Patrícia Valente Araújo Jacques Gonçalves

APLICAÇÃO DA TERAPIA FOTODINÂMICA NA REDUÇÃO DE PATÓGENOS CARIOGÊNICOS

Belo Horizonte
Faculdade de Odontologia
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Tese apresentada ao Programa de Pós-Graduação da Faculdade de Odontologia da Universidade Federal de Minas Gerais como parte dos requisitos para a obtenção do título de Doutor em Odontologia

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“Nunca ande pelo caminho traçado, pois ele conduz somente até aonde os outros já foram”

Alexander Graham Bell

Aos meus pais, **MARIA EUGÊNIA** e **JEFERSON**, e meus irmãos **TITI** e **FELIPE**, pelo apoio e amor incondicional. Difícil traduzir em palavras o que vocês significam para mim!!

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grande amor e companheiro. Sua
compreensão, carinho e cumplicidade tornaram meu caminho mais fácil!!

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Dedico este trabalho

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RESUMO

O objetivo deste estudo foi avaliar o efeito da terapia fotodinâmica (PDT) sobre patógenos cariogênicos *in vitro* e *in vivo*, testando-se este procedimento como um coadjuvante ao tratamento usual das lesões de cárie. Como procedimentos *in vitro*, várias associações entre fontes de luz e agentes fotossensibilizantes foram testadas. O tratamento que apresentou melhor desempenho na fotossensibilização letal de suspensões de *Streptococcus mutans* (ATCC 25175) foi definido como protocolo de aplicação da PDT *in vivo*. Previamente à realização desta etapa, o estudo foi aprovado pelo Comitê de Ética em Pesquisa da UFMG. Foram selecionadas crianças portadoras de molares com lesões cariosas cavitadas de dentina. Amostras de dentina cariada foram removidas antes e após a aplicação da PDT e submetidas à avaliação microbiológica, através dos métodos e cultura e PCR em tempo real. Como protocolo de aplicação da PDT, após cinco minutos de contato entre o agente fotossensibilizante azul de metileno (25mg/L) e a cavidade, esta foi irradiada por um minuto por um laser vermelho (Grupo I, n=10) ou por um aparelho fotoativador de luz halógena (Grupo II, n=10). Na análise dos resultados da cultura de microorganismos, a PDT,associando o azul de metileno a um laser vermelho, não apresentou potencial antimicrobiano quando utilizada sobre lesões de cárie. A associação do azul de metileno + fonte de luz halógena mostrou diferenças estatisticamente significantes entre os grupos controle e os grupos teste e uma redução acima de 95% foi observada, confirmando a eficácia desta terapia. Através do PCR em tempo real, ao se comparar os resultados dos grupos controle com os resultados dos grupos teste, observou-se que a PDT não foi capaz de reduzir o número de cópias de DNA de *S. mutans,* quando a luz halógena foi utilizada. Outros estudos são necessários antes de se propor a aplicação clínica da PDT no tratamento das lesões de cárie.

ABSTRACT

The aim of this study was to evaluate the effect of photodynamic therapy (PDT) on caries lesions *in vitro* and *in vivo*, testing this procedure as an alternative to the usual treatment of caries. For *in vitro* procedures, associations between different light sources and photosensitizers agents were tested. The treatment which showed better performance in the lethal photosensitization of suspensions of *Streptococcus mutans* (ATCC 25175) was defined as a clinical protocol for the application of PDT *in vivo*. The study was approved by the Ethics Committee of the Federal University of Minas Gerais. Molars with deep active carious were selected from children of both genders aged between three years and nine years. Samples of carious dentin were removed before and after application of PDT and subjected to microbiological analyses by culture and real time PCR methods. As a clinical protocol of PDT, after five minutes of contact between the photosensitizer methylene blue (25mg/L) and the cavity, this cavity was irradiated for one minute by a red laser (Group I, n = 10) or an halogen light source (Group 2, n = 10). The results of the culture method showed that in the Group I, which associated the methylene blue to a red laser, the PDT showed no antimicrobial potential when used on caries lesions. For Group II (methylene blue + halogen light source), the statistical analysis showed differences between control and test groups and a reduction above 95% was observed, confirming the effectiveness of this therapy. When comparing the results of control groups with the results of the test groups by real time PCR, it was observed that PDT was not able to reduce the number of copies of DNA from *S. mutans*, when the halogen light was used. Further studies are needed before proposing a clinical application of PDT in the treatment of carious lesions.

