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FACULDADE DE ODONTOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA
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ALESSANDRA CESAR TRINDADE

TERAPIA FOTODINÂMICA COMO COADJUVANTE AO TRATAMENTO ENDODÔNTICO: ANÁLISE DA LITERATURA E ESTUDO EM RATOS

PHOTODYNAMIC THERAPY ASSOCIATED TO ENDODONTIC THERAPY: LITERATURE REVIEW AND A STUDY IN RATS

Prof. Dr. João Batista Blessmann Weber
Orientador

PORTO ALEGRE
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ALESSANDRA CESAR TRINDADE

Orientador: Prof. Dr. João Batista Blessmann Weber.

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Resumo

RESUMO

Os objetivos deste estudo foram: (a) estabelecer, através de revisão da literatura, o estado da arte na aplicabilidade da terapia fotodinâmica para a desinfecção do sistema de canais radiculares bem como as perspectivas futuras; (b) avaliar a resposta dos tecidos periapicais de dentes de ratos, portadores de periodontite apical, à terapia fotodinâmica como coadjuvante ao tratamento endodôntico, através de análise histológica, microbiológica e radiográfica. Para tanto, 32 molares inferiores esquerdos de ratos foram expostos ao meio bucal por 21 dias, para indução da periodontite apical. Após, foram divididos aleatoriamente em quatro grupos conforme o tratamento proposto: tratamento endodôntico em sessão única (G1); tratamento endodôntico em duas sessões com medicação intracanal à base de hidróxido de cálcio [$Ca(OH)_2$] por 14 dias (G2); tratamento endodôntico em sessão única, associado à terapia fotodinâmica (PDT) (G3) e tratamento endodôntico em duas sessões com $Ca(OH)_2$ por 14 dias, associado à PDT (G4). Após 28 dias, os animais foram mortos e as mandíbulas preparadas para avaliação. Para avaliação microbiológica foram realizadas coletas antes e após cada etapa de tratamento. As quantificações de UFC/mL foram analisadas através de modelos mistos lineares, mostrando significativa redução microbiana após todos os tratamentos propostos, quando comparados à coleta inicial, não havendo porém diferença significativa entre os grupos. Os resultados radiográficos foram submetidos a ANOVA, não havendo diferença estatisticamente significativas entre os grupos. Para os dados histológicos, foi utilizado o teste de Kruskall-Wallis seguido por Mann-Whitney U para aqueles casos onde diferenças foram detectadas. Estas diferenças foram encontradas nas populações de neutrófilos e eosinófilos nos grupos onde a PDT foi utilizada e para macrófagos e células gigantes quando o $Ca(OH)_2$ foi utilizado, independentemente da PDT. Os dados parecem apontar para uma melhor condição de reparo quando da utilização da PDT, pelo efeito biomodulador do *laser* nos tecidos periapicais. A associação da PDT com MIC com $Ca(OH)_2$ parece propiciar os melhores resultados, sendo necessários mais estudos para determinar esta relação.

Palavras-chave (termos MeSH e DeCS): Fotoquimioterapia, Lasers, Fármacos Fotossensibilizantes, Cavidade pulpar, Biofilmes, Endodontia

Abstract

ABSTRACT

The aims of this study were: (a) establish, through a review of the literature, the state of the art regarding the use of photodynamic therapy for the disinfection of the root canal system as well as future prospects, (b) evaluate the periapical tissues response on rats' teeth with apical periodontitis, to photodynamic therapy as an adjunct to endodontic treatment through histological, microbiological and radiological analysis. Therefore, 32 left lower rats' molars were exposed to the oral environment for 21 days to induce apical periodontitis. The animals were randomly divided into four groups according to proposed treatment: endodontic treatment in one session (G1); endodontic treatment in two sessions with with calcium hydroxide [$Ca(OH)_2$] dressing for 14 days (G2) ; endodontic treatment in one session associated with photodynamic therapy (PDT) (G3) and endodontic treatment in two sessions with $Ca(OH)_2$ for 14 days, associated with PDT (G4). After 28 days, the animals were killed and the jaws prepared for evaluation. For microbiological analysis samples were collected before and after each treatment step. Quantification of CFU/mL were analyzed using linear mixed models, showing significant microbial reduction after all proposed treatments, compared to the initial collection, although no significant difference between groups were found. The radiographic results were submitted to ANOVA, showing no statistically significant difference between groups. For histological data, Kruskal-Wallis test were used, followed by Mann-Whitney U test for those cases where differences were detected. These differences were found in neutrophils and eosinophils populations in groups where PDT was used and macrophages and giant cells when $Ca(OH)_2$ was used, with or without PDT. These data seem to point to a better repair quality when PDT is applied, caused by laser biomodulator effect in periapical tissues. The association of PDT with an inter-appointment dressing with $Ca(OH)_2$ produces better conditions to stimulate repair, being more research needed to determine this relationship.

Keywords (MeSH and DeCS terms): Photochemotherapy, Photosensitizing Agents, Lasers, Dental Pulp Cavity, Biofilms, Endodontics

Sumário

SUMÁRIO

1. Introdução Geral

1. INTRODUÇÃO GERAL

É fato que os microrganismos e seus produtos desempenham papel fundamental no desenvolvimento das doenças pulpares e periapicais, relacionando diretamente o sucesso da terapia endodôntica a sua permanência ou eliminação do sistema de canais radiculares, podendo comprometer o prognóstico dos dentes tratados endodonticamente. Devido às características da microbiota e às variações anatômicas nos sistemas de canais radiculares, principalmente na região apical, dada sua complexidade anatômica, somente o preparo químico-mecânico não é suficiente para assegurar a completa desinfecção dos canais radiculares contaminados (NAIR, SJÖGREN, FIGDOR, 1999; NAIR, 2004; GOMES *et al.* 2004). Agentes químicos antimicrobianos, como hipoclorito de sódio, digluconato de clorexidina e hidróxido de cálcio são utilizados no intuito de proporcionar a adequada descontaminação do sistema de canais radiculares.

Também com o propósito de reduzir a contaminação das superfícies dentárias ou de lesões de tecidos moles, passou-se a empregar a tecnologia LASER (acrônimo para *Light Amplification by Stimulated Emission of Radiaton*, ou "amplificação de luz por meio da emissão estimulada de radiações"), incorporada à prática clínica odontológica na década de 1990, e que ganha força nas últimas décadas, como coadjuvante às terapias convencionais.

Com a utilização de lasers de alta potência (Er:YAG, Er,Cr:YSGG, Nd:YAG, Ho:YAG e diodo), que provocam a morte de microorganismos por elevação da temperatura, através da desnaturação proteica, a redução microbiana pode ultrapassar

99% (GUTKNECHT *et al.* 1996a; GUTKNECHT *et al.* 1996b). Resultados tão promissores levam à indicação do uso de tal tecnologia como coadjuvante ao tratamento endodôntico convencional. No entanto, riscos de injúrias aos tecidos dentais e vizinhos, como carbonização da dentina, promoção de anquilose, derretimento de cemento, reabsorção radicular e necrose perirradicular, estão associados à utilização dos *lasers* de alta potência quando o fator térmico não é controlado (HARDEE *et al.*, 1994; KOBA *et al.*, 1999).

Lasers de baixa potência não produzem efeito térmico, pois a variação de temperatura é de aproximadamente 0,5ºC. Tampouco promovem alterações morfológicas na estrutura dentária, além de serem mais portáteis, de mais fácil manuseio e ter custo reduzido em relação aos *lasers* de alta potência. Como a dose da radiação é facilmente calculada, a área a ser tratada pode ser bem delimitada e a radiação pode ser transmitida por fibras ópticas, que podem receber adaptação para atingir o alvo (ACKROYD *et al.*, 2001), esta terapia tem encontrado lugar nas mais diversas áreas da Odontologia (WALSH, 1997a; WALSH, 1997b).

A utilização de lasers de baixa potência ou LEDs, associados a fotossensibilizadores, é a base da terapia fotodinâmica (PDT, do inglês PhotoDynamic Therapy), também chamada desinfecção fotoativada (PAD, Photo-Activated Disinfection) ou ainda quimioterapia antimicrobiana fotodinâmica (PACT, PhotoDynamic Antimicrobial Chemotherapy) (WAINWRIGHT, 1998; KONOPKA, GOSLINSKI, 2007).

Os efeitos antimicrobianos da terapia fotodinâmica na desinfecção do sistema de canais radiculares vêm sendo estabelecidos nos últimos anos, por uma série de

estudos *in vitro* (SEAL *et al.*, 2002; WILLIAMS, PEARSON, COLLES, 2006; SILVA GARCEZ *et al.*, 2006; SOUKOS *et al.*, 2006) e ensaios clínicos (BONSOR *et al.*, 2006a; BONSOR *et al.*, 2006b; GARCEZ *et al.*, 2008), com excelentes resultados na redução de diferentes microrganismos, inclusive aqueles resistentes à antibioticoterapia (GARCEZ *et al.*, 2010).

A avaliação histopatológica da reação dos tecidos periapicais a diferentes protocolos para o tratamento endodôntico substanciam a prática clínica baseada em evidências. Os efeitos biológicos de novas substâncias ou procedimentos empregados na terapia endodôntica devem ser pesquisados através de métodos que reproduzam as condições clínicas, como a reação inflamatória e a reabsorção de tecidos mineralizados na região periapical (TANOMARU *et al.*, 2008). A resposta biológica dos tecidos periapicais pode ser avaliada em dentes de ratos, onde a periodontite apical é induzida (KAKEHASHI, STANLEY, FITZGERALD, 1965; EURASQUIN, MURUZABAL, 1967; SATO, ANTONIAZZI, 1993; ANAN, AKAMINE , MAEDA, 1993; STASHENKO *et al.*, 1994).

