010;109(4):634-641.

51. SEDGLEY CM, NAGEL AC, HALL D, APPLEGATE B. Influence of irrigant needle depth in removing bioluminescent bacteria inoculated into instrumented root canals using real-time imaging in vitro. **International endodontic journal** 2005;38(2):97-104.

52. SEN BHB, B. G. Fungi in endodontic infections. In: **Endodontic Microbiology** 2009:164-194.

53. SILVA NR, COELHO PG, VALVERDE GB, BECKER K, IHRKE R, QUADE A, et al. Surface characterization of Ti and Y-TZP following non-thermal plasma exposure. Journal of biomedical materials research. Part B, **Applied biomaterials** 2011;99(1):199-206.

54. SIQUEIRA JF, JR., ROCAS IN, PAIVA SS, MAGALHAES KM, GUIMARAES-PINTO T. Cultivable bacteria in infected root canals as identified by 16S rRNA gene sequencing. **Oral microbiology and immunology** 2007;22(4):266-271.

55. SIQUEIRA JF, JR., SEN BH. Fungi in endodontic infections. **Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics** 2004;97(5):632-641.

56. SLADEK REJ, FILOCHE SK, SISSONS CH, STOFFELS E. Treatment of Streptococcus mutans biofilms with a nonthermal atmospheric plasma. **Letters in applied microbiology** 2007;45(3):318-323.

57. SLADEK REJ, STOFFELS E, WALRAVEN R, TIELBEEK PJA, KOOLHOVEN RA. Plasma treatment of dental cavities: A feasibility study. **Ieee T Plasma Sci** 2004;32(4):1540-1543.

58. SLADEK REJIOPOPTFDC. nvestigation of possibilities of plasma treatment for dental caries. **14th IEEE International Pulsed Power Conference** 2003;1-2:1109-1111.

59. SOARES BM, DA SILVA DL, SOUSA GR, AMORIM JC, DE RESENDE MA, PINOTTI M, et al. In vitro photodynamic inactivation of Candida spp. growth and adhesion to buccal epithelial cells. **Journal of photochemistry and photobiology.** B, Biology 2009;94(1):65-70.

60. SOUZA RC, JUNQUEIRA JC, ROSSONI RD, PEREIRA CA, MUNIN E, JORGE AO. Comparison of the photodynamic fungicidal efficacy of methylene blue, toluidine blue, malachite green and low-power laser irradiation alone against Candida albicans. **Lasers in medical science** 2010;25(3):385-389.

61. STOFFELS E, KIEFT IE, SLADEK REJ, VAN DEN BEDEM LJM, VAN DER LAAN EP, STEINBUCH M. Plasma needle for in vivo medical treatment: recent developments and perspectives. **Plasma Sources Sci** T 2006;15(4):S169-S180.

62. SUN P, PAN J, TIAN Y, BAI N, WU HY, WANG LX, et al. Tooth Whitening With Hydrogen Peroxide Assisted by a Direct-Current Cold Atmospheric-Pressure Air Plasma Microjet. **Ieee T Plasma Sci** 2010;38(8):1892-1896.

63. TANG YZ, LU XP, LAROUSSI M, DOBBS FC. Sublethal and killing effects of atmospheric-pressure, nonthermal plasma on eukaryotic microalgae in aqueous media. **Plasma Process Polym** 2008;5(6):552-558.

64. TURNER SR, LOVE RM, LYONS KM. An in-vitro investigation of the antibacterial effect of nisin in root canals and canal wall radicular dentine. **International endodontic journal** 2004;37(10):664-671.

65. UPADYA MH, KISHEN A. Influence of bacterial growth modes on the susceptibility to light-activated disinfection. **International endodontic journal** 2010;43(11):978-987.

66. VAHABI S, FEKRAZAD R, AYREMLOU S, TAHERI S, ZANGENEH N. The effect of antimicrobial photodynamic therapy with radachlorin and toluidine blue on streptococcus mutans: an in vitro study. **J Dent (Tehran)** 2011;8(2):48-54.

67. VAZIRI S, KANGARLOU A, SHAHBAZI R, NAZARI NASAB A, NASERI M. Comparison of the bactericidal efficacy of photodynamic therapy, 2.5% sodium hypochlorite, and 2% chlorhexidine against Enterococcous faecalis in root canals; an in vitro study. **Dental research journal** 2012;9(5):613-618.

68. WALTIMO TM, SEN BH, MEURMAN JH, ORSTAVIK D, HAAPASALO MP. Yeasts in apical periodontitis. Critical reviews in oral biology and medicine : an official publication of the **American Association of Oral Biologists** 2003;14(2):128-137.

69. WANG QQ, ZHANG CF, CHU CH, ZHU XF. Prevalence of Enterococcus faecalis in saliva and filled root canals of teeth associated with apical periodontitis. **International journal of oral science** 2012;4(1):19-23.

70. WANG RZ, H.; SUN, P.; WU, H.; PAN, J.; ZHU, W.; ZHANG, J.; FANG, J.. The effect of an atmospheric pressure plasma microjet on tooth root canal, denyinal tubules infection and reinfection prevention. **Plasma Med** 2011;1:143-155.