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ARTIGO 1

LISTA DE GRÁFICOS

LISTA DE ABREVIATURAS E SIGLAS

°C - Graus Celsius

% - por cento

$^1O_2$ - oxigênio singleto

$AlPcS_2$- ftalocianina dissulfonada de alumínio

ART - atraumatic restorative treatment / tratamento restaurador atraumático

ARTm - tratamento restaurador atraumático modificado

ATCC - *American Type Culture Collection*

BHI - *brain heart infusion* / infusão cérbro coração

Cells/ml - células por mililitro

CFU-UFC - *counting forming units* / unidades formadoras de colônias

CLSM - *confocal laser scanning microscopy* / microscopia confocal a laser

COEP – Comitê de Ética em Pesquisa

dATP – desoxi-adenosina trifosfato

dCTp – desoxi-citidina trifosfato

dGTp – desoxi-guanosina trifosfato

DNA - ácido desoxirribonucleico

dUTp - desoxi-uridina trifosfato

FDA - *Food and Drug Administration*

FO-UFMG – Faculdade de Odontologia da Universidade Federal de Minas Gerais

GaAlAs – arseneto de gálio-alumínio

h - hora

HeNe - hélio neônio

HHP - *hand held photopolimerizer* / aparelho fotopolimerizador

J - Joule

J/cm^2 - Joule por centímetro quadrado

kHz - Kilohertz

L - Litro

Laser - *light amplification by stimulated emission of radiaton* / amplificação da luz por emissão estimulada de radiação

LED - *light emmiting diode* /diodo emissor de luz

Log – logaritmo

µg/ml – micrograma por mililitro

µl - microlitro

µm – micrometro

µM - micromolar

MB - *methylene blue* / azul de metileno

MeSh – Medical Subject Headings

mg - miligrama

mg/L - miligrama por litro

$MgCl_2$ - cloreto de magnésio

min - minuto

ml - mililitro

mm - milímetro

mm^2 - milímetro quadrado

mW - miliWatt

mW/cm^2 - miliWatt por centímetro quadrado

NCTC - *National Collection of Type Cultures*

ng - nanograma

nm - nanometro

ns - nanosegundo

PCR - *polymerase chain reaction* / reação em cadeia da polimerase

PDT - *photodynamic therapy* / terapia fotodinâmica

PS - *photosensitiser* / agente fotossensibilizante

ROS - *reactive oxigen species* / espécies reativas do oxigênio

s - segundo

S_1 - estado singleto

SEM - *scanning electron microscopy*/ microscopia eletrônica de varredura

S. mutans - *Streptococcus mutans*

T1 - estado tripleto

TBO - *toluidine blue O* / azul de toluidina

TEM - *transmission electron microscopy* / microscopia eletrônica de transmissão

TPI/ PIT – tempo pré-irradiação / *pre irradiation time*

TSB - *tryptic soy broth* / caldo de tripsina de soja

W - watt

SUMÁRIO

1 INTRODUÇÃO

1 INTRODUÇÃO

A terapia fotodinâmica, do inglês *photodynamic therapy* (PDT), é um tratamento que engloba a ação simultânea de uma fonte de luz e de um agente fotossensibilizante (PS), na presença do oxigênio dos tecidos. Individualmente cada uma destas substâncias é inócua e quando interagem são capazes de originar espécies citotóxicas que levam à morte celular (KONOPKA e GOSLINSKI, 2007).

A ação fotodinâmica se dá quando o agente fotossensibilizante (PS) absorve fótons da fonte luz e seus elétrons passam a um estado excitado singleto S_1 (GARCEZ *et al.*, 2003). Subseqüentemente, o PS pode tanto voltar para seu estado inicial, com emissão de fluorescência, ou sofrer uma transição para um estado tripleto altamente energético (KONOPKA e GOSLINSKI, 2007). Através de um processo de cruzamento inter-sistemas, caracterizado pela inversão do *spin* eletrônico, a molécula do PS pode passar do estado S_1 para o estado excitado tripleto (T_1), caracterizado pelo tempo de vida muito mais longo que o estado S_1.

A interação do PS em seu estado tripleto excitado com o oxigênio endógeno das células alvo resulta nos efeitos citotóxicos. Tais interações podem ser por dois mecanismos principais de reação: tipo I ou tipo II (O'RIORDAN, AKILOV E HASAN, 2005).

Na reação Tipo I, o PS reage diretamente com o substrato, que pode ser a membrana celular ou uma molécula, para formar radicais livres. Estes radicais irão posteriormente reagir com o oxigênio, produzindo as espécies reativas do oxigênio (ROS- *reactive oxigen species*) como os radicais superóxido, hidroxila e peróxido. Na reação tipo II, o PS transfere sua energia diretamente para o oxigênio molecular, para formar o oxigênio singleto. A vida útil do oxigênio singleto em sistemas biológicos é menor que 40ns e seu raio de ação é da ordem de 20 nm. Devido à alta

reatividade e vida útil curta do oxigênio singleto e ROS, apenas moléculas e estruturas que estiverem próximas da sua área de produção são diretamente afetadas pela PDT (CASTANO, DEMIDOVA E HAMBLIN, 2004).

Ambas as reações podem ocorrer simultaneamente e a razão entre elas é influenciada pelas características do PS, dos substratos intracelulares e da concentração de oxigênio no meio. Porém, a presença do oxigênio singleto parece ser o principal fator para ocorrência de citotoxicidade (MACHADO, 2000).