A presente tese consiste em dois artigos científicos que investigam os efeitos da PDT na desinfecção do sistema de canais radiculares. No primeiro artigo é feita uma ampla revisão de literatura sobre o tema, buscando estabelecer o estado da arte no que tange a utilização da terapia fotodinâmica em endodontia. O mecanismo de ação e capacidade antimicrobiana da PDT em patógenos endodônticos, assim como os parâmetros aplicados e busca por otimização também são abordados. O segundo artigo avalia e compara, através de análise histológica e radiográfica, o reparo dos tecidos

periapicais em dentes de ratos, portadores de periodontite apical induzida, submetidos a:

a. tratamento endodôntico em sessão única;
b. tratamento endodôntico em sessão única associado à terapia fotodinâmica;
c. tratamento endodôntico em duas sessões, com intervalo de 14 dias, usando o hidróxido de cálcio como medicação intracanal;
d. tratamento endodôntico em duas sessões, com intervalo de 14 dias, usando o hidróxido de cálcio como medicação intracanal, associado à terapia fotodinâmica;

A redução microbiana intracanal, nos diferentes tratamentos propostos, também é avaliada.

2. Capítulo I

2. Capítulo I

Artigo 1

Photodynamic Therapy in Endodontics: State of the Art

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Photodynamic Therapy in Endodontics: State of the Art

AC Trindade[1], JAP De Figueiredo[1], L Steier[2], JBB Weber[1].

[1]Postgraduate Program in Dentistry, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil, [2]Postgraduate Dental Education Unit, University of Warwick, Warwick University Medical School, Coventry, United Kingdom

Corresponding Author:

Dr. Alessandra Cesar Trindade

Av. Ipiranga, 6681 - Prédio 6

CEP: 90619-900

Porto Alegre - RS - Brazil

55 (51) 3320-3538

endoale@gmail.com

Abstract

Recently, several *in vitro* and *in vivo* studies demonstrated promising results about the use of PDT during root canal system disinfection. However there is no consensus on a standard protocol to its incorporation during root canal treatment. The purpose of this study was to summarize the results of researches on PDT in endodontics published in peer-reviewed journals. A review of pertinent literature was carried out in PubMed database and data obtained were categorized into sections in terms of relevant topics. Studies carried out in recent years highlighted the antimicrobial potential of PDT. However most of these studies were not able to confirm a significant improvement in root canal disinfection, for PDT as a substitute to current disinfection methods. Its indication as an excellent adjunct to conventional endodontic therapy is well documented, though. Data suggest the need for protocol adjustments or new photosensitizers formulations to enhance PDT predictability.

Keywords (MeSH and DeCS terms): Biofilms, Dental Pulp Cavity, Endodontics, Lasers, Photochemotherapy, Photosensitizing Agents.

Keywords: biofilm, photodynamic therapy, photosensitizers, phototherapy, reactive oxygen species, root canal disinfection

Introduction

Microorganisms and their byproducts play a key role in pulpal and periapical diseases development, directly linking the success of endodontic therapy to their complete elimination from root canal system (Nair *et al.* 1999, Nair 2004, Gomes *et al.* 2004). Although cleaning and shaping (Sedgley 2004), application of an inter-appointment antibacterial dressing, and sealing of the root canal (Bystrom & Sundqvist 1981), are accepted procedures to achieve and maintain root canal disinfection, the complete elimination of microorganisms from the root canals system seems to be an impossible task (Sedgley 2004). Due to microflora characteristics and anatomical variations in root canal system, especially at apical region, given its complexity, these procedures may not suffice to ensure complete disinfection (Nair *et al.* 1999, Nair 2004, Gomes *et al.* 2004).

Aiming for complete disinfection, high-power lasers were used, leading to 99% of bacterial elimination by temperature rising and protein denaturation (Gutknecht 1996a, Gutknecht 1996b). However, damages to dental and surrounding tissues, such as ankylosis, cement and dentin melting or carbonization, root resorption and perirradicular necrosis, can be associated with high-power lasers (Hardee *et al.* 1994, Koba *et al.* 1999).

Due to its very low increase in temperature, approximately 0.5ºC (10), low-power lasers do not promote morphological changes in tooth structure (Hardee *et al.* 1994, Koba *et al.* 1999), but also do not promote disinfection when used alone. When associated to exogenous photosensitizers, a cascade of photochemical events starts, resulting in the production of reactive oxygen species, toxic to tumor cells, bacteria and fungi (Konopka & Goslinski 2007). This is the mechanism of action of photodynamic therapy (PDT), also called photo-activated disinfection (PAD), light-activated disinfection (LAD) or photodynamic antimicrobial chemotherapy (PACT) (Wainwright 1998). PDT has found a place in various fields of dentistry, including endodontics (Konopka & Goslinski 2007).

Many *in vitro* and *in vivo* studies in recent years showed the excellent antimicrobial potential of PDT in root canal system disinfection, especially against *Enterococcus faecalis*. The purpose of this study was to summarize the results of researches on photodynamic therapy in endodontics published in peer-reviewed journals.

Methods

A review of pertinent literature was carried out in PubMED by inserting the following keywords and Boolean operators: photodynamic therapy OR photoactivated disinfection OR photodynamic antimicrobial chemotherapy OR photodynamic inactivation OR light activated disinfection OR photo-activated disinfection AND endodontics. All articles retrieved form search were considered, with no restriction to time period applied, but only articles in English were included. Forty-nine articles were retrieved and thirty-three were selected after applying exclusion criteria for reviews and cytotoxicity studies. Data obtained from this review will be presented in the following sections in terms of relevant topics, added by relevant articles on the basis of PDT.

Review

Photodynamic Therapy

The action mechanism of PDT relies on the topical or systemic administration of a nontoxic photosensitizer, followed by low dose irradiation with visible light of a suitable wavelength. Absorption of the light triggers excitation of the photosensitizer which, in presence of oxygen, leads to a cascade of photochemical effects, resulting in the production of high reactive oxygen species (ROS), toxic to tumor cells, bacteria and fungi. These reactions can occur by electron transfer to hydrogen, leading to the production of free radicals (type I reaction) or by energy transfer to oxygen (type II reaction), resulting on the production of singlet oxygen (Konopka & Goslinski 2007).

Light Sources

Photosensitizer activation has been accomplished by using various light sources, such as argon lasers, Nd:Yag, gold or copper vapor lasers, all complex and expensive

equipment (Konopka & Goslinski 2007). With the advent of diode lasers, these have become the most used for its low cost and portability. Other light sources, such as light emitting diodes (LED) or conventional halogen light, have also been used with good results (Bouillaguet *et al.* 1996a, Bouillaguet *et al.* 1996b, Schlafer *et al.* 2010, Rios *et al.* 2011). The use of intracanal optical fibers has also been studied as a way to increase the effectiveness of PDT (Foschi *et al.* 2007, Fimple *et al.* 2008, Ng *et al.* 2011, Nunes *et al.* 2011, Garcez *et al.* 2013). Basically, the light source correct calibration as well as the radiation and photosensitizer delivery should be monitored and the resonance between the light source wavelength and the selected photosensitizer must be observed (Wainwright 1998) as it will be explained hereinafter.

Photosensitizers

The desired properties of an optimal photosensitizer include favorable photo-physical, chemical and biological characteristics, low citotoxicity, short-time photosensitivity, absorption peaks in the low-loss transmission window of biological tissues, simplicity in formulation, reproducibility, high stability and high affinity and penetration on bacterial cell in detriment of healthy tissues (selectivity) (Konopka & Goslinski 2007).

Although the photochemical principle for cancer and antimicrobial PDT is the same, there are important differences in the structures of photosensitizers and cellular targets. For cancer treatment, porphyrins, chlorins, phthalocyanines and bacteriochlorins are the indicated photosensitizers, for their tumor location and low toxicity in the absence of light in mammalian cells. To eradicate microorganisms, the most studied photosensitizer belong to the groups of halogenated xanthene, phenothiazines, acridines and conjugated chlorin (Wainwright 1998).

Hamblin and Hasan (2004) have mentioned that photosensitizers for antimicrobial purposes can be divided into three groups: those that strongly bind and penetrate the microorganisms (chlorin e_6, for example), those that bind weakly (toluidine blue and methylene blue) and those that do not demonstrate binding (Rose Bengal). This is because, in bacterial cells, outer membrane damage plays a important role, differently

from mammalian cells, where the main targets for PDT are lysosomes, mitochondria and plasma membranes.

In endodontics, photosensitizers derived from phenothiazines have been widely used (Seal *et al.* 2002, Bonsor *et al.* 2006a, Bonsor *et al.* 2006b, Silva Garcez *et al.* 2006, Soukos *et al.* 2006, Williams *et al.* 2006, Foschi *et al.* 2007, Garcez *et al.* 2007, George & Kishen 2007, Bergmans *et al.* 2008, Fimple *et al.* 2008, Fonseca *et al.* 2008, Garcez *et al.* 2008, George & Kishen 2008, Lim *et al.* 2009, Garcez *et al.* 2010, Kishen *et al.* 2010, Pagonis *et al.* 2010, Souza *et al.* 2010, Upadya & Kishen 2010, Ng *et al.* 2011, Nunes *et al.* 2011, Cheng *et al.* 2012, Shrestha *et al*. 2012, Shrestha & Kishen 2012, Silva *et al*. 2012, Bago *et al*. 2013, Eldeniz *et al*. 2013, Garcez *et al.* 2013, Komine & Tsujimoto 2013, Stojicic *et al*. 2013). Phenothiazines show intense absorption at 600-660nm wavelength (red light), a useful spectrum in PDT, known as the therapeutic window required for efficient light penetration in biological tissues (Wainwright 1998). Both tumor cells and bacterial strains resistant to multiple antibiotics are sensitive to methylene blue (MB) and ortho-toluidine blue (TB).

According to Bouillaguet *et al*. (2010a, 2010b), blue light sources (380 - 520 nm), routinely used in dental offices for resin-based materials photocuring, are attractive options for PDT in dentistry. But, despite this potential advantage, the use of blue light for PDT may be limited by a lack of appropriate photosensitizers. They point Riboflavin, chlorin e_6 and pheophorbide-a polylysine as suitable photosensitizers for blue light sources, but suggest further additional tests before its clinical indication.

Pre-irradiation time and irradiation dose

Pre-irradiation time (PIT) corresponds to the time elapsed between the photosensitizer application and its activation by light. This time is necessary to allow photosensitizer uptake by the target before irradiation, since it is expected to bind or even translocate cell membrane.

According to Wainwright (1998), a photosensitizer that is slowly uptaken by the microorganism may at first cause only cell wall photodamage whereas nucleic acid

strand breakage, for example, will be apparent on longer incubation times.