71. WILSON BC, PATTERSON MS. The physics, biophysics and technology of photodynamic therapy. **Physics in medicine and biology** 2008;53(9):R61-109.

72. WOOD S, METCALF D, DEVINE D, ROBINSON C. Erythrosine is a potential photosensitizer for the photodynamic therapy of oral plaque biofilms. **J Antimicrob Chemother** 2006;57(4):680-684.

73. ZANIN IC, GONCALVES RB, JUNIOR AB, HOPE CK, PRATTEN J. Susceptibility of Streptococcus mutans biofilms to photodynamic therapy: an in vitro study. **J Antimicrob Chemother** 2005;56(2):324-330.

Anexos

Formulário de Aprovação do Comitê de Ética em Pesquisa do Estudo com Dentes Humanos do Capítulo 4

New York University • University Committee on Activities Involving Human Subjects
665 Broadway, Suite 804• New York, NY 10012-2331 • www.nyu.edu/ucaihs/
(212) 998-4808 • fax: (212) 995-4304 • ask.humansubjects@nyu.edu

Checklist for Determining Whether a Project Involves Human Subjects Research
Version: 1.2
Date: 05/05/10

This application replaces the applications previously used for Class B, P and S data, and does not need to be submitted to the UCAIHS Office for a determination. Please file a copy with your appropriate research protocol.

Please note that this application does not replace the submission of an application to determine if the research is exempt from human subjects research regulations. Federal regulations do not provide investigators with the authority to make an independent determination that an activity meets one or more of the categories of exempt human subjects research. Investigators who intend to conduct activities that might involve human subjects research must submit a formal application to the NYU University Committee on Activities Involving Human Subjects (UCAIHS) for approval before the research can begin. Guidance on this issue is available in the NYU policies Institutional Authorization for Determining whether Research or Other Activities Represent Human Subjects Research and UCAIHS policy on Publicly Available Data, De-Identified Data, and Biological Specimens.

I. Research Title: PHOTODYNAMIC CHEMOTHERAPY AND TISSUES TOLERABLE PLASMA AS ALTERNATIVE THERAPIES AGAINST PATHOGENIC ENDODONTIC BIOFILMS

II. Personnel

A. Principal Investigator

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B. Faculty Sponsor – required when PI is a student

Name (Last, First)	Degree(s)	University Status/Title
Department		College
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III. Activities Determined by UCAIHS Not to Represent Human Subjects Research

Indicate whether any of the below describe the activities associated with your project.

☐ Your project is limited to accessing one or more of the following public use datasets: Inter-University Consortium for Political and Social Research (ICPSR), U.S Bureau of the Census, National Center for Health Statistics, National Center for Educational Statistics, U.S. Bureau of Labor Statistics, National Election Studies, National Crime Victimization Survey: School Crime Supplement, 2003, National

Epidemiologic Survey on Alcohol and Related Conditions (NESARC), National Survey of America's Families (NSAF), National Sample Survey of Registered Nurses (NSSRN), Federal Election Commission Website, and PRAMS.

☐ Your project is limited to course-related activities designed specifically for educational or teaching purposes; where data is collected from and about human subjects as part of a class exercise or assignment and is not intended for use outside of the classroom.

☐ Your activity is a case report involving the observation of a single patient whose novel condition or response to treatment was guided by the care provider's judgment regarding the best interest of the individual.

☐ Your project involves research that is limited to death records, autopsy materials, or cadaver specimens (provided that the cadaveric tissues/cells are not used for clinical investigations).

If you checked any of the above boxes – STOP. Your activity is in a category that UCAIHS has determined ***not*** *to represent* human *subjects research. The requirement for submission to UCAIHS of an application for human subjects research has been waived. You can skip to page 3, Section V, sign and retain this form in your files to document this determination. UCAIHS strongly recommends that investigators (faculty) document this determination by placing a copy of this completed application in your files to address any future queries about the project.*

If your data set is not listed above, complete the remainder of this application or contact UCAIHS to determine if you must complete an application for IRB review.

IV. Criteria for Research Involving Human Subjects

A. Does the activity meet the definition of research?

Answer yes or no to the following:

- The activity employs a systematic approach involving predetermined methods for studying a specific topic, answering a specific question, testing a specific hypothesis, or developing a theory.
  ☒ Yes ☐ No
- The activity is intended to contribute to generalized knowledge by extending the results beyond a single individual or an internal unit (e.g., publications or presentations).
  ☒ Yes ☐ No

B. Does the activity involve human subjects according to the definition?

Answer yes or no to the following:

- The investigator obtains specimens or data through intervention or interaction with a living individual (e.g., interviews, surveys, physical procedures, manipulations of the subject's environment, private or limited access internet sites, or any other direct contact or communication with a subject).
  ☐ Yes ☒ No
- The investigator is obtaining identifiable private information about living individuals (e.g. chart reviews, lab studies on tissues or specimens, information from data or tissue repository).
  ☐ Yes ☒ No
- The data or specimens are received by or provided to the investigator with identifiable private information.
  ☐ Yes ☒ No

- The data or specimens are coded and the investigator has access to a link that would allow the data or samples to be identified.
  ☐ Yes ☒ No

Note: If you answered "yes" to both questions in Section IV.A **AND** "yes" to at least one question in Section IV.B. **STOP**. Your activity meets the definition of human subjects research, and either exempt, expedited, or convened review is required. Proceed to the UCAIHS website and complete the Application for Review by UCAIHS (http://www.nyu.edu/ucaihs/forms/).