Várias classes de compostos químicos, incluindo as fenotiazinas, ftalocianinas e porfirinas, as quais apresentam propriedades fotoativas têm sido efetivas na terapia fotodinâmica (MAISH *et al.*, 2007). Um agente fototerapêutico clinicamente adequado deve possuir no seu estado tripleto excitado um tempo de vida de longa duração, podendo reagir eficientemente tanto com moléculas vizinhas, como com o oxigênio. Deve também apresentar elevada absortividade molar na região espectral compreendida entre 600 e 1000 nm, conhecida como “janela fototerapêutica”, onde a membrana celular apresenta considerável transparência à radiação eletromagnética (MACHADO, 2000). Quanto maior o comprimento de onda incidente, maior é o seu grau de penetração no tecido. Radiações de comprimento de onda menores sofrem maior espalhamento e são absorvidas pelos cromóforos endógenos (substâncias que absorvem luz) nos tecidos, fazendo com que a penetração de luz seja menor. Por outro lado, acima de 800 nm ocorre absorção da radiação pela água o que restringe o comprimento de onda nesse limite superior (SIMPLICIO, 2002).

Qualquer fonte de luz que tenha características espectrais apropriadas pode ser utilizada em PDT, tais como lâmpadas de tungstênio ou halogênio, laser ou LED (PAULINO *et al.,* 2005). A fonte de luz deve ser centrada na banda de absorção do PS utilizado, isto é, na região do

espectro na qual a luz pode excitar o PS transferindo energia a fim de que a reação fotodinâmica seja desencadeada (FERREIRA, 2003).

Atualmente a PDT é utilizada no tratamento de vários tipos de câncer, porém, vários estudos têm demonstrado que a terapia fotodinâmica também apresenta propriedades antimicrobianas (KONOPLA e GOSLINSKI, 2007). De acordo com Hope e Wilson (2006) uma das vantagens da PDT em relação à antibioticoterapia convencional é que o efeito antimicrobiano fica confinado apenas às áreas cobertas pelo corante e irradiadas pela luz. Além disso, de acordo com Wainwright (1998) a resistência bacteriana à PDT é improvável, pois o oxigênio singleto e os radicais livres formados interagem com várias estruturas celulares bacterianas e diferentes caminhos metabólicos.

De acordo com Zeina *et al.* (2002), a seletividade da PDT pelas bactérias é devido a diferenças no tamanho/volume da célula e na quantidade de organelas intra-celulares a serem afetadas. Soukos *et al.* (1998) afirmam que ROS geradas pela PDT tem menor capacidade de se difundir para uma organela intracelular de uma célula eucarionte do que no caso das bactérias, que apresentam uma organização celular bem mais simples, sem uma membrana nuclear, por exemplo.

O uso da PDT na Odontologia tem recebido considerável investigação. Vários estudos *in vitro* já foram conduzidos utilizando a terapia fotodinâmica com sucesso na eliminação de bactérias orais, como as responsáveis pela cárie e doença periodontal (ZANIN *et al.*, 2006; ZANIN *et al.*, 2005; WOOD *et al.*, 2006; KOMERIC *et al.*, 2003).

Por se tratar de uma infecção localizada, se as bactérias dentro das lesões de cárie puderem ser erradicadas pela PDT *in situ,* ter-se-iam conseqüências benéficas para a saúde dental. O agente fotossensibilizante

poderia ser aplicado com uma seringa, e a luz irradiada via fibra óptica (ZANIN *et al.*, 2006).

Uma vez que vários estudos têm demonstrado o potencial da PDT em promover a morte de patógenos cariogênicos *in vitro*, o objetivo deste trabalho foi avaliar o efeito da terapia fotodinâmica sobre patógenos cariogênicos *in vitro* e *in vivo*, testando-se este procedimento como uma alternativa ao tratamento usual das lesões de cárie.

2 JUSTIFICATIVA E RELEVÂNCIA

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Em virtude da possibilidade de aplicação tópica e de seu efeito localizado, a utilização da terapia fotodinâmica para o tratamento de lesões de cárie tem sido bastante discutida na literatura. A terapia fotodinâmica é uma alternativa promissora para o tratamento de infecções locais, pois trata-se de uma técnica pouco invasiva, de baixo custo e fácil aplicação.

Se as bactérias dentro das lesões de cárie puderem ser erradicadas pela PDT *in vivo*, poderíamos ter conseqüências benéficas para a saúde dentária. A dentina infectada e desorganizada poderia ser mais bem preservada, tornando o tratamento mais fácil tanto para o dentista quanto para o paciente, pois permitiria que as lesões fossem restauradas com remoção mínima de tecido e com um prognóstico de longo prazo mais favorável. A justificativa na realização desta terapêutica na clínica odontológica busca uma simplificação de procedimentos curativos, promovendo supressão da doença de forma biológica e conservadora.