The total energy applied by the light source to the photosensitizer may also interfere with the chemical reactions and ROS release, changing the outcome of PDT. To be able to understand and control the irradiation it is necessary to know some physical parameters. Energy (E) may be defined as the light amount deposited on target and is defined by the relation between the light source power (P) and application time (t) (E = P x t) and is expressed in Joules (J).

Fluency is the parameter that causes more confusion, since some authors suggest its calculation should take the light source cross-sectional area (Soukos *et al.* 2006), while others consider an estimated area of $1cm^2$ where light would be acting. In both cases it is the rate at which energy is deposited in a determined area and is expressed in J/cm^2.

Bactericidal effects of PDT

There is a fundamental difference in susceptibility to antimicrobial PDT between Gram-positive (G+) and Gram-negative (G-) bacteria. In general, G+ bacteria are more susceptible than G-, thus the structural characteristics of different bacterial types must be observed (Wainwright 1998, Usacheva *et al.* 2001).

High susceptibility of G+ species can be explained by their physiology. Cytoplasmic membrane is surrounded by a relatively porous layer of peptidoglycan and lipoteichoic acid, which allows the photosensitizer to cross. G- bacteria have an inner cytoplasmic membrane and an outer membrane, separated by a peptidoglycan-containing periplasm that forms a physical and functional barrier between the cell and its environment. Several different proteins are present in outer membrane, some of which function as pores to allow the passage of nutrients, whereas others have enzymatic function or are involved in maintaining the structural integrity of the outer membrane (Hamblin & Hasan 2004). Neutral or anionic photosensitizers molecules are effective in binding and inactivating G+ bacteria. In G- bacteria these molecules bind only with the outer membrane, to a greater or lesser extent, not being able to completely inactivate them after illumination (Hamblin & Hasan 2004). To inactivate a bacterial cell, the

photosensitizer must be absorbed by the cell membrane and/or be translocated to the cytoplasm, leading to inhibition of further DNA, RNA and protein synthesis (Usacheva *et al.* 2001).

According to Wainwright (1998), phenothiazines are more effective against G+ than G- species. Due to its hydrophilic nature, low molecular weight and positive charge that allows passage through the porin-protein channels in the outer membrane of G- bacteria, MB interacts predominantly with lipopolysaccharide anionic macromolecules. In an *in vitro* study by Usacheva *et al*. (2001), TB interacted with G- LPS significantly more than MB, which can be one of the main determining factors in the photo-oxidative effect against G- bacteria.

***in vitro* / *ex vivo* studies**

Data referring to study type, microrganisms involved and bacterial reduction, when available, are summarized in Table 1. Parameters used for PDT in each study can be found in Table 2. This section meant to discuss some relevant topics regarding different methodologies and results.

It seems to be well established that neither the photosensitizer or light source only are able to produce significant bacterial reduction. It is the combination of both that can activate the PDT mechanism and lead to bacterial death (Seal GJ, *et al.* 2002, Silva Garcez *et al.* 2006, Williams *et al.* 2006, Foschi *et al.* 2007, Bergmans *et al.* 2008, Fimple *et al.* 2008, Schlafer *et al.* 2010, Rios *et al.* 2011)

Light sources other than lasers (LEDs or non-coherent lights) do not affect PDT outcome. Bacterial reduction can be achieved regardless the LS, since its wavelength is compatible to the photosensitizers absorption range (Kishen *et al.* 2010, Schlafer *et al.* 2010, Upadya & Kishen 2010, Rios *et al.* 2011, Cheng *et al.* 2012, Eldeniz *et al.* 2013). Various photosensitizer concentrations were evaluated and phenothiazines (TB or MB) at 0.0125/0.01% seem to have more evidence available regarding its efficacy (Seal *et al.* 2002, Williams *et al.* 2006, Schlafer *et al.* 2010, Komine & Tsujimoto 2013) with no statistical differences between them (Souza *et al.* 2010).

All bacterial strains tested seem to be sensitive to PDT, although in different levels (Seal *et al.* 2002, Bonsor *et al.*2006a, Bonsor *et al.* 2006b, Silva Garcez *et al.* 2006, Soukos *et al.* 2006, Williams *et al.* 2006, George & Kishen 2007, Garcez *et al.* 2007, Foschi *et al.* 2007, Bergmans *et al.* 2008, Fimple *et al.* 2008, Fonseca *et al.* 2008, Garcez *et al.* 2008, George & Kishen 2008, Lim *et al*. 2009, Garcez *et al.* 2010, Kishen *et al.* 2010, Pagonis *et al.* 2010, Schlafer *et al.* 2010, Souza *et al.* 2010, Upadya & Kishen 2010, Ng *et al*. 2011, Nunes *et al.* 2011, Rios *et al.* 2011, Cheng *et al.* 2012, Shrestha *et al.* 2012, Shrestha & Kishen 2012, Silva *et al.* 2012, Bago *et al.* 2013, Garcez *et al.* 2013, Komine & Tsujimoto 2013, Stojicic *et al.* 2013), with exception of *Candida albicans* (Eldeniz *et al.* 2013). PDT is less effective in root canals biofilms than in planktonic bacteria suspensions, even in younger biofilms, so substrates or tissue inhibitors appear to have an important effect on PDT process (Schlafer *et al.* 2010, Silva Garcez *et al.* 2006, Williams *et al.* 2006, Shrestha & Kishen 2012). Mature biofilms seems to be more difficult to inactivate and ways to improve its disrupting and inactivation have been studied (Bonsor *et al.* 2006b, Lim *et al*. 2009).

Studies evaluating the effects of PDT alone usually found great bacterial reduction (Foschi *et al.* 2007, Fimple *et al.* 2008, Schlafer *et al.* 2010, Williams *et al.* 2006, Soukos *et al.* 2006, Bergmans *et al.* 2008, Fonseca *et al.* 2008). Those comparing PDT with sodium hypochlorite (NaOCl) (3 to 6%) or conventional chemomechanical preparation (CMP), normally showed better results for NaOCl or CMP alone (Seal *et al.* 2002, Lim *et al*. 2009, Rios *et al.* 2011, Cheng *et al.* 2012). Lower NaOCl concentrations (i.e. 0.5 and 2.5%) are less effective than PDT alone (26; 46). The association of PDT and NaOCl or CMP, even with 2.5% NaOCl, seems to achieve the best results in bacterial reduction (Garcez *et al.* 2007, Lim *et al*. 2009, Souza *et al.* 2010, Ng *et al*. 2011, Rios *et al.* 2011).

in vivo studies

Since *in vivo* studies are known to produce better clinical evidence, they are discussed more profoundly. Four *in vivo* studies were performed in patients with

irreversible pulpitis or apical periodontitis, where endodontic treatment was indicated. Microbiological samples were obtained after each step for CFU counting.

Two studies associated PDT to conventional CPM (Bonsor *et al.*2006a, Garcez *et al.* 2008), finding reduction ranges from 87.7 to 91% for CPM alone and 96.7 to 98.5% when PDT was performed after CPM. Garcez *et al.* (2008) went further and performed a second session of therapies, after 1 week with calcium hydroxide ($Ca(OH)_2$) as inter-appointment intracanal medication. The total first plus second reduction (99.9%) was significantly different from the first combination ($p=0.00006$). Results of these clinical trials suggest that PDT added to endodontic treatment may lead to an enhanced decrease of bacterial load and be an appropriate approach for the treatment of endodontic infections.

The antimicrobial effect of PDT in patients with antibiotic-resistant microflora was reported (Garcez *et al.* 2010). Initial samples showed that all patients had at least one microorganism resistant to antibiotics. CMP alone produced a significant reduction in numbers of microbial species but only 3 teeth were bacteria-free, whereas the combination of CMP with PDT eliminated all drug-resistant species.

Bonsor *et al.* (2006b) also compared two protocols, where PDT was performed after coronal preparation with a chelating agent, previously to CPM with NaOCl, and after complete CMP. The authors found a greater proportion of bacteria-free teeth when PDT was performed after coronal preparation then after complete CMP. Results indicate that the use of a chelating agent acting as a cleaner and disrupter of the biofilm, associated with PDT, is an effective alternative to the use of hypochlorite as a root canal cleaning system.

A histopathological study, where apical periodontitis was induced on dogs, was performed by Silva *et al.* (2012). PDT-treated groups showed moderately/severely enlarged periapical region with no inflammatory cells, moderate neoangiogenesis and fibrogenesis, and the smallest periapical lesions. Although apical closure by mineralized tissue deposition was not achieved, these findings suggest that PDT can be a promising

adjunct therapy to cleaning and shaping procedures in teeth with apical periodontitis undergoing one-session endodontic treatment.

Optimizing PDT efficacy

To increase the antimicrobial activity of PDT, different photosensitizers formulations and associations and/or PDT protocols have been suggested.

The amount of singlet oxygen ($^1O_2$) generated from different MB concentrations was examined by Komine and Tsujimoto (2013). The largest amount of $^1O_2$ was generated from 0.01% MB and was increased by laser irradiation in a dose-dependent manner. Associations of MB with 0.5% hydrogen peroxide (H_2O_2) and 0.05% chlorhexidine (CHX), 0.5% H_2O_2 and 0.05% EDTA or 0.05% EDTA and 0.05% CHX were tested by Stojicic at al. (2013). In modified PDT, up to 100% of suspended *E. faecalis* and mixed plaque bacteria were killed. Up to twenty times more biofilm bacteria were killed by modified PDT than by conventional PDT with MB alone ($P<0.001$).

Formulations of MB, dissolved in a mixture of glycerol:ethanol:water (MIX) (George & Kishen 2007, Lim *et al*. 2009, Upadya & Kishen (2010) and in an emulsion of perfluoro-decahydro-naphthalene:H_2O_2:triton-X100 (PF4) were proposed (George & Kishen 2008, Upadya & Kishen 2010). According to the authors, these modified photosensitizer formulations effectively enhanced photo-oxidation and singlet oxygen generation and facilitated comprehensive inactivation of biofilm bacteria. An improved dual-staged PDT technique, utilizing MIX-based MB and/or PF4 as photosensitizer and the oxygen carrier perfluoro-decahydro-naphthalene (PF) as irradiation medium, was suggested (George & Kishen 2007, George & Kishen 2008, Lim *et al*. 2009, Upadya & Kishen 2010). This dual-staged protocol consists in removing photosensitizer excess after PIT and filling the root canal with PF, previously to irradiation, in order to achieve more thorough disinfection.