For all projects that do NOT meet the definition of human subjects research, obtain appropriate signatures in Section V below and retain for your files.

For additional information regarding the review of human subjects research, please refer to the UCAIHS website www.nyu.edu/ucaihs/.

V. SIGNATURES

Signature of Principal Investigator: _____ **Date 01/12/2014**

Signature of Faculty Sponsor: _____ **Date 01/12/2014**

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MICROBIOLOGY (M KLEIN, SECTION EDITOR)

Photodynamic and Tissue Tolerable Plasma Therapies as Alternatives to Antimicrobials to Control Pathogenic Biofilms

Marco Aurelio Paschoal & Thereza Cristina Botelho Dantas & Simone Duarte

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Abstract The need for novel antimicrobial techniques has become critical for a number of reasons, including the emergence of resistant strains because of excessive prescription and misuse of antibiotics. The mouth is colonized by a large number of microorganisms. When these microorganisms are organized as biofilms they can contribute to chronic diseases in the human population, such as dental caries, candidiasis, and periodontal disease. Hence, alternative antimicrobial approaches have risen to facilitate the treatment of these dis- eases. As novel therapies, photodynamic antimicrobial chemotherapy and tissue-tolerable plasma are promising methods that offer the possibility of microbial decontamination with decreased odds for the traditional side effects. Thus, the pres- ent review aims to offer an overview and future directions of these new approaches to control pathogenic oral biofilms.

Keywords Photochemotherapy · Photodynamic antimicrobial chemotherapy · Tissue-tolerable plasma · Microorganism · Biofilm

This article is part of the Topical Collection on Microbiology

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Introduction

The increased microbial resistance against commercially available antimicrobial drugs and substances have cooperated with the search for alternative treatments for the control of pathogenic biofilms involved with diseases that affect the body, including biofilm-dependent oral diseases [1, 2]. Photodynamic antimicrobial chemotherapy (PACT) is an alternative therapy that involves the concomitant combination of photosensitizers (PS) under proper light activation, aiming to generate toxic species of oxygen resulting in microbial death, while still being safe for the

mammalian cells [3]. Following the same path, another novel therapy with a similar mechanism of action is tissue-tolerable plasma (TTP), an ionized gas produced by subjecting one or more gases to an electric field, of either constant or alternating amplitude [4]. The advantages of PACT and TTP over other antibiotic applications are that they can be used for site-specific treatment. they provide almost instantaneous bacterial response, and there is less chance for the development of bacterial resistance and minimal side effects [1]. It is generally assumed that oxygen and nitrogen-based radicals are the most significant contributors to the sterilizing effects of PACT and TTP [5]. Hence, this review aims to provide an overview of (i) in vitro and in vivo studies of both PACT and TTP therapies to treat oral biofilms, and (ii) additional applications in dentistry, as well as to discuss their mechanisms of action and future directions for the field.

Photodynamic Antimicrobial Chemotherapy in Dentistry

Treatment using light and PS activated by light were men- tioned in ancient times and were used to treat a wide variety of disorders or diseases [6]. Different nomenclatures are given to this process. PACT, when there is a combination of a chemical compound and light, or simply photodynamic inactivation (PDI) or photodynamic therapy (PDT), are the most common definitions [7, 8]. PACT/PDI/PDT can be defined as the administration of a non-toxic drug or dye known as PS, either systemically, locally, or topically, to a patient with a localized lesion (including some types of cancer), followed by illumination with visible light (e.g. light-emitting diode [LED] or lasers) at a proper wavelength, which will activate the PS and produce cytotoxic species and, consequently, the breakdown of cellular microstructures, infected tissue destruction, and cell death [9]. This approach is considered a minimally invasive procedure and can be repeated several times, with minimal chances of causing resistance due to different mechanisms of action and targets [1]. Furthermore, PACT/PDI ideally causes limited or no damage to the host tissues [10]. A substantial number of studies state that the photodynamic approach can also be used to kill microorganisms, and is considered a promising alternative to treating oral biofilms involved in several major oral diseases such as candidiasis [11, 12], dental caries [13], and periodontal disease [14]. In recent years, PACT/PDI has been proposed as an alternative treatment for localized bacterial infections [15].

A PACT/PDI/PDT regimen can be beneficial for the elimination of microorganisms during systematic therapies of biofilm-dependent diseases, and is able to inactivate some of the known antibiotic-resistant microbial pathogens [15]. Therefore, this approach would be beneficial for the treatment of biofilm-dependent oral diseases, considering the high accumulation of bacteria in the oral environment and the easy access to the site of the disease. This alternative therapy is already known to be effective for some localized microbial infections, such as oral candidiasis [11, 12], chronic wounds, dental caries [13], periodontal disease [14], and treatment of endodontic lesions [16].