Embora vários estudos já tenham demonstrado o potencial antimicrobiano da PDT *in vitro*, em suspensões bacterianas, em amostras de placa dental ou em biofilmes produzidos laboratorialmente, não há relatos na literatura que confirmem a eficácia desta terapia em lesões de cárie *in vivo*. Este trabalho tem a importância de nos revelar os primeiros resultados da aplicação da PDT *in vivo*. Como as bactérias presentes na cárie dental podem ser menos susceptíveis à PDT devido à limitada penetração do agente fotossensibilizante ou devido à dificuldade da luz se propagar através da estrutura dentinária, torna-se imprescindível que os resultados satisfatórios encontrados nos estudos *in vitro* sejam confirmados a fim de se propor esta terapia como uma alternativa ao tratamento convencional das lesões de cárie.

3 OBJETIVOS

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3.1 Objetivos Gerais

Avaliar o efeito da terapia fotodinâmica sobre patógenos cariogênicos *in vitro* e *in vivo*, testando-se este procedimento como uma alternativa ao tratamento usual das lesões de cárie.

3.2 Objetivos Específicos

Os objetivos específicos deste trabalho foram:

– Avaliar a influência do tipo de agente fotossensibilizante (azul de metileno ou azul de toluidina) no processo de redução do *S. mutans in vitro;*

– Avaliar a influência da concentração (25, 10 e 5 mg/L) dos agentes fotossensibilizantes no processo de redução do *S. mutans in vitro;*

– Avaliar a influência das fontes de luz (laser vermelho com comprimento de onda de 660nm ou luz halógena com espectro de emissão entre 450 e 750 nm) no processo de redução do *S. mutans in vitro;*

– Definir um protocolo clínico para aplicação da terapia fotodinâmica *in vivo;*

– Aplicar a terapia fotodinâmica *in vivo* em dentes decíduos ou permanentes com lesões cariosas extensas de pacientes em tratamento nas clínicas de Odontopediatria da FO-UFMG e realizar uma análise quantitativa de amostras de dentina cariada antes e após a terapia, através da cultura de microorganismos do PCR em tempo real.

4 ARTIGOS

4 ARTIGOS

A abordagem da literatura é convencionalmente realizada em um capítulo de revisão de literatura. Neste trabalho a faremos sob o título “Artigos” e na forma dos trabalhos enviados para publicação fazendo-se relevância apenas àqueles em que a metodologia de alguns experimentos foi fortemente embasada.

A sequência de apresentação será um primeiro artigo de revisão sistemática com o objetivo de abordar a literatura relacionada ao tema. A seguir, serão apresentados os artigos relacionados aos experimentos *in vitro* e *in vivo*, que respondem aos objetivos específicos propostos e definem a parte experimental. As normas de publicação das respectivas revistas se encontram no Anexo 1.

Este primeiro artigo foi enviado para a Revista *Journal of Laser Applications* estando em fase de correções sugeridas pelo editor.

Artigo 1

PHOTODYNAMIC THERAPY IN CARIOGENIC AGENTS: A SYSTEMATIC REVIEW

Abstract

The purpose of this systematic review was to evaluate the current status of the photodynamic therapy (PDT) against cariogenic microorganisms. Publications with abstracts, published in English and available online in PubMed were included. To identify relevant publications, all abstracts obtained from the search in PubMed were read independently by the authors. In total, 19 studies were reviewed. It was difficult to compare the results among all the studies

because of their heterogeneity regarding photosensitizer, light source, exposure time and photosensitizer incubation period before the irradiation. All but one *in-vitro* studies assessed showed significant killing of microorganisms demonstrating the bactericidal effect of PDT against cariogenic bacteria. Due to heterogeneity in the treatment parameters, there are no available clinical protocols to apply the PDT *in-vivo* and further works are now being undertaken to make this treatment clinically accessible and to determine the efficacy of PDT on carious dentin *in vivo*.

Keywords: photodynamic therapy (PDT), cariogenic agents, photosensitizer, light source, systematic review.

1. Introduction

Photodynamic therapy (PDT) can be defined as the administration of a non-toxic agent known as a photosensitizer (PS), followed after some time by the illumination of the lesion with visible light, which leads to the generation of cytotoxic species (superoxide anion, singlet oxygen, triplet oxygen) and consequently to cell death and tissue destruction (1).

Over the past 30 years PDT has been used clinically in the treatment of many different localized cancerous and precancerous conditions and there are numerous clinical trials that are on-going. PDT has also been investigated in the treatment of skin diseases of non-neoplastic origin including psoriasis, scleroderma and acne. Successful treatment of age related macular degeneration employing PDT has resulted in FDA approval for this therapy as a first line treatment in the year 2000 (2).

The use of PDT in dental research has received considerable investigation. Various methodologies using substrates as bacterial suspensions, human dental plaque samples, collagen matrix that resembled demineralized dentine or extracted carious teeth have been used to demonstrate the antibactericidal effect of PDT against cariogenic bacteria (2).