A broadly recognized component of microbial resistance to many classes of antibiotics, efflux pumps are found in both G+ and G- bacteria. Kishen *et al.* (2010) investigated the role of a specific microbial efflux pump inhibitor (EPI), verapamil

hydrochloride, in the MB-mediated PDT of *E. faecalis* biofilms. The ability to inactivate biofilm bacteria was enhanced when the EPI was associated with MB ($P<0.001$).

It has been pointed that the combination of photosensitizer with bioactive natural polymers such as chitosan, with inherent ability to permeate bacterial cell membrane and interact with bacterial cell biofilm structure, could further improve the anti-biofilm efficacy of PDT. A Rose Bengal-conjugated chitosan (CSRB) as photosensitizer, activated by green light, was evaluated (Shrestha *et al.* 2012). The CSRB particles may be a synergistic multifunctional treatment approach with lower cytotoxicity and effective anti-biofilm activity as well as the ability to reinforce the dentin collagen to enhance resistance to degradation and improve mechanical properties. This may be a targeted treatment strategy to deal with infected dentin in a clinical scenario where both disinfection and structural integrity need to be addressed concomitantly.

Recently, nanoparticles based on metals or polymers are being assessed for augmenting the endodontic disinfection methods (Pagonis *et al.* 2010, Shrestha & Kishen 2012). The antibacterial activity of chitosan nanoparticles (CSnps) and PDT with RB and MB as photosensitizer were tested on the presence of various tissue inhibitors (Shrestha & Kishen 2012). The tissue inhibitors existing within the root canal affected the antibacterial activity of CSnps and PDT at varying degrees, suggesting further research to enhance their antimicrobial efficacy in an endodontic environment.

The uptake and distribution of poly(lactic-co-glycolic acid) (PLGA) nanoparticles loaded with MB by *E. faecalis* in suspension was investigated (Pagonis *et al.* 2010). Transmission electron microscopy showed nanoparticles concentrated mainly on the cell walls of microorganisms. To evaluate its effect in infected root canals after PDT, contents were sampled by flushing the root canals with a coronal application of 1 mL of BHI broth, for CFU counting. Survival fractions were 15.2% and the authors suggest that the PDT effects were probably affected by the presence of serum proteins in BHI broth.

Although the great majority of studies retrieved for this review mention the use of an intracanal optical fiber (ICF) (Table 2), its necessity was a specific subject of two studies (Nunes *et al.* 2011, Garcez *et al.* 2013). Both studies used extracted single-

rooted teeth contaminated with *E. faecalis*, MB as photosensitizer and similar radiation doses. In the study performed by Nunes *et al*. (2011), all teeth had the crown removed and they achieved bacterial reduction from 99.41 to 99.65% without or with ICF respectively, suggesting that PDT was effective regardless of the use of an intracanal optical fiber. Garcez *et al*. (2013) used teeth with and without the crown. All teeth with crowns previously removed showed a reduction of two logs (99%). Teeth whose crowns were kept, showed a reduction of one log (85% and 97%, depending on the size of the laser tip) and four logs (99.99%) when ICF was used. These results suggest that, at clinical conditions, when teeth has its crowns, the use of the ICF is better than when the laser light is placed on the pulp chamber.

Ng *et al.* (2011) suggested future studies exploring the use of ultrasonic waves for enhancement of the transdentinal movement and penetration of MB in canal biofilms. It has been already pointed that high-intensity focused ultrasound produces collapsing cavitation bubbles that can deliver antibacterial nanoparticles into the dentinal tubules, improving root canal disinfection (Shrestha *et al.* 2009). This may be a great way to enhance PDT effects and should be investigated in future experiments.

Conclusions

Studies carried out in recent years highlighted the antibacterial potential of photodynamic therapy (PDT), although most of these studies were not able to confirm significant improved disinfection when compared to conventional chemomechanical preparation with NaOCl. Planktonic bacteria are way more sensitive to PDT than bacteria in biofilms. Different photosensitizers, different irradiation doses and light sources, with different wavelengths and power were used. Hence it is almost impossible to reach a consensus about a protocol to be clinically recommended. Considering literature reviewed, most recommended PIT is 1 to 5 minutes and phenothiazines and their associations/modifications are the most tested photosensitizers. Low level lasers emitting red light (600-660 nm) appear as the most used light source but LEDs or non-coherent lights can also be used. Irradiation dose can vary from 1.2 to 159 J and the use of an intracanal fiber may increase uniformity of light distribution along the root canal,

improving PDT efficiency. Further studies for adjustments in PDT protocol or photosensitizers formulation, in order to optimize PDT outcome, as well as more *in vivo* studies, are suggested.

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TABLE 1. Study type, microorganisms studied and bacterial reduction after PDT in root canals

Ref.	Study type	Microorganisms	Bact. ↓(% or log)
Seal *et al.* 2002	ex vivo	*S. intermedius*	5log10
Bonsor *et al.* 2006a	in vivo	polymicrobial/naturally infected teeth	96.7
Bonsor *et al.* 2006b	in vivo	polymicrobial/naturally infected teeth	91
Williams *et al.* 2006	in vitro/ex vivo	*F. nucleatum, P. micros, P. intermedia and S. intermedius*	99
Silva Garcez *et al.* 2006	ex vivo	*E. faecalis*	99.2
Soukos *et al. 2006*	in vitro/ex vivo	*P. gingivalis, P. intermedia, F. nucleatum, P. micros, P. endodontalis, E. faecalis*	97
Gerge & Kishen 2007	in vitro/ex vivo	*E. faecalis, A. actinomycetemcomitans*	100/99.77
Garcez *et al.* 2007	ex vivo	*P. mirabilis, P. aeruginosa*	98
Foschi *et al.* 2007	ex vivo	*E. faecalis*	77.5
Fimple *et al.* 2008	ex vivo	*A. israelii, F. nucleatum, P. gingivalis, P. intermedia*	> 80
Garcez *et al.* 2008	in vivo	polymicrobial/naturally infected teeth	99.9
Bergmans *et al.* 2008	ex vivo	*S. anginosus, E. faecalis, F. nucleatum*	93.8/88.4/98.5
Gerge & Kishen 2008	in vitro/ex vivo	*E. faecalis*	100
Fonseca *et al.* 2008	ex vivo	*E. faecalis*	99.9
Lim *et al.* 2009	ex vivo	*E. faecalis*	99.99
Pagonis *et al.* 2010	in vitro/ex vivo	*E. faecalis*	84.8
Souza *et al.* 2010	ex vivo	*E. faecalis*	> 99.48
Upadya & Kishen 2010	in vitro	*E. faecalis, P. aeruginosa*	100/99
Kishen *et al. 2010*	in vitro	*E. faecalis*	✪
Garcez *et al.* 2010	in vivo	polymicrobial/naturally infected teeth	100
Schlafer *et al. 2010*	in vitro/ex vivo	*E. coli, C. albicans, E. faecalis, F. nucleatum, S. intermedius*	99.75
Rios *et al.* 2011	ex vivo	*E. faecalis*	99.9
Ng *et al.* 2011	ex vivo	polymicrobial/naturally infected teeth	70
Nunes *et al.* 2011	ex vivo	*E. faecalis*	> 99.41
Garcez *et al.* 2012	ex vivo	*E. faecalis*	99.99
Cheng *et al. 2012*	ex vivo	*E. faecalis*	96.96
Shrestha *et al.* 2012	in vitro	*E. faecalis*	100
Shrestha & Kishen 2012	in vitro/ex vivo	*E. faecalis*	27 to 98
Silva *et al.* 2012	in vivo	polymicrobial/naturally infected teeth	✪
Eldeniz *et al.* 2013	ex vivo	*C. albicans*	✪
Stojicic *et al. 2013*	in vitro	*E. faecalis*, mixed plaque	0 to 100
Bago *et al.* 2013	ex vivo	*E. faecalis*	99.99
Komine & Tsujimoto 2013	in vitro	*E. faecalis*	>99.0