The mechanism by which microbial cells die when ex- posed to PACT-

generated reactive oxygen species (ROS) has been the subject of intense investigations. Some hypotheses are that after PS activation by light in a proper wavelength (corresponding to its absorption window), there is a transfer of energy from the activated PS and the available oxygen gives rise to the formation of highly reactive oxygen species, such as singlet oxygen and free radicals that oxidize biological substrates [17]. Another hypothesis is that after activation, PS can initiate a photochemical type I or II reaction [18, 19]. For the type I reaction, the activated PS reacts by transferring either an electron or hydrogen to an oxygen molecule or other adjacent molecule to form an anion or cation radical, respectively [18, 19]. These radicals are likely to react with molecular oxygen, resulting in ROS production [18, 19]. Examples of ROS include superoxide anion (O_2^-), hydroxyl radical (OH), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O^2$). The type II reaction is an energy transfer that occurs at the ground state of molecular oxygen and allows the production of singlet oxygen which is capable of reacting with cellular components, generating membrane permeability, irreversible damage, and microbial death [19, 20]. Enzymes such as superoxide dismutase, catalase, per- oxidase, and a number of secondary scavengers are able to control the levels of oxidative species [16]. In addition, critical sites of action for singlet oxygen in PACT/PDI/ PDT include mitochondria, DNA, and lipid membranes [9]. As established by the literature, radiation and chemo- therapy tend to damage the DNA and lead to apoptosis via cell–cycle checkpoints, growth arrest, and p53 activation [21, 22]. Alternatively, PACT/PDI/PDT tends to act via acute stress response involving mitochondrial damage, cytochrome C release, and formation of an apoptosome caspase [21, 22]. Acute challenges can affect the expression of proteins, especially transcription factors, which regulate the transcription of specific genes causing cell deregulation followed by cell death. Another possible mechanism of action is related to transcription factors; synthesis and expression of proteins are often induced by acute challenges and bind to certain regions on the DNA, resulting in transcription of genes and, consequently, production of different proteins causing cell deregulation followed by cell death [23].

An ideal PS should have a low level of dark toxicity (e.g. in the absence of light does not present any damage effect), absorb light in the corresponding therapeutic window, null activity related to mutagenicity/carcinogenicity, and be water soluble and easily eliminated by the body [24, 25]. PS are derived from the tetrapyrrole aromatic nucleus found in many naturally occurring pigments such as hemecholophyll and bacteriochlorophyll [24]. Such examples are represented by porphyrins and chlorins, which were the first dyes studied [24]. Both groups present an absorption band between 630 and 690 nm that can be activated by red light sources [19].

Synthetic, non-naturally conjugated pyrrolic ring dyes, and a non-tetrapyrrolic (naturally occurring or synthetic dye) are represented by rose bengal/erythrosine, toluidine blue, and methylene blue dyes, respectively. Most of these dyes can be excited with wavelengths between 400 and 500 nm, corresponding to light sources in the blue region [26, 27].

Different approaches and protocols have been used to determine the concentration of PS required to have the desired antimicrobial effect, which is established by the targeted microorganisms, their growth mode (planktonic or biofilm), and PS solubility [19, 28].

In general, PS are activated by complementary wave- lengths. For example, phenothiazine dyes (e.g. toluidine blue and methylene blue) are classified at the blue broadband and are correctly activated by red lights at 600-660nm that corresponds to complementary wavelength [29,30]. On this same way, red dyes, such as rose bengal, erythrosine, Photogem/Photosan can be irradiated with wavelengths between 400-500 nm that corresponds to the blue light spectrum [31,32].

The literature presents three main categories of clinical PACT light sources: LEDs, lasers, and halogen lamps [33•]. Lasers demonstrate some advantages, such as monochromaticity, high efficiency of light delivery without substantial power loss, and coupling of single optical fibers, but their costs are usually high when compared with other light sources [33•]. On the other hand, LEDs are most suitable for PACT and are easier to use presenting low costs when compared with lasers [34]. Also, in terms of wavelengths, LEDs and halogen lamps are not as specific as lasers, there- fore LEDs can be used for several PS with similar activation peaks [34]. Hence, the use of halogen lamps and blue LEDs that present lower potencies and lower tissue penetration are desired.

In the dental field, the usual PACT applications are related to accessible areas where light does not need to penetrate very deep (such as complementary to caries removal, caries prevention, herpes simplex, candidiasis, and periodontal disease) [35]. Although the current studies report on the success of the use of the aforementioned PS/lights, further studies are necessary to gain a better understanding of PACT mechanisms of action and toxicity.

The oral cavity sustains more than 700 species of microorganisms, few of which participate in the pathogenesis of various oral diseases, such as dental caries and candidiasis [35, 36]. The main challenge regarding the treatment of these diseases resides in antimicrobial resistance, reoccurrence (treatment fail), interactions with other medical regimens, organ toxicity, staining potential, taste alteration, and compliance of the patients to achieve therapy success [36].