Dental caries development is considered to involve a triad of indispensable factors: bacteria (dental plaque), carbohydrates (the diet), and susceptible teeth (the host) (3). In theory, dental caries can be prevented by eliminating cariogenic bacteria, especially *Strep. mutans*, from the mouth, as well as by increasing the resistance of teeth and modifying the diet. Other bacteria found in actively progressive carious lesions are considered to be secondary invaders, probably forming symbosis with *Strep. mutans* with regard to their physiological activities. Only a limited number of bacterial species other than *Strep. mutans* are occasionally found to be cariogenic in experimental animals (4).

Current treatment of the carious lesion involves the removal of all desmineralized dentine (‘drilling’) and restoration of the tooth (‘filling’) with any of a variety of materials. KIDD et al.(5) have proposed the removal of only the softened and wet dentine and the effective sealing of the cavity with a restorative as being sufficient to achieve ‘healing of the lesion’. The difficulties in determining the amount of tissue removal necessary clinically and the inadequacies of most restorative materials currently available in effectively achieving a longterm seal means that an effective way of disinfecting both the infected and affected tissue is highly desirable before completion of treatment (6).

A more attractive proposition is to kill the organisms in situ and then restoring the site without the removal of the softened and demineralized dentine. This is clinically attractive, as it would reduce the amount of tooth tissue required to be removed (7).

As the chemical antibacterial agents are difficult to maintain at a therapeutic concentration in the oral cavity and can be rendered ineffective by resistance development in the target organisms, PDT could be considered an alternative antimicrobial approach. Bacteria and other microbes can be sensitized to light through prior treatment with a chemical photosensitising agent. Various classes of chemical compounds, including phenothiazines, phthalocyanines, and porphyrines, with photoactive properties have been successfully tested as photoinactivating agents against Gram-positive and Gram-negative bacteria (8).

According to PAULINO et al. (9), most PDT applications are associated with a laser light, although non-laser light sources have also been used. The photoactivated dye technique can be undertaken with a range of visible red and near infrared lasers, systems using low power visible semiconductor diode laser or visible light sources. The dye or light alone should not be citotoxy, at least not in the phototherapeutical active doses (10).

The specificity of PDT is aided by the fact that singlet oxygen is the dominant bactericidal molecule which has a short life span and a limited diffusion distance of 100 nm (11). The advantages of this approach are that bacteria can be irrradicated in very short periods of time (seconds or minutes) and damage to adjacent host tissues and disruption of the normal microflora can be avoided. This approach may be a useful alternative to antibiotics and antiseptics in eliminating cariogenic bacteria (8).

The aim of this study was to evaluate the current status of the photodynamic therapy against cariogenic microorganisms, specifically *Strep. mutans*, by means of a systematic review.

2. Material and Methods

To achieve a systematic approach, the literature search was conducted as described by GOODMAN (12) and cited by OLSSON et al. (13): (i) specify the problem; (ii) formulate a plan for the literature search; (iii) conduct a literature search and retrieve publications; and (iv) interpret and assess the evidence from the literature retrieved.

2.1. Specification of the problem

The following questions define the problem: Is the photodynamic therapy an effective treatment for cariogenic agents? Is there a definitive protocol to apply for this therapy in vivo?

2.2. Systematic literature search

According to MeSH, the term “Photochemotherapy” or “Photodynamic Therapy” is defined as “a therapy using oral or topical photosensitizing agents with subsequent exposure to light” and was introduced in MeSH in 1978 (http://www.nlm.nih.gov/mesh).

Publications were retrieved from PubMed using MeSH-terms as presented in Table 1. Only publications with abstracts, published in English and with full text available online were included. The search was limited to in vitro researches. The PubMed search was combined with a search performed in The Cochrane Controlled Trials Register (CENTRAL) using the phrase ‘photodynamic therapy in dentistry’. To identify relevant publications, all abstracts obtained from the search

in PubMed were read independently by the authors. Following publications were included:

1 Original scientific studies:

• in vitro studies that evaluate the potential for photodynamic therapy to inactivate oral cariogenic bacteria. Oral bacteria associated with other diseases, such as surgery, endodontics or periodontal events were excluded;

• the cariogenic agents could be presented in carious lesions of extracted teeth, biofilms or bacterial suspensions.

2 Reviews:

• the phrase “photodynamic therapy in dentistry” should be written in the title or the abstract;

• the publication type should be indexed as Review.

The limits were Publication dates from January 1966 to May 2008 and indexed as ‘only items with abstracts’ and ‘English’. The search was performed on the 25 May 2008. All the intended readers are dentists.

2.3. Interpretation and assessment of the publications included

All abstracts are read and a selection of relevant publications was made according to the inclusion criteria above mentioned. When an abstract was considered by at least one author to be relevant, the publication was ordered in full text. All the intended readers are dentists.

Reference lists of the reviews and of the included original scientific studies were hand searched to find additional original scientific studies.