✪ uninformed

TABLE 2. PDT parameters adopted per study

Ref.	PS	PS Concentration	PIT (sec.)	LS	λ (nm)	ICF	RD (J or J/cm ²)
Seal *et al.* 2002	TB	12.5, 25, 50, 100μgmL–1	30	laser	632.8	no	2.1 to 21 J
Bonsor *et al.* 2006a	TB	✪	60	laser	633 +/- 2	yes	12 J
Bonsor *et al.* 2006b	TB	✪	60	laser	633 +/- 2	yes	12 J
Williams *et al.* 2006	TB	10.0, 20.0 mg L-1	0/30/60	laser	633 +/- 2	yes	9.6 to 15.8 J
Silva Garcez *et al.* 2006	azulen-based paste	✪	300	laser	685	yes	9 J
Soukos *et al. 2006*	MB	25 μg/ml	300	laser	665	yes	70 J
Gerge & Kishen 2007	MIX	✪	1800	laser	664	no	36 J
Garcez *et al.* 2007	PEI+CE6	✪	600	laser	660	yes	9.6 J
Foschi *et al.* 2007	MB	6.25 μg/ml	300	laser	665 +/- 2	yes	18.96 J
Fimple *et al.* 2008	MB	25 μg/mL	600	laser	665	yes	30 J/cm²
Garcez *et al.* 2008	PEI+CE6	✪	120	laser	660	yes	9.6 J
Bergmans *et al.* 2008	PEI+CE6	12.7 mg mL-1	60	laser	635	yes	15 J
Gerge & Kishen 2008	PF4	✪	600	laser	664	no	31.84 J/cm²
Fonseca *et al.* 2008	TB	.0125%	300	laser	660	yes	6.4 J
Lim *et al.* 2009	MIX	✪	0/1200	laser	660	no	36 J
Pagonis *et al.* 2010	MB+PGLA	6.25 μg/ml	900	laser	665	yes	30 J/cm²
Souza *et al.* 2010	MB/TB	15 μg/mL	120	laser	660	yes	9.6 J
Upadya & Kishen 2010	MIX/PF4	✪	900	noncoherent	660	no	2 to 40 J
Kishen *et al. 2010*	RB/MB/MB+EPI	100 μM EPI 0.49 mg mL-1	900	noncoherent	540±15 for RB/660±15 for MB	no	2 to 30 J/cm²
Garcez *et al.* 2010	PEI+CE6	✪	120	laser	660	yes	9.6 J
Schlafer *et al. 2010*	TB	100 μg/mL	60	LED	600 to 660 (628 peak)	yes	30 J
Rios *et al.* 2011	TB	✪	30	LED	600 to 660 (628 peak)	yes	30 J
Ng *et al.* 2011	MB	50 μg/mL	300	laser	665	yes	30 J/cm²
Nunes *et al.* 2011	MB	.01%	300	laser	660	yes	8.1 and 16.2 J
Garcez *et al.* 2012	MB	60μM	600	laser	660	yes	9.6 J
Cheng *et al. 2012*	MB	.01 mg/ml	0	laser	660	yes	12 J
Shrestha *et al.* 2012	CSRB	✪	900	✪	540	✪	5 to 60 J/cm²
Shrestha & Kishen 2012	CSnps/RB/MB	✪	900	✪	✪	✪	5 and 10 J/cm²
Silva *et al.* 2012	phenothiazine chloride	10 mg/ml	60	laser	660	yes	3.3 J/cm²
Eldeniz *et al.* 2013	TB	0.1 mg/ml	60	LED	620 to 640 (630 peak)	yes	30 J
Stojicic *et al. 2013*	MB+	15μM/L^{-1}	0/300	laser	660	no	1.2/2.4/7.2 J
Bago *et al.* 2013	TB/phenothiazine chloride	155μg mL-1/ 10 mg mL-1	60/120	laser	660	yes	6 J
Komine & Tsujimoto 2013	MB	.01%	0	laser	660	no	53/106/159 J/cm²

PS, photosensitizer; PIT, pre-irradiation time; LS, light source, λ, wavelenght; ICF, intracal fiber; RD, radiation dose
TB=Toluidine blue; MB=Methilene blue; MIX=MB in glycerol:ethanol:water (30:20:50); PEI+CE6=polyethylenimine and chlorin-e6 conjugate;
PF4=MB +perfluoro-decahydro-naphthalene:H2O2:triton-X100; MB+PGLA=MB-loaded nanoparticles; RB=Rose Bengal;
MB+EPI=associated with efflux pump inhibitor (EPI), verapamil hydrochloride; CSRB=Rose Bengal-conjugated chitosan; CSnps=chitosan nanoparticles;
MB+=different associations
✪ uninformed

Table 2.

3. Capítulo II

3. Capítulo II

Artigo 2

Histopathological, Microbiological and Radiographic Analysis of Antimicrobial Photodynamic Therapy for the Treatment of Teeth with Apical Periodontitis: a Study in Rats' Molars

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Histopathological, Microbiological and Radiographic Analysis of Antimicrobial Photodynamic Therapy for the Treatment of Teeth with Apical Periodontitis: a Study in Rats' Molars

Alessandra Cesar Trindade, DDS, MSc[1], José Antônio Poli de Figueiredo, DDS, MSc, PhD[1], Silvia Dias de Oliveira, PhD[2], Valdir Cristóvão Barth Junior, BSc[2], Carina Follmann, DDS, MSc[1], Liviu Steier, DMD[3], Carlos Frederico Brilhante Wolle, DDS, MSc, PhD[1], João Batista Blessmann Weber, DDS, MSc, PhD[1].

1. Postgraduate Program in Dentistry, Pontifical Catholic University of Rio Grande do Sul – PUCRS
2. Laboratory of Immunology and Microbiology, Faculty of Biosciences, Pontifical Catholic University of Rio Grande do Sul – PUCRS
3. Postgraduate Dental Education Unit, University of Warwick, Warwick University Medical School, Coventry, United Kingdom

Corresponding Authors:

Alessandra Cesar Trindade / João Batista Blessmann Weber
Av. Ipiranga, 6681 - Prédio 6
CEP: 90619-900
Porto Alegre - RS - Brazil
(51) 3320-3562/3573
(51) 3320-3626/3609

endoale@gmail.com / jweber@pucrs.br

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Abstract

Objective: The aim of this study was to evaluate, *in vivo*, by histological and radiographic analysis, the response of apical tissues of rats' teeth with experimentally induced apical periodontitis, after one and two-session endodontic treatment with and without PDT. A microbiological analysis was performed in order to verify bacterial reduction after each treatment.

Methods: Thirty-two rats' root canals with experimentally induced apical periodontitis were assigned to 4 groups: conventional endodontic therapy in one session; conventional endodontic therapy in two sessions with calcium hydroxide dressing for 14 days; conventional endodontic therapy in one session with PDT prior to root canal filling; conventional endodontic therapy in two sessions with PDT prior to calcium hydroxide dressing for 14 days. For microbiological evaluation, samples were collected, before and after the proposed treatments, and processed for CFU/mL counting. For radiographic and histological analysis the animals were killed after 28 days and the mandibles surgically removed.

Results: PDT associated to conventional endodontic therapy is able to promote bacterial reduction in root canals with induced apical periodontitis. This reduction had no significant difference compared to conventional endodontic therapy alone. Although radiographic evaluation showed no significant differences, histological analysis showed lower scores for neuthophils/eosinophils in PDT treated groups and macrophages/giant cells in calcium hydroxide groups.

Conclusion: The use of low level laser as light source may optimize tissue repair by modulating inflammatory process. PDT may be indicated as an adjunct to conventional endodontic therapy for teeth with apical periodontitis and the association of PDT with an inter-appointment dressing with calcium hydroxide produces better conditions to stimulate repair.

Keywords (MeSH and DeCS terms): Photochemotherapy, Photosensitizing Agents, Lasers, Dental Pulp Cavity, Biofilms, Endodontics

Keywords: photodynamic therapy, laser, photosensitizers, root canal disinfection, biofilm, endodontics

Introduction

The permanence of bacteria and their products within the root canal system may compromise the prognosis of endodontically treated teeth. Due to microflora characteristics and anatomical variations in root canal system, especially at apical region, given its anatomical complexity, conventional endodontic procedures may not suffice to ensure complete disinfection (1-3).

Studies carried out in recent years highlighted the antibacterial potential of photodynamic therapy (PDT) when associated to conventional endodontic therapy (4-6). The action mechanism of PDT relies on the administration of an exogenous photosensitizer, associated to a low dose irradiation with visible light of a suitable wavelength. Light absorption triggers excitation of the photosensitizer which, in presence of oxygen found in cells, leads to a cascade of photochemical effects, resulting in the production of high reactive oxygen species (ROS), toxic to tumor cells, bacteria and fungi (7).

Different photosensitizers, irradiation doses and light sources, with various wavelengths have been used in researches. Hence it is almost impossible to reach a consensus on a protocol to be clinically recommended. Phenothiazines are the most tested photosensitizers and low level lasers emitting red light (600-660 nm) the most used light source (8).

Although the antimicrobial effect of PDT is well established, there is a lack of studies evaluating tissue response to PDT in teeth with pulp necrosis and periapical lesion. The aim of this study was to evaluate *in vivo*, through histological and radiographic analysis, the response of apical tissues of rats' teeth with experimentally induced apical periodontitis, after one and two-session endodontic treatment with and without PDT. A complementary microbiological analysis was performed in order to verify bacterial reduction after each treatment.

Materials and Methods

This study was approved by Pontifical Catholic University of Rio Grande do Sul Institutional Animal Care and Use Committees (Protocol 09/00140).

Animal Models of Periapical Lesions

Thirty-two male Wistar rats (200-240g weight) were used. The animals were anesthetized by an intraperitoneal injection of combined xylazine (10mg/kg) and ketamine (100mg/kg). Periapical lesions were induced as described previously with minor adaptations (9). Pulp exposure was performed at the mesial fossa of the left mandible first molar, followed by instrumentation of the mesial canal with #10 K-file (Dentsply-Maillefer®, Ballaigues, Switerzland), under irrigation with sterilized saline solution. Root canals were left open to the oral environment for 21 days, to allow the periapical lesions formation (9-11). Due to possible systemic effect of laser irradiation (12) we have opted for using only one tooth per animal.

Root Canal Treatment

After the periapical lesion induction period, the animals were anesthetized and, under 16x and 25x magnification, using a surgical microscope (Alliance Microscopia®, São Paulo, Brazil), the mesial root canal of left mandibular molar were prepared up to the #30 K-file (13-14), with 2,5% sodium hypochlorite as irrigating solution, followed by EDTA 17% for 3 minutes, and a final flush with sterilized saline solution. The canals were then dried with #25 sterilized paper points (Endopoints®, Endo Points Industry and Commerce, Paraíba do Sul, Brazil) and teeth distributed into 4 groups of 8 teeth, according to the proposed treatment: group 1 (one session): chemomechanical preparation (CMP) with immediate root canal filling (RCF); group 2 (two sessions): CMP and calcium hydroxide ($Ca(OH)^2$) dressing (Calen®, SS White, Rio de Janeiro, Brazil), placed into root canal with a 27G needle and a proper syringe, for 14 days; group 3 (one session/PDT): CMP + photodynamic therapy (PDT), with immediate RCF; group 4 (two sessions/PDT): CMP + PDT and calcium hydroxide dressing for 14 days. After 14 days, for subsequent RCF, the calcium hydroxide dressing was removed by abundant

irrigation with sterilized saline solution. Root canal was dried and filled with a 25.06 gutta-percha point and AH Plus® sealer (Dentsply-DeTrey®, Konstanz, Germany). The coronal access was sealed with amalgam (Permite®, SDI Brasil Industria e Comércio LTDA., São Paulo, Brazil).