The efficacy of PACT for different pathogens is dependent on the type and concentration of the PS, as well as on the interaction of the PS with the light source [33•]. In the biofilm, the presence of an extracellular matrix allows stability and structural integrity, possibly limiting the diffusion of sub- stances throughout the biofilm, and protecting the bacteria from inimical influences of antimicrobials and other environ- mental challenges [37]. Recent studies related to PACT application over Candida albicans [38] and Streptococcus mutans [39] using methylene blue/red LED (630 nm) and erythrosine/ laser (532 nm), respectively, achieved a high photokilling rate. Dovigo et al. [40] reported the inactivation of Candida sp. biofilms formed by

clinical isolate strains after the biofilms were exposed to Photodithazine®. Nastri et al. [41] state that the association of toluidine blue and diode laser light at 830 nm was effective against bacterial strains involved in periodontal disease, even though they were organized in biofilms, indicating it can be a coadjuvant method for the treatment of periodontal pockets. Araujo et al. [42] reported that curcumin in association with blue light was effective on the decontamination of dentine carious lesions contaminated with S. mutans and Lactobacillus spp. biofilms. However, possibly because of the limitation of light penetration and drug diffusion, microorganisms were less affected in the biofilm phase than the planktonic counterparts (references). Moreover, Lee et al. [43] were able to achieve significant cell death using erythrosine as a photosensitizing agent and a dental halogen light on S. mutans biofilm. Nevertheless, for dental use, several aspects, including energy, light irradiation times, and distance to the targeted area, must be carefully analyzed, aiming at tissue-tolerable temperature in the dentin, pulp, and other oral tissues.

Tissue-Tolerable Plasma in Dentistry

Plasma is an electrical discharge produced by subjecting one or more gases to an electrical field, either as a constant or alternating amplitude [4]. The term ‘plasma’ comes from Greek language and was introduced by Irving Lanmuir, an American chemist and physicist, in 1928. There are three main states of matter—solid, liquid, and gas. When a gas receives more energy, particles of the gas collide with each other. As a result, electrons and ions are produced and the gas becomes charged. This state of matter is called ‘plasma’, which is known as the fourth state of matter [44].

Plasma has several appealing features, including the ability to reach the gas phase without the need for elevated temperature [45, 46]. The electrons then collide with the gas, and enhance the level of dissociation, excitation, and ionization. The ions and neutrons continue reasonably cold, therefore TTP does not cause thermal damage to the surfaces [47, 48]. Bare hands may touch a TTP device without causing any heating, electrical shock, or other painful sensation [49]. Al- though TTP is not as well established as PACT, these characteristics open up the possibility of using plasmas for the treatment of heat-sensitive materials, including biological matter such as cells and tissues.

Plasma-based dental applications have attracted much attention in dental biomaterial modification [50, 51], tooth whitening [52, 53], dental material adhesion [54], and dental caries [55], and as an effective anti-oral biofilm agent [56, 57].

TTP may improve disinfection performance because this approach can kill several microorganisms, including bacteria and viruses [55, 58–63].

Scanning electron microscopy (SEM) was used to study how TTP exposure can impact Escherichia coli cell morphology [64]. The results show that E. coli cells presented severe morphological changes, including lysis (splitting of cells), which was attributed to one of two processes: membrane rupture caused by the charge build-up on

the cells, or chemical attack by radicals such as O_2^- and/or OH. Microorganisms exposed to plasma are also exposed to an intense bombardment by these short-living reactive species, with a higher oxidation potential, probably provoking surface levels that the living cell cannot repair sufficiently quickly. In this sense, we can say that TTP destroys microorganisms by disrupting the cell wall using these highly reactive free radicals, without the use of heat, chemicals, or pressure [65]. This may explain why cells are, in many cases, destroyed very quickly [66].

Sterilization by TTP usually depends on the synergism of a few bacterial agents: energetic photons in the ultraviolet (UV) B and UVC range; reactive species, including oxygen free radicals; and charged species such as electrons and ions. Their relevance is dependent on the type of plasma source and the sample preparation, in addition to how the UV photons get absorbed in atmospheric air [67]. The main plasma sterilization mechanisms are etching, oxidation, and UV radiation. Thus, bacterial size and structure may affect plasma effective- ness in bacterial deactivation. Yang et al. [68] reported the bactericidal effect of TTP on S. mutans and L. acidophilus, which are major pathogens in dental caries. The results indicated that TTP treatment was very effective in the disinfection of oral bacteria. They found that L. acidophilus killing needed a longer TTP exposure when compared with S. mutans. One of the hypotheses is that L. acidophilus presents a larger cell (≅1 μm × 3 μm), which could mean higher plasma tolerance when compared with the smaller S. mutans(≅1 μm in diameter) [68]. To accomplish the same killing intensity, up to 5 min of TTP exposure was necessary to deactivate L. acidophilus, which is contrary to the 10 s of TTP exposure needed to kill S. mutans.

In the same study [68], free-floating planktonic S. mutans and L. acidophilus were exposed to argon plasma for different periods of time. Leakage of intracellular proteins and nucleic acid related to bacterial killing were accessed by a UV-visible spectrometer. The absorbance peak intensity was the highest amount of intracellular proteins and DNAs. According to the findings, a very short plasma exposure of only 1 s significantly increased the killing of both bacteria, which was demonstrated by the dramatic leakage of the intracellular proteins and DNAs, which usually occurs when the cell membranes are damaged.