3. Results

3.1. Systematic literature search

After a search using the combined MeSh terms listed in Table 1, a total of 62 studies were found, but 12 of them were repeated and 38 of them were not evaluated because of the excluded criteria (case reports, evaluation of oral bacteria not associated with carious lesions, scientific articles without abstract or in another language different from English or with full text unavailable online). A total of 12 studies were then selected (8 original scientific studies and 4 reviews). After the hand search of the reference lists of these studies, we found seven additional original scientific studies. In total, 19 studies were assessed and critically appraised.

The number of original studies that were excluded and reasons for exclusion are listed in Table 2. The search in The Cochrane Controlled Trials Register did not identify any additional publications.

3.2. Interpretation and assessment of the included studies

Fifteen studies were assessed and data extraction regarding each one are presented in Tables 3 to 7. The other four studies were review articles. Different types of photosensitizers such as toluidine blue, methylene blue, Rose Bengal or phtalocyanines were examined. The light sources used, the pre-irradiation time and exposure time also presented a heterogeneity. The pre irradiation time is defined as the time which the photo-agent is in contact with the substrate, before the light exposure. According to BEVILLACQUA (14), pre-irradiation time is important to help achieve a robust PDT's antibacterial effect.

Conventional methods for determining the effects of PDT involve exposing bacteria to light in the presence of a photosensitizer

followed by counting number of viable bacteria remaining in the sample. The control conditions which are normally incorporated into these studies include the use of the photosensitizer without light, light without the photosensitizer and neither light nor the photosensitizer. When the PS was associated with a light source, lethal photosensitization were frequently reported, except in one study.

4. Discussion

According to HAMBLIN & HASSAN (26), PDT for localized infections could be carried out by local delivery of the PS into the infected area by methods such as topical application, interstitial injection or aerosol delivery. The key issues to be addressed are the effectiveness of the treatment in destroying sufficient numbers of the disease-causing microorganisms, effective selectivity of the PS for the microbes, thus avoiding an unacceptable degree of PDT damage to host tissue in the area of infection.

Almost all in vitro studies assessed showed significant killing of cariogenic microorganisms when they were exposed to both photosensitizer and light. WILLIAMS et al. (6) reported a reduced antibacterial effect in a collagen matrix compared to planktonic suspension. In this study, for an energy dose of 2.4 J an average log reduction of 9.53 in planktonic suspension was found, compared to a 1.01 log reduction in shredded collagen. It may be inferred that photo-activated disinfection is affected by physical constraints. Since the process seems to be time dependent, it is likely to be the rate at which the dye diffuses into the collagen plug. Although the effect was reduced, it was noted that PDT could be effective to reduce the bacteria counts even when the bacteria were embedded in a collagen matrix or in carious dentin (7).

PAULINO et al (9) showed that bacteria were 10 times more sensitive to PDT than the fibroblasts which supposedly can be a reflection of the protection and repair systems in the eukaryote. These authors demonstrated the photoinduced destruction of *Strep. mutans* without cellular death of the fibroblasts, supporting the selectivity of this therapy. Due to its localized and noninvasive nature, the side effects associated with many antibiotics (e.g., gastrointestinal disturbance) are unlikely to occur with PDT (27).

PDT is an inherently complex technology that depends on multiple variables including the chemical and photochemical properties of the PS, the PS dosage and delivery vehicle, the drug-light time interval, the wavelength, energy dose, power density and pulse structure of the light, and the oxygenation state of the tissue. It was difficult to compare the results of the studies assessed in this review because of the heterogeneity of the above mentioned variables. But it was noted that in all but one studies demonstrated the antibactericidal effect of PDT against cariogenic bacteria, independently of the combination light source -PS tested.

According to WILLIAMS et al. (20) the energy dose is the most important factor in killing bacteria. WILSON et al. (23) describes that the energy dose could be calculated from the equation: exposure time (s) multiplied by the laser output power (mW). By increasing the power, the energy dose applied to the bacteria could be increased without altering exposure time, as it is important for clinical convenience to have a short exposure time. Besides that, it is necessary that the photosensitizer have an absorption spectrum near to emission spectrum of the lamp source (10). Therefore, taking into consideration these parameters, it is possible that various combinations of different light sources and PS could be effective in lethal photosensitization of bacteria.

As reported by ZANIN et al. (18), dental caries may be a disease well suited to photosensitization therapy. If bacteria within carious lesions could be eradicated in-vivo by photosensitization, there would be beneficial consequences for dental health. Caries are often localized infections, and the photosensitizer could be applied to the lesion by means of a localized injection and light could then be delivered via an optical fibre upon the localized region. Infected or damaged dentine could be better preserved, thereby making patient treatment easier (for both dentist and patient) by enabling lesions to be restored with minimal tissue removal, and improving the long-term prognosis for the repaired tooth.

The in vitro studies evaluated here showed a satisfactory result of PDT against cariogenic microorganisms, mainly *Strep. mutans*. Since the causative microorganisms in a carious lesion are invariably unknown, a relatively non specific bactericidal effect of the PDT on a range of cariogenic microorganisms is desired because targeting of specific bacterial strains would be less satisfactory and may result in a limited effect clinically (24).