Photodynamic Therapy

A GaAlPIn diode laser (Flash Lase III® – DMC Equipamentos, São Carlos, São Paulo), with 100mW and red (660-690nm wavelength) continuous emission, with an intra canal fiber attached, was used. The photosensitizer was 0.01% methylene blue (MB). Prior to irradiation, the canals belonging to one session/PDT and two sessions/PDT groups were filled with MB for 5 minutes, as pre-irradiation time (PIT), and then irradiated for 90 seconds, with the intra canal fiber placed as close as possible to the working length, corresponding to a total dose of 9J, as recommended by manufacturer.

Microbiological Analysis

For microbiological evaluation, root canals were filled with sterilized saline solution and the contents collected with #25 sterilized absorbent paper points. Sample 1 (S1) was collected right before the proposed treatments and sample 2 (S2) after CMP (one session and two sessions groups) or CMP + PDT (one session/PDT and two sessions/PDT groups). The absorbent paper points were placed as close as possible to the working length and transferred to 1.5mL microcentrifuge tubes, containing 1 mL of sterile saline solution. The tubes were then vortexed to suspend attached bacteria into the solution. To estimate the number of colony-forming units per milliliter (CFU/mL), serial decimal dilutions (up to 10^{-3}) were prepared. Aliquots of 100µL of each dilution and the original suspension were spread onto the surface of blood agar in duplicate and incubated, under aerobical and microaerophilic conditions, at 37ºC for 24 hours.

Radiographic Analysis

For radiographic and histological analysis the animals were killed, 28 days after the final filling, by deep anesthesia with isoflurane. The mandibles were then surgically removed, dissected, and placed in 10% neutral-buffered formalin solution. Radiographs were taken right after mandibles dissection. The x-ray cylinder was adjusted at a focal distance of 30 cm, and in a perpendicular angle with the buccal surface of the first molar. The x-ray unit (Gnatus®, Ribeirão Preto, SP, Brazil) operated at 7mA at 70kVp and an exposure time of 0.2 seconds. A digital sensor system (CygnusRay MPS®, Cygnus Technologies L.L.C., Scottsdale, AZ, USA) was used and the captured images saved as a JPEG format. Images were analyzed by a calibrated examiner (ICC = 0.9414) blinded to the experimental groups using Adobe Photoshop CS6® (Adobe Systems Incorporated, San Jose, CA, USA), as previously described (15). The average of 3 measurements was submitted to statistical analysis.

Histological Analysis

After radiographs were obtained, samples were fixed with buffered 10% paraformaldehyde for 24 hours. The specimens then were decalcified with 17% EDTA for 8 weeks, dehydrated in ascending concentrations of ethanol, and embedded in paraffin. Semi-serial sections of 6µm were stained with hematoxylin-eosin. Images of the central portion of the roots, including apex and periapical tissues were taken, at 40, 100, 200 and 400x magnification, with a Moticam 5 camera (Motic, Hong Kong, China) attached to an Olympus® optical microscope (Olympus Corporation, Tokyo, Japan), imported to Motic Plus 2.0® software (Motic, Hong Kong, China) and saved as tif format.

A blinded calibrated (kappa = 0.746) senior examiner evaluated the periapical tissue events as previously described (16). Briefly, scores were determined as follows:

1. absent (inflammatory cells absent or within vessels; periodontal fibers attached to cementum);
2. mild (inflammatory cells or restricted to the apex; thickened periodontal ligament and few fibers arranged irregularly);

3. moderate (sparsely distributed inflammatory cells not restricted to the vicinity of the apex but still presenting a more contained distribution; periodontal fibers arranged irregularly)
4. intense (a heavy presence of inflammatory cells, widely distributed throughout the area adjacent to the root apex; severe disorganization of the periodontal support structures).

Additional cell events were assigned grouping neutrophils/eosinophils, lymphocytes/plasmacytes, macrophages/giant cells. For those, scores were adapted from Figueiredo *et al.* (17) using the following criteria:

1. absence of these cells;
2. cells are present but few and sparsely distributed;
3. cells are viewed in restricted areas;
4. cells are forming infiltrates and are the dominating ones within the lesion.

Statistical Analysis

The sample size was established on the basis of previous literature studies data (9-11; 13-14) and confirmed through statistical program GraphPad InStat (GraphPad Software Inc, San Diego, CA, USA) for $p<0.05$. Data of microbiological analysis were subjected to linear mixed models for correlated data. Radiographic data were subjected to analysis of variance (one-way analysis of variance). For histological features, Kruskall-Wallis test was used followed by the Mann-Whitney U test for those cases in which statistically significant differences were detected. The significance level was set at 0.05.

Results

A total of five animals were excluded from radiographic and histological analysis due to tooth fracture, being 2 on two sessions group and 3 on two sessions/PDT group. Two more pieces on one session group were lost during histologic preparation and were excluded from histological analysis. For microbiological analysis all samples were considered.

Microbiological Analysis

Highly significant bacterial reduction (S1 and S2) occurred in all groups ($P < 0.001$) in both aerobical and microaerophilic conditions. No differences were found between groups, with P values of 0.28 for aerobical conditions and 0.36 for microaerophilic conditions. Table 1 depicts the mean and percent reduction of CFU/mL between S1 and S2 for all groups. The ability for each procedure on reducing CFUs was compared by an intragroup analysis.

Radiographic Analysis

Data depicted in Figure 1A show mean lesion area for each group, regarding the number of sessions and PDT application. No differences were observed between groups but an interaction of 2 sessions and PDT could be observed for two sessions/PDT group ($P = 0.026$). Representative radiographic findings are provided in Figure 1B-E.

Histological Analysis

The overall inflammatory mean scores are shown in Figure 2A, where no significant differences were found. Figure 2B shows mean scores for inflammatory events. The groups differed only when the items compared were neutrophils/eosinophils and macrophages/giant cells. A significant lower presence ($P = 0.047$) of neutrophils/eosinophils were observed in PDT groups whereas macrophages/giant cells population decreased on two session groups, where $Ca(OH)_2$ was used as intracanal medication ($P = 0.009$). Figure 3A-D shows some parameters used in assessing the results.

Discussion

Microorganisms and their byproducts play a key role in pulpal and periapical disease development, directly linking the success of endodontic therapy to their complete elimination from root canal system (1-3). Due to microflora characteristics and anatomical variations in root canal system, especially at apical region, cleaning and

shaping, application of an inter-appointment antibacterial dressing and sealing of the root canal may not ensure complete disinfection (18-19). Persistent apical periodontitis occurs when root canal treatment procedures have not reached a satisfactory standard for the control and elimination of intracanal infection (2).

Many *in vitro* studies in recent years showed the antibacterial potential of PDT in root canal system disinfection, especially against *Enterococcus faecalis* (5-6; 20-24). It has also been reported that bacteria in biofilms are less susceptible to PDT than in planktonic suspension (25). There are few studies about the PDT effects on polimicrobial infections, besides clinical trials (26-29). In the present study, the efficacy of PDT on reducing CFU/mL of polimicrobial infection was confirmed. The reduction was similar to those found in other studies associating PDT and CMP with NaOCl (4-6; 8; 30).

A histopathological study, where apical periodontitis was induced dogs (8) showed that PDT-treated groups had moderately/severely enlarged periapical region with no inflammatory cells, moderate neoangiogenesis and fibrogenesis, and the smallest periapical lesions. Although in the present study no differences were found regarding the overall inflammatory process, neutrophils/eosinophils lower population in PDT treated groups may indicate a biostimulatory effect promoted by the laser irradiation. Low level laser therapy (LLLT) optimizes tissue repair (32) by modulating inflammatory process (33). Macrophages/giant cells decrease in two session groups is possibly linked to the osteoclast formation inhibition provided by inter-appointment $Ca(OH)_2$ dressing, since it has been reported that destruction of the LPS lipid A side chain by $Ca(OH)_2$ might account for the inability of LPS to stimulate osteoclast formation (34). The association of PDT and inter-appointment $Ca(OH)_2$ dressing is a reasonable explanation to the significant difference on the interaction result found for group 4 on radiographic analysis.

The small size of the teeth and difficulties related to access pulp chamber increased fragility of the dental structure (35). Thus, re-intervention and longer period favored tooth fracture, affecting coronal sealing, as observed in two sessions and two sessions/PDT groups. Apart from oral bacterial flora and apical response to pulp

exposure in rats being similar to that observed in humans (9-11), in addition to faster biological response (36), technical problems like those described above plus the impossibility to isolate operative field, difficulties to take trans-operatory radiographs and establish a proper working length are critical aspects that must be observed.

Conclusions

Although PDT associated to conventional endodontic therapy is able to promote bacterial reduction in root canals with induced apical periodontitis, this reduction had no significant difference compared to conventional endodontic therapy with NaOCl alone. The use of low level laser as light source may promote a biostimulatory effect on apical tissues, optimizing tissue repair by modulating inflammatory process. PDT may be indicated as an adjunct conventional endodontic therapy for teeth with apical periodontitis and the association of PDT with an inter-appointment dressing with $Ca(OH)_2$ produces better conditions to stimulate repair.

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Table 1. Bacterial reduction in aerobical and microaerophilic conditions

		Aerobical conditions			Microaerophilic conditions		
Group	**n**	**S1**	**S2**	**Δ%**	**S1**	**S2**	**Δ%**
One Session / PDT-	8	14.739 (3.406; 63.770)	28 (6; 124)	-99.8	8.183 (1.558; 42.982)	8 (1; 43)	-99.9
One Session / PDT+	8	14.388 (3.325; 62.251)	14 (3; 62)	-99.9	10.593 (2.016; 55.642)	6 (1; 31)	-99.9
Two Sessions / PDT-	8	25.242 (5.273; 120.831)	28 (5; 134)	-99.9	33.996 (5.772; 200.226)	30 (5; 181)	-99.9
Two Sessions / PDT+	8	23.064 (4.818; 110.407)	330 (68; 1.580)	-98.6	11.087 (1.882; 65.299)	96 (16; 568)	-99.1

Data are depicted as geometric means (lower and upper bounds). Highly significant bacterial reduction (S1 and S2) occurred in all groups ($P < .001$) in both aerobical and microaerophilic conditions. No differences were found between groups, with P values of .28 for aerobical conditions and .36 for microaerophilic conditions. Δ%: Bacterial reduction after each proposed treatment.

A
Lesion Size (Pixels/cm)
2500
2000
1500
1000
500
0
PDT-
PDT+
One Session
Two Sessions
B
C
D
E

Figure 1.