Lu et al. [49] showed that TTP could efficiently kill Enterococcus faecalis grown in biofilms, one of the main types of bacterium causing failure of root canal treatment, in several minutes. Pan et al. [69] also investigated the in vitro feasibility of using cold plasma against E. faecalis in infected root canals, and observed a significant decrease in the number of colony- forming units, after a prolonged exposure time of 8–10 min. Yamazaki et al. [70•] carried out experiments to evaluate the sterilizing effects of the low-frequency atmospheric pressure plasma on oral pathogenic microorganisms (S. mutans, C. albicans and E. faecalis) and to determine its potential for clinical application. The major findings were that the plasma was effective in sterilizing all three oral pathogenic microorganisms in a short exposure time.

Furthermore, the highest concentration of plasma energy occurs 5–6 mm beyond the plasma needle [71]. The plasma needle produces bactericidal agents locally and no excess of radicals remained at the end of the treatment period [71].

Sladek et al. [55] evaluated the antimicrobial activity of TTP against biofilm cultures of a key cariogenic bacterium, S. mutans, comparing TTP with 0.12 % chlorhexidine mouth rinse. The authors suggested that TTP may benefit caries treatment not only because of its antimicrobial properties but also because TTP is a gas that acts in a non-contact way, which could implicate that it can diffuse toward the surface, allowing the treatment of surface irregularities, including cracks and fissures. Under the conditions of this study, TTP presented higher inhibitory effects against S. mutans biofilm when com- pared with 0.12 % chlorhexidine. Moreover, the effectiveness of TTP is also dependent on the type of bacteria, culture medium, and input power of plasma.

Furthermore, considering dental material adhesion is one of the main concerns in dentistry, TTP has also been used to modify the surface of polymers and to deposit an inert protective layer on it (reference). The plasma processes can increase wettability, biocompatibility, and durability without influencing bulk material properties (reference). This feature of plasma may have great potential for dental application [72••].

Recently, there has been increasing interests on the effects of TTP. Since TTP has a bactericidal effect on oral pathogens, although it could not replace rotary instruments, it is expected that TTP will play an important role in the prevention and treatment of dental caries. Likewise, TTP is a potential method for disinfection of root canals and for the treatment of other infectious diseases of the oral cavity. There have been at- tempts to apply plasma technology in the various fields of dentistry. Although many studies are still in the early stages, the potential value of plasma for dental applications has been demonstrated [44, 57, 60]. In addition to safety concerns, the fundamental principles of how TTP interferes with tissues, cells, and the entire living body must be investigated. To enlarge the scope of plasma applications and place relevant research to practical use, interdisciplinary research with participation of dental professionals is required.

Conclusions and Future Directions

Although both PACT and TTP therapies are very promising, they are still not ready to replace classic therapy for treatment of oral diseases. However, these alternative approaches may improve, accelerate, and lower the cost of prevention and treatment of these diseases. Further studies should take into account aspects such as concentration of dye and high- potency light sources (important factors to be considered for PACT), as well as optimizing the exposure time and substance used to generate the TTP stream to further facilitate the elimination of oral biofilm bacteria.

Compliance with Ethics Guidelines

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Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Papers of particular interest, published recently, have been highlighted as:

- • Of importance
- •• Of major importance

1. Anderson GG, O'Toole GA. Innate and induced resistance mechanisms of bacterial biofilms. Curr Top Microbiol Immunol. 2008;322:85–105.

2. Fux CA et al. Survival strategies of infectious biofilms. Trends Microbiol. 2005;13(1):34–40.

3. Konan YN, Gurny R, Allemann E. State of the art in the delivery of photosensitizers for photodynamic therapy. J Photochem Photobiol B. 2002;66(2):89–106.

4. Moisan M et al. Low-temperature sterilization using gas plasmas: a review of the experiments and an analysis of the inactivation mechanisms. Int J Pharm. 2001;226(1–2):1–21.

5. Maisch T. Anti-microbial photodynamic therapy: useful in the future? Lasers Med Sci. 2007;22(2):83–91.

6. Allison RR, Mang TS, Wilson BD. Photodynamic therapy for the treatment of nonmelanomatous cutaneous malignancies. Semin Cutan Med Surg. 1998;17(2):153–63.

7. Hamblin MR, Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? Photochem Photobiol Sci. 2004;3(5):436–50.

8. Komerik N, MacRobert AJ. Photodynamic therapy as an alternative antimicrobial modality for oral infections. J Environ Pathol Toxicol Oncol. 2006;25(1–2):487–504.

9. Konopka K, Goslinski T. Photodynamic therapy in dentistry. J Dent Res. 2007;86(8):694–707.

10. Meisel P, Kocher T. Photodynamic therapy for periodontal diseases: state of the art. J Photochem Photobiol B. 2005;79(2):159–70.

11. Calzavara-Pinton P et al. Photodynamic antifungal chemotherapy. Photochem Photobiol. 2012;88(3):512–22.

12. Souza RC et al. Comparison of the photodynamic fungicidal efficacy of methylene blue, toluidine blue, malachite green and low- power laser irradiation alone against Candida albicans. Lasers Med Sci. 2010;25(3):385–9.

13. Teixeira AH et al. Effect of photodynamic antimicrobial chemo- therapy on in vitro and in situ biofilms. Caries Res. 2012;46(6): 549–54.

14. Goulart Rde C et al. Photodynamic therapy in planktonic and biofilm cultures of Aggregatibacter actinomycetemcomitans. Photomed Laser Surg. 2010;28 Suppl 1:S53–60.