Many studies used high cost light sources (7, 19, 22, 23, 24, 25), which are inaccessible to the majority of dentists. Other manuscripts used a high pre-irradiation time or even high exposure times (16, 17, 19, 24) both not compatible with a clinical treatment. There isn't a protocol for clinical application of PDT in vivo yet and further studies should focus on making this treatment clinically acceptable, because according to BURNS et al. (24) the facility for killing the bacteria within the lesion would allow more objective decisions as to the amount of dentine to be removed. The practitioners could apply the photo agent dye to the carious lesion within the cavity, irradiate the site and then a lining to cover the dental cavity wall protecting the pulp may be placed. This would reduce the risk of recurrent caries

while providing the clinician with a more objective means of removing those bacteria which are known to contribute to spread of caries in dentine, at the same time maintaining the demineralized dentine structure. This, in turn, would reduce the amount of tissue to be removed during cavity preparation.

In conclusion, photodynamic therapy (PDT) is a technique that has been shown to be effective in vitro against cariogenic bacteria. A wide range of photosensitizers are available with differing compositions and light-absorption properties. A wide range of light sources are available, ranging from state-of-the-art laser technology to basic tungsten-filament lamps (28). PDT is proposed as a potential, low-cost approach to the treatment of oral infection and efforts should be undertaken to make this treatment clinically accessible and to determine the efficacy of PDT on carious dentin in-vivo.

Acknowledgments: Capes, CNPq and Fapemig

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Table 1: Combination of MeSh terms and number of publications retrieved

Mesh Terms	Number of papers found
Photochemo/ Photodynamic therapy + carious lesions	2
Photochemo/Photodynamic therapy + cariogenic bacteria	2
Photochemo/Photodynamic terapy + *streptococcus mutans*	7
in vitro + antimicrobial photochemo/ photodynamic therapy	22
In vitro + photochemo/dynamic therapy + oral bacteria	14
in vitro + photochemo/dynamic therapy + *streptococcus mutans*	3
photodynamic therapy in dentistry	12

Table 2- Reasons for exclusion and number of excluded studies

Case report	1
Unavailable online	6
Oral bacteria associated with another diseases	31
TOTAL	38

TABLE 3- Data from in vitro studies using PDT against cariogenic microorganisms. Unclear data reported with "?"

Author name/year	**Microorganism**	**Light source**	**Photosensitizer (PS), PS concentration (PSC); pre-irradiation time (PIT) and exposure time (ET)**	**Methods**	**Results**
MULLER et al., 2007 (14)	*A.naeslundi* *Veilonella díspar* *F.nucleatum* *S.sobrinus* *S.oralis* *C.albicans*	Soft laser (Helbo TheraLite Laser) 660nm 75mW	PS: methylene blue PSC: ? PIT: 60s ET: 60s	Multi species biofilms-coated discs of bovine teeth were treated with gasiform vacuum ozone or vacuum alone, PDT, laser alone or PS alone. Chlorexidine or hypoclorite served as a (+) control and untreated samples served as a (-) control. Number of CFU on blood agar was counted.	Only the 5% hypoclorite solution was able to totally eliminate the microorganisms in the biofilm. Gasiform ozone and PDT failed to demonstrate an effective reduction of microbiota in a multi species biofilm.
BEVILA-CQUA et al., 2007 (15)	*Strep. mutans* (ATCC 25175)	LED; wavelength range 600–670nm, with a peak at 640nm. Energy density: 2,18J/cm^2	PS: TBO PSC: 0,01mg/L PIT: 5min ET: 180s	Preparation of the *Strep. mutans* inoculum was per the standard 0.5 McFarland scale, with a final concentration of 1.5 x 10^8 cells/ml of sucrose broth. The samples were made of glass capillary tubes and divided into five groups: (1) control group (-) without bacterial plaque formation); (2) control group (+) only with bacterial plaque formation; (3) inoculum +TBO; (4) inoculum + LED and (5) inoculum + LED + TBO. The biofilm that formed on the glass surfaces was analyzed by SEM and colony count.	100% of the bacteria were killed following irradiation with LED and TBO. It was demonstrated that PDT was efficient at killing microorganisms and preventing the formation of biofilms. SEM analysis showed that there was reduction or elimination of bacterial plaque in the samples from group 5 (treated with PDT), and in samples from group 1 (negative control).
METCALF et al., 2006 (16)	*Strep. mutans* (NCTC 10449)	White light, tungsten filament lamp (400W). Average light intensity: 22,7mW/cm^2	PS: erythrosine PSC: 22µM PIT:15 min in the dark. ET: 0, 1, 2, 5, 10, 15 or 30min for continuous irradiation. Light fractionation: pulsed for 5 x 1min or for 10 x 30s.	*Strep. mutans* biofilms were grown and submitted to PDT using continuous irradiation times or light fractionation. Surviving cells were counted on Columbia agar supplemented with 5%(v/v) horse blood.	98% of cell killing occurred in the first 5min of irradiation. The two light fractionation regimes were also superior to longer continuous irradiation times of up to 30min.