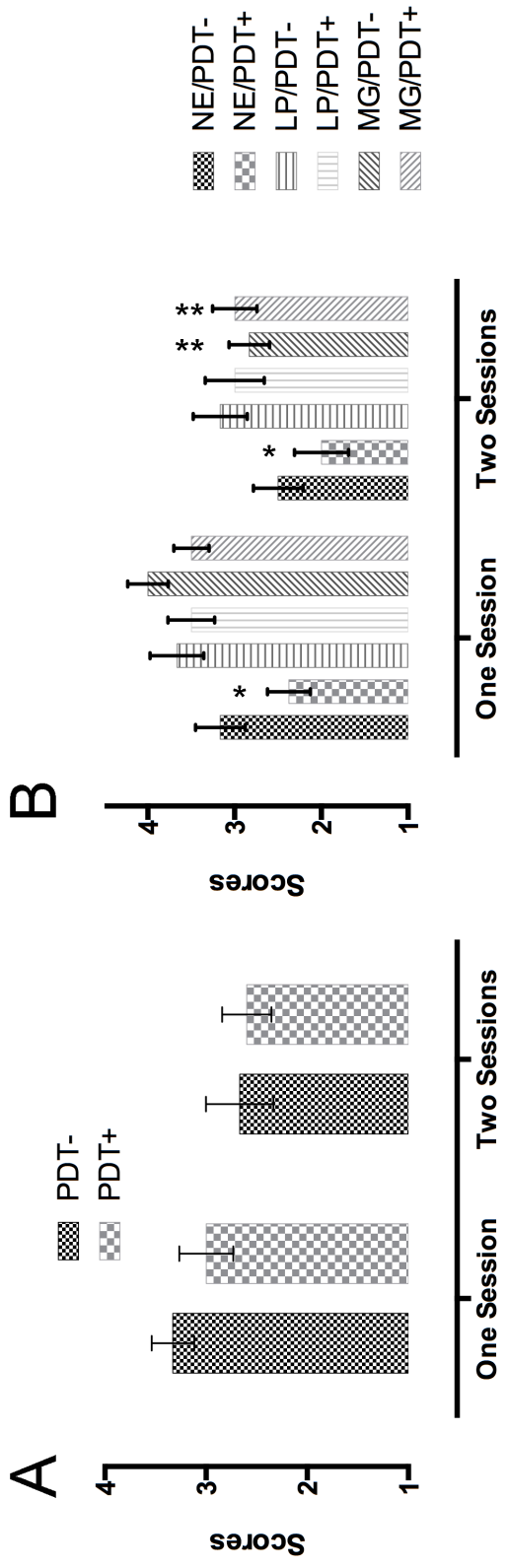
A
Scores
4
3
2
1
PDT-
PDT+
One Session
Two Sessions
B
Scores
4
3
2
1
NE/PDT-
NE/PDT+
LP/PDT-
LP/PDT+
MG/PDT-
MG/PDT+
*
*
**
**
One Session
Two Sessions

Figure 2.

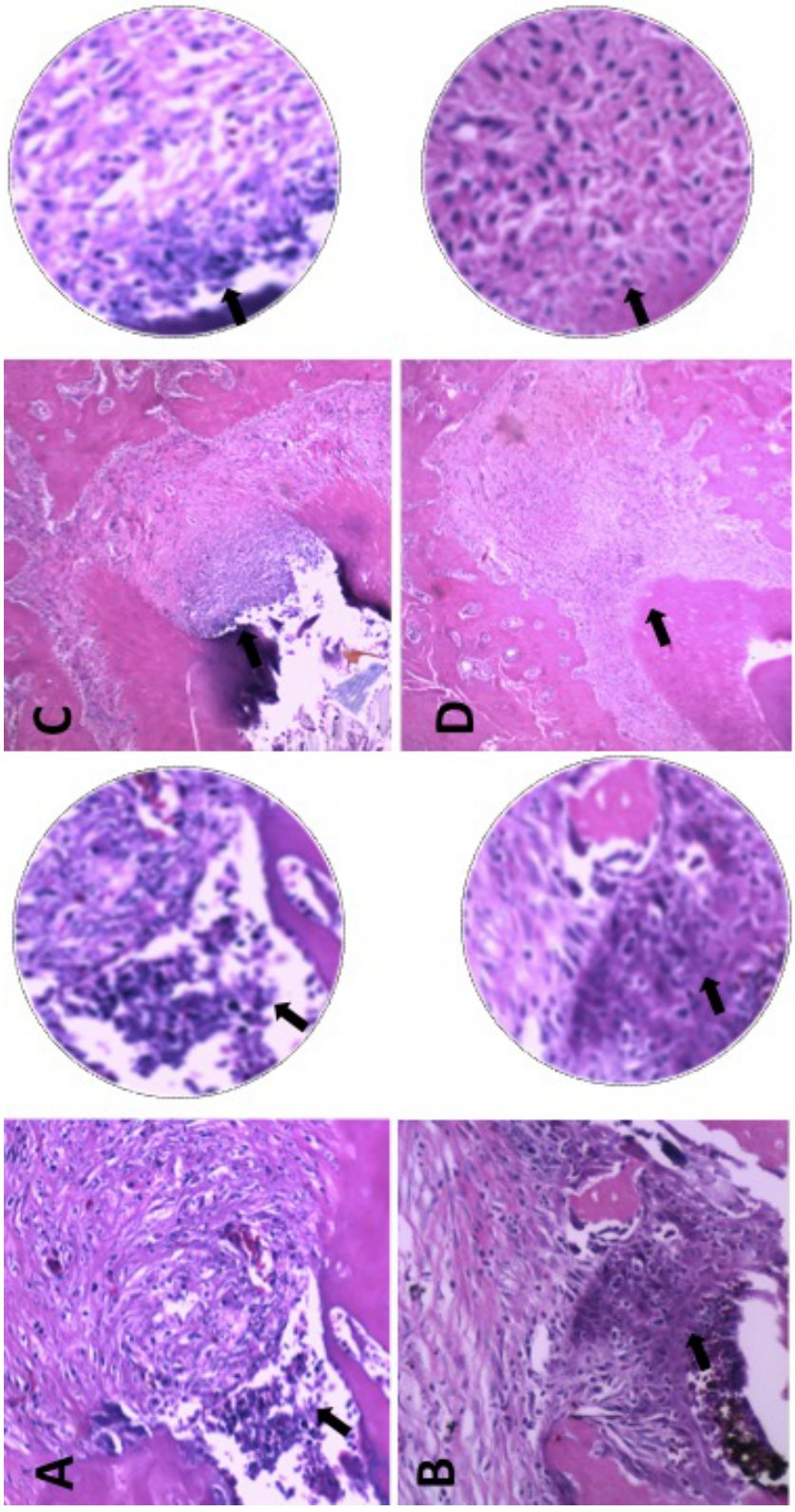

Figure 3.

Figure Legends

Figure 1. A – Graph displaying lesion size according to use of PDT and number of sessions. **B –** one session / PDT- ; **C –** two sessions/ PDT- ; **D –** one session / PDT+ ; **E** – two sessions / PDT+

Figure 2. Graphs with overall scores for inflammatory response to treatments **(A)** and with specific cell events **(B).** A significant lower presence (*P = 0.047) of neutrophils/eosinophils were observed in PDT groups whereas macrophages/giant cells population decreased on two session groups, where $Ca(OH)_2$ was used as intracanal medication (**P = 0.009). NE/PDT- = neutrophils and eosinophils in groups where PDT were not performed; NE/PDT+ = neutrophils and eosinophils in groups where PDT were performed; LP/PDT- = lymphocytes and plasmacytes in groups where PDT were not performed; LP/PDT+ = lymphocytes and plasmacytes in groups where PDT were performed; MG/PDT- = macrophages and giant cells in groups where PDT were not performed; MG/PDT+ = macrophages and giant cells in groups where PDT were performed.

Figure 3. - Histologic events observed amongst groups: **A –** one session / PDT- ; note the infiltrate of neutrophils; **B –** two sessions / PDT- ; lymphocytes and plasmacytes together with macrophages and giant cells are infiltrating towards the apex; **C –** one session / PDT+ ; an infiltrate of lymphocytes and plasmacytes is observed in an area limited to the apex; **D –** two sessions / PDT+; fibroblasts are surrounding the apical area with sparse lymphocytes, plasmacytes and macrophages.

4. Discussão Geral

4. DISCUSSÃO GERAL

A permanência de bactérias e seus produtos no interior do sistema de canais radiculares pode comprometer o prognóstico dos dentes tratados endodonticamente (NAIR, SJÖGREN, FIGDOR, 1999; NAIR, 2004; NAIR, 2006; SIQUEIRA, 2001; SIQUEIRA & RÔÇAS, 2008).

O *E. faecalis*, uma bactéria anaeróbia facultativa, é um dos microrganismos mais comumente encontrado em infecções endodônticas persistentes, lesões refratárias e biofilmes periapicais (SIQUEIRA, DE UZEDA, FONSECA, 1996; LOVE, 2001), sendo capaz de sobreviver por longos períodos sem nutrição. É capaz ainda de penetrar nos túbulos dentinários (SIQUEIRA, DE UZEDA, FONSECA, 1996; LOVE, 2002) ficando protegido da ação das soluções irrigantes e medicações intracanal usuais, tornando-se um dos microrganismos de mais difícil eliminação.

Os efeitos da terapia fotodinâmica na desinfecção do sistema de canais radiculares contaminados com o *E. faecalis* tem sido alvo da maior parte dos estudos sobre PDT na endodontia (SOUKOS *et al*.,2006; SILVA GARCEZ *et al.*, 2006; FOSCHI *et al.*, 2007; GEORGE, KISHEN, 2007; BERGMANS *et al.*, 2008; FONSECA *et al.*, 2008; LIM *et al.*, 2009; SOUZA *et al.*, 2010; SCHLAFER *et al.* 2010; UPADYA, KISHEN, 2010; RIOS *et al.* 2011; NUNES *et al.*, 2011; CHENG *et al.*, 2012; SHRESTHA *et al.*, 2012; GARCEZ *et al.*, 2013; BAGO *et al.*, 2013; KOMINE, TSUJIMOTO, 2013).