15. Tseng SP et al. Toluidine blue O photodynamic inactivation on multidrug-resistant Pseudomonas aeruginosa. Lasers Surg Med. 2009;41(5):391–7.

16. Souza LC et al. Photodynamic therapy with two different photo- sensitizers as a supplement to instrumentation/irrigation procedures in promoting intracanal reduction of Enterococcus faecalis. J Endod. 2010;36(2):292–6.

17. Tuite EM, Kelly JM. Photochemical interactions of methylene blue and analogues with DNA and other biological substrates. J Photochem Photobiol B. 1993;21(2–3):103–24.

18. Dougherty TJ et al. Photodynamic therapy. J Natl Cancer Inst. 1998;90(12):889–905.

19. Castano AP, Demidova TN, Hamblin MR. Mechanisms in photo- dynamic therapy: part one-photosensitizers, photochemistry and cellular localization. Photodiagnosis Photodyn Ther. 2004;1(4): 279–93.

20. Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). J Antimicrob Chemother. 1998;42(1):13–28.

21. Boulikas T, Vougiouka M. Cisplatin and platinum drugs at the molecular level. Oncol Rep. 2003;10(6):1663–82.

22. Pruschy M et al. Key targets for the execution of radiation-induced tumor cell apoptosis: the role of p53 and caspases. Int J Radiat Oncol Biol Phys. 2001;49(2):561–7.

23. Villard J. Transcription regulation and human diseases. Swiss Med Wkly. 2004;134(39–40):571–9.

24. Detty MR, Gibson SL, Wagner SJ. Current clinical and preclinical photosensitizers for use in photodynamic therapy. J Med Chem. 2004;47(16):3897–915.

25. Allison RR et al. Photosensitizers in clinical PDT. Photodiagnosis Photodyn Ther. 2004;1(1):27–42.

26. Stockert JC et al. Photodynamic damage to HeLa cell microtubules induced by thiazine dyes. Cancer Chemother Pharmacol. 1996;39(1–2):167–9.

27. Bottiroli G et al. Enzyme-assisted cell photosensitization: a propos- al for an efficient approach to tumor therapy and diagnosis. The rose bengal fluorogenic substrate. Photochem Photobiol. 1997;66(3): 374–83.

28. Paschoal MA et al. Streptococcus mutans photoinactivation by combination of short exposure of a broad-spectrum visible light and low concentrations of photosensitizers. Photomed Laser Surg. 2014;32(3):175–80.

29. Wood S et al. Erythrosine is a potential photosensitizer for the photodynamic therapy of oral plaque biofilms. J Antimicrob Chemother. 2006;57(4):680–4.

30. Vahabi S et al. The effect of antimicrobial photodynamic therapy with radachlorin and toluidine blue on streptococcus mutans: an in vitro study. J Dent (Tehran). 2011;8(2):48–54.

31. Paulino TP et al. Use of hand held photopolymerizer to photoinactivate Streptococcus mutans. Arch Oral Biol. 2005;50(3):353–9.

32. Bolean M et al. Photodynamic therapy with rose bengal induces GroEL expression in Streptococcus mutans. Photomed Laser Surg. 2010;28 Suppl 1:S79–84.

33.• Nagata JY et al. Antibacterial photodynamic therapy for dental caries: evaluation of the photosensitizers used and light source properties. Photodiagnosis Photodyn Ther. 2012;9(2):122–31. This manuscript provides a current review of photodynamictherapy components applied to dental caries treatment.

34. Wilson BC, Patterson MS. The physics, biophysics and technology of photodynamic therapy. Phys Med Biol. 2008;53(9):R61–R109.

35. Zanin IC et al. Susceptibility of Streptococcus mutans biofilms to photodynamic therapy: an in vitro study. J Antimicrob Chemother. 2005;56(2):324–30.

36. Rezusta A et al. In vitro fungicidal photodynamic effect of hypericin on Candida species. Photochem Photobiol. 2012;88(3): 613–9.

37. Paes Leme AF et al. The role of sucrose in cariogenic dental biofilm formation: new insight. J Dent Res. 2006;85(10):878–87.

38. de Souza SC et al. Photosensitization of different Candida species by low power laser light. J Photochem Photobiol B. 2006;83(1):34–8.

39. Ishiyama K et al. Bactericidal action of photogenerated singlet oxygen from photosensitizers used in plaque disclosing agents. PLoS ONE. 2012;7(5):e37871.

40. Dovigo LN et al. Photodynamic inactivation of clinical isolates of Candida using Photodithazine®. Biofouling. 2013;29(9):1057–67.

41. Nastri L et al. Effects of toluidine blue-mediated photodynamic therapy on periopathogens and periodontal biofilm: in vitro evalu- ation. Int J Immunopathol Pharmacol. 2010;23(4):1125–32.

42. Araujo NC et al. Photodynamic effects of curcumin against cariogenic pathogens. Photomed Laser Surg. 2012;30(7):393–9.

43. Lee YH et al. The photodynamic therapy on Streptococcus mutans biofilms using erythrosine and dental halogen curing unit. Int J Oral Sci. 2012;4(4):196–201.

44. Kim JH et al. Plasma in dentistry: a review of basic concepts and applications in dentistry. Acta Odontol Scand. 2014;72(1):1–12.

45. Kunhardt EE. Generation of large-volume, atmospheric-pressure, non- equilibrium plasmas. IEEE Trans Plasma Sci. 2000;28(1):189–200.

46. Kogelschatz U. Filamentary, patterned, and diffuse barrier dis- charges. IEEE Trans Plasma Sci. 2002;30(4):1400–8.

47. Laroussi M, Lu X. Room-temperature atmospheric pressure plasma plume for biomedical applications. Appl Phys Lett. 2005;87(11).

48. Stoffels E et al. Plasma needle: a non-destructive atmospheric plasma source for fine surface treatment of (bio)materials. Plasma Sources Sci Technol. 2002;11(4):383–8.

49. Lu XP et al. An RC plasma device for sterilization of root canal of teeth. IEEE Trans Plasma Sci. 2009;37(5):668–73.

50. Silva NRFA et al. Surface characterization of Ti and Y-TZP follow- ing non-thermal plasma exposure. J Biomed Mater Res B Appl Biomater. 2011;99B(1):199–206.

51. Dantas MCC et al. Comparison between the effect of plasma and chemical treatments on fiber post

surface. J Endod. 2012;38(2):215–8.

52. Pan J et al. A novel method of tooth whitening using cold plasma microjet driven by direct current in atmospheric-pressure air. IEEE Trans Plasma Sci. 2010;38(11):3143–51.

53. Sun P et al. Tooth whitening with hydrogen peroxide assisted by a direct-current cold atmospheric-pressure air plasma microjet. IEEE Trans Plasma Sci. 2010;38(8):1892–6.

54. Cokeliler D et al. Modification of glass fibers to improve reinforce- ment: a plasma polymerization technique. Dent Mater. 2007;23(3): 335–42.

55. Sladek REJ et al. Treatment of Streptococcus mutans biofilms with a nonthermal atmospheric plasma. Lett Appl Microbiol. 2007;45(3):318–23.

56. Du TF et al. Evaluation of antibacterial effects by atmospheric pressure nonequilibrium plasmas against Enterococcus faecalis biofilms in vitro. J Endod. 2012;38(4):545–9.

57. Koban I et al. Antimicrobial efficacy of non-thermal plasma in comparison to chlorhexidine against dental biofilms on titanium discs in vitro: proof of principle experiment. J Clin Periodontol. 2011;38(10):956–65.

58. Kuo SP et al. A microwave-augmented plasma torch module. Plasma Sources Sci Technol. 2006;15(2):266–75.

59. Tang YZ et al. Sublethal and killing effects of atmospheric-pressure, nonthermal plasma on eukaryotic microalgae in aqueous media. Plasma Process Polym. 2008;5(6):552–8.

60. Goree J et al. Killing of S-mutans bacteria using a plasma needle at atmospheric pressure. IEEE Trans Plasma Sci. 2006;34(4):1317– 24.

61. Sladek REJ et al. Plasma treatment of dental cavities: a feasibility study. IEEE Trans Plasma Sci. 2004;32(4):1540–3.

62. Stoffels E et al. Plasma needle for in vivo medical treatment: recent developments and perspectives. Plasma Sources Sci Technol. 2006;15(4):S169–80.

63. Sladek REJ, et al. Investigation of possibilities of plasma treatment for dental caries. Ppc-2003: 14th IEEE International Pulsed Power Conference, 2003. Digest of Technical Papers. Vols 1 and 2:1109- 1111.

64. Laroussi M. Nonthermal decontamination of biological media by atmospheric-pressure plasmas: review, analysis, and prospects. IEEE Trans Plasma Sci. 2002;30(4):1409–15.

65. Laroussi M. Sterilization of contaminated matter with an atmo- spheric pressure plasma. IEEE Trans Plasma Sci. 1996;24(3): 1188–91.

66. Moreau M, Orange N, Feuilloley MGJ. Non-thermal plasma tech- nologies: new tools for bio-decontamination. Biotechnol Adv. 2008;26(6):610–7.

67. Vleugels M et al. Atmospheric plasma inactivation of biofilm- forming bacteria for food safety control. IEEE Trans Plasma Sci. 2005;33(2):824–8.

68. Yang B et al. Oral bacterial deactivation using a low-temperature atmospheric argon plasma brush. J Dent. 2011;39(1):48–56. 69. Pan J et al. Cold plasma therapy of a tooth root canal infected with enterococcus faecalis biofilms in vitro. J Endod. 2013;39(1):105–10. 70.• Yamazaki H et al. Microbicidal activities of low frequency atmospheric pressure plasma jets on oral pathogens. Dent Mater J. 2011;30(3):384–91. This manuscript is important because it describes a new research prototype model suggesting the use of plasma in small and difficult to reach environments.

71. Kim SJ, Chung TH, Bae SH. Striation and plasma bullet propagation in an atmospheric pressure plasma jet. Phys Plasmas. 2010;17(5).

72.•• Kim JH et al. Plasma in dentistry: a review of basic concepts and applications in dentistry. Acta Odontol Scand. 2014;72(1):1– 12. This manuscript is of major importance because it is the most recent review regarding the use of plasma in dentistry. The authors broadly describe the most important concepts to support the use of TTP against oral pathogens.