TABLE 4 - Data from in vitro studies using PDT against cariogenic microorganisms. Unclear data reported with "?"

Author name/year	**Microorganism**	**Light source**	**PS, PSC, PIT, ET**	**Methods**	**Results**
WOOD et al., 2006 (17)	*Strep. mutans* (NCTC 10449)	400W tungsten filament lamp. For erythrosine 22.7 mW/cm^2, 500-550nm. MB / photofrin: 22.5 mW/cm^2 600-650nm	PS: erythrosine, MB and photofrin PSC: 22µM for all PIT: 15min in the dark. ET: 15 min.	Biofilm-containing pans were removed in triplicate at 48, 120, 168, 216 and 288h after inoculation, placed in vials containing the dyes and irradiated with light. Bacterial survival was enumerated by serially diluting samples and plating in triplicate. To examine the localization of erythrosine in the biofilms, intact biofilms were examined using a confocal laser scanning microscope (CLSM).	PDT using MB and irradiation resulted in log10 reductions in CFU of between 1.5 (48 h biofilms) and 2.6 (288 h biofilms). Photofrin-mediated PDT had less effect on *Strep. mutans* biofilm cell viability than the other photosensitizers, with 0.5 to 1.1 $\log_{10}$ CFU reductions observed, again with the older biofilms being more susceptible to photoinactivation.
ZANIN et al., 2006 (18)	*S.mutans* *S. sobrinus* *S. sanguinis*	LED (Laserbeam) wavelength: 620 to 660nm, 32mW Energy density:85,7J/cm^2	PS: toluidine blue O (TBO) PSC: 0,1mg/ml PIT: 5 min ET: 7min	Enamel slabs from bovine incisors were immersed in BHI broth and inoculated with microorganism's overnight culture to form a 5 days biofilm which was submitted to PDT. The number of viable microorganisms, the concentration of water insoluble polysaccharide and the mineral loss were analysed.	A significant reduction of ~95% in viability was observed for *S mutans* and *S. sobrinus* biofilms and a reduction of 99% was observed for *S. sanguinis*, only in the groups that TBO was combined with light source. The extent of desmineralization and the level of water insoluble polysaccharide production increased in older biofilms.
PAULINO et al., 2005 (9)	*Strep. mutans* (ATCC: 25175)	Hand held photopolymerizer: 400-500nm and light emmited of 350 – 500mJ/ cm ^{2})	OS: Rose Bengal PSC: 0 a 50µM PIT:? ET: 0 a 40s	Bacterial suspensions adjusted at 10^{-3} CFU/ml and fibroblasts were treated with different concentrations of Rose Bengal and irradiated with light. The control groups evaluated the dye toxicity per se in the dark and the light toxicity without any dye. The CFU/ml was calculated for *Strep. mutans* and the cells viability was calculated for fibroblasts.	In concentrations below 5µg/ml, Rose Bengal does not show toxicity to *Strep. mutans* as well to fibroblasts. In concentrations above this value, an exponencial increase of toxicity occurs for both types of cells. At 0,5µg/ml 100% of cell death for *Strep. mutans* was observed, without affect the fibroblasts.

TABLE 5- Data from in vitro studies using PDT against cariogenic microorganisms. Unclear data reported with "?"

Author name/year	**Microorganism**	**Light source**	**Photosensitizer (PS), PS concentration (PSC); pre-irradiation time (PIT) and exposure time (ET)**	**Methods**	**Results**
ZANIN et al., 2005 (19)	*Strep. mutans* (NCTC: 10449)	Helium/neon gas laser (632,8nm) and a LED (620 a 660nm) Power output of both light sources:32 mW	PS: Toluidine blue (TBO) PSC: 100mg/L PIT: 5min ET: 5,15 or 30min to HeNe or LED light	Hydroxyapatite discs containing *Strep. mutans* biofilms were submitted to PDT. Control groups consisted of biofilms treated with TBO without light and with light without TBO. Ten-fold serial dilutions were carried out and aliquots plated onto BHI agar, incubated for three days and the number of viable organisms was enumerated. The biofilms were examined in a CLSM.	Significant decreases in the viability of *Strep. mutans* biofilms were only observed when biofilms were exposed to both TBO and light (99,9% of reduction for both sources). CSLM images suggested that lethal photo sensitization occurred mainly in the outermost layers of the biofilms.
WILLIAMS et al., 2004 (6)	*Strep. mutans* (NCTC 10449)	Laser (Denfotex Light System) (633nm; 60 or 80mW power output)	PS: TBO PSC: 10mg/L PIT: 30 ou180s ET: 30, 60 or 180s for bacteria/collagen suspension and 30 or 60s for carious tissue	Bacterial suspension mixed in a collagen matrix was submitted to PDT. Eight extracted human teeth were used for each contact time. All softened carious tissue was removed with an excavator and divided into two similar portions (control group and photo-activated disinfection technique-PAD).	Although antibacterial effects were less than those obtained using planktonic