Poucos são os estudos disponíveis sobre os efeitos da PDT em infecções polimicrobianas, sendo um estudo *ex vivo* (NG *et al.*, 2011) e um *in vivo* em dentes de cães (SILVA *et al.*, 2012), além dos ensaios clínicos já citados (BONSOR *et al.*, 2006a;

BONSOR *et al.*, 2006b; GARCEZ *et al.*, 2008; GARCEZ *et al.*, 2010). De acordo com Upadya e Kishen (2010) bactérias em suspensão planquitônica são mais suscetíveis à PDT que aquelas organizadas em biofilmes. Por ser uma prática muito recentemente introduzida na endodontia, diferentes protocolos para a PDT tem sido propostos. Para o presente estudo, o protocolo sugerido pelo fabricante foi selecionado e a eficácia da PDT em reduzir a quantidade de unidades formadoras de colônias por mL (UFC/mL) em infecções polimicrobianas foi confirmada, não havendo porém diferenças estatísticas entre os grupos. A redução percentual foi similar à encontrada em outros estudos onde a PDT foi associada ao preparo químico-mecânico com hipoclorito de sódio (NaOCl) (NG *et al.*, 2011; GARCEZ *et al.*, 2007; SOUZA *et al.*, 2010; RIOS *et al.*, 2011; LIM *et al.*, 2009).

Com relação aos achados histológicos, embora não tenha havido diferença estatisticamente significativa no grau de inflamação geral, foram encontradas diferenças nas populações de neutrófilos e eosinófilos nos grupos tratados com PDT ($P = 0.047$). Este efeito pode ser explicado pelo efeito biomodulador promovido pela utilização do *laser*. Laserterapia de baixa intensidade (LLLT) sabidamente otimiza o reparo tecidual (PRETEL *et al.*, 2007) por modulação do processo inflamatório (KARU, 1999). Decréscimo significativo na presença de macrófagos e células gigantes também foi observado, desta vez nos grupos onde o tratamento foi realizado em duas sessões com o uso do hidróxido de cálcio ($Ca(OH)_2$) como medicação intracanal, independentemente do uso da PDT. De acordo com a literatura, o $Ca(OH)_2$ é capaz de destruir a cadeia lipídica A do lipopolissacarídeo (LPS) bacteriano, tornando-o incapaz de estimular a formação de osteoclastos (JIANG *et al.*, 2003). Neste sentido, a associação da PDT

com a medicação intracanal com $Ca(OH)_2$ parece ser a causa da interação (P = 0.026) observada no grupo 4, tratado em duas sessões associado à PDT. Para os demais dados da análise radiográfica, não foram encontradas diferenças significativas entre os grupos.

Cinco animais foram excluídos das avaliações radiográfica e histológica por fratura do dente, sendo 2 no grupo 2 e 3 no grupo 4. Outros dois espécimes foram perdidos durante o processamento histológico, sendo excluídos da análise histológica. Para a análise microbiológica, todas as amostras foram consideradas. As perdas de dentes pertencentes aos grupos onde o tratamento endodôntico foi realizado em duas sessões, com a utilização de $Ca(OH)_2$ como medicação intracanal podem estar relacionadas ao enfraquecimento da estrutura dentária provocada pelo $Ca(OH)_2$, que reduz significativamente a resistência à fratura (BATUR *et al.*, 2013; ZAREI *et al.*, 2013).

De acordo com estudos prévios (KAKEHASHI, STANLEY & FITZGERALD, 1965; STASHENKO *et al.*, 1994), a exposição pulpar de molares de ratos ao meio bucal leva a alterações periapicais bastante semelhantes às observadas em humanos. Métodos para o preparo químico-mecânico de dentes de ratos também podem ser encontrados na literatura (EURASQUIN, MURUZABAL, 1967; SATO, ANTONIAZZI, 1993; ANAN, AKAMINE, MAEDA, 1993). Apesar das características da flora bacteriana oral e resposta dos tecidos apicais em ratos serem bastante semelhantes às observadas em humanos, além de uma resposta biológica mais rápida (MURUZÁBAL & EURASQUIN, 1970), algumas dificuldades técnicas devem ser levadas em consideração quando da seleção do modelo animal. O tamanho reduzido dos dentes e o difícil acesso à câmara pulpar podem fragilizar a estrutura dental, levando a fraturas. Re-intervenções e

períodos experimentais mais longos, como nos grupos 2 e 4 do presente estudo, podem favorecer a fratura e/ou a perda do selamento coronário. A impossibilidade de realizar o isolamento absoluto do campo operatório e tratamento de todos os canais radiculares, assim como efetuar tomadas radiográficas no transoperatório e dificuldades em se estabelecer o comprimento de trabalho adequado devem ser observados.

5. Conclusões

5. CONCLUSÕES

A partir dos resultados do presente estudo pode-se concluir:

1. Os dados disponíveis na literatura sugerem a necessidade de ajustes no protocolo da PDT ou formulação dos fotossensibilizadores, a fim de melhorar sua eficácia. No entanto, sua indicação como coadjuvante à terapia endodôntica convencional está bem documentada.

2. A PDT associada ao tratamento endodôntico convencional é capaz de promover redução bacteriana em canais radiculares com periodontite apical induzida, não sendo porém esta redução estatisticamente significativa quando comparada ao tratamento convencional de forma isolada;

3. Embora a análise radiográfica não tenha demonstrado diferenças estatisticamente significativas entre os grupos, os escores histológicos mais baixos para neutrófilos e eosinófilos nos grupos tratados com PDT podem sugerir melhores condições para o reparo apical;

4. O uso do $Ca(OH)_2$ parece reduzir o infiltrado de do macrófagos e células gigantes, também favorecendo o reparo;

5. A utilização de *lasers* de baixa potência como fonte de luz para a aplicação da PDT pode otimizar o reparo tecidual pela modulação do processo inflamatório;

6. A PDT pode ser indicada como coadjuvante ao tratamento endodôntico convencional, no tratamento de dentes portadores de periodontite apical e sua associação ao preparo químico-mecânico e medicação intracanal com $Ca(OH)_2$ parece produzir as melhores condições para o reparo tecidual.

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7. Anexos

Anexo A: Ofício de aprovação da Comissão Científica e de Ética da Faculdade de Odontologia da PUCRS.

Comissão Científica e de Ética
Faculdade da Odontologia da PUCRS

Porto Alegre 26 de novembro de 2009

O Projeto de: Tese

Protocolado sob nº:	0046/09
Intitulado:	Análise histológica, bacteriológica e radiográfica da resposta dos tecidos periapicais de dentes de ratos, portadores de periodontite apical, à terapia fotodinâmica como coadjuvante ao tratamento endodôntico
Pesquisador Responsável:	Prof. Dr. João Batista Blessmann Weber
Pesquisadores Associados	Alessandra Cesar Trindade; José Antônio Poli de Figueiredo
Nível:	Doutorado

Foi ***aprovado*** pela Comissão Científica e de Ética da Faculdade de Odontologia da PUCRS em *26 de novembro de 2009.*

Este projeto deverá ser imediatamente encaminhado ao CEUA/PUCRS

Prof. Dr. Eraldo Luiz Batista Júnior
Presidente da Comissão Científica e de Ética da
Faculdade de Odontologia da PUCRS

Av. Ipiranga, 6681, Prédio 06 sala 209
Porto Alegre /RS – Brasil – Cx. Postal:1429
90619-900

Fone/Fax: (51) 3320-3538
e-mail: odontologia-pg@pucrs.br

Anexo B: Ofício de aprovação do Comitê de Ética para o Uso de Animais da PUCRS.

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA PARA O USO DE ANIMAIS

Ofício 059/10 – CEUA

Porto Alegre, 22 de abril de 2010.

Senhor Pesquisador:

O Comitê de Ética para o Uso de Animais apreciou e aprovou seu protocolo de pesquisa, registro CEUA 09/00140, intitulado: **"Análise histológica, microbiológica e radiográfica da resposta dos tecidos periapicais de dentes de ratos, portadores de periodontite apical, à terapia fotodinâmica como coadjuvante ao tratamento endodôntico".**

Sua investigação está autorizada a partir da presente data.

Atenciosamente,

Profa. Dra. Anamaria Gonçalves Feijó
Coordenadora do CEUA – PUCRS

Ilmo. Sr.
Prof. Dr. João Batista B. Weber
Faculdade de Odontologia
N/Universidade

PUCRS
Campus Central
Av. Ipiranga, 6690 – Prédio 60, sala 314
CEP: 90610-000
Fone/Fax: (51) 3320-3345
E-mail: ceua@pucrs.br

Anexo C: Submissão do artigo ***"Photodynamic Therapy in Endodontics: State of the Art"*** periódico *International Endodontic Journal*

Dear Dr. Trindade

Your manuscript entitled "Photodynamic Therapy in Endodontics: State of the Art" has been successfully submitted online to the International Endodontic Journal.

Your manuscript ID is IEJ-13-00710.

Please mention the above manuscript ID in all future correspondence or when calling the Editorial Office for questions. If there are any changes in your postal or e-mail address, please log in to ScholarOne Manuscripts athttp://mc.manuscriptcentral.com/iej and edit your user information as appropriate.

You can also view the status of your manuscript at any time by checking your Author Centre after logging in tohttp://mc.manuscriptcentral.com/iej.

Thank you for submitting your manuscript to the International Endodontic Journal.

Kind regards

Paul Dummer
Editor, International Endodontic Journal
iejeditor@cardiff.ac.uk

Anexo D: Submissão do artigo ***"Histopathological, Microbiological and Radiographic Analysis of Antimicrobial Photodynamic Therapy for the Treatment of Teeth with Apical Periodontitis: a Study in Rats' Molars"*** periódico *Journal of Endodontics*

Dear Dr. Trindade,

Your submission entitled "Histopathological, Microbiological and Radiographic Analysis of Antimicrobial Photodynamic Therapy for the Treatment of Teeth with Apical Periodontitis: a Study in Rats' Molars" has been received by the Journal of Endodontics.

You will be able to check on the progress of your paper by logging on to the Journal of Endodontics web site as an author.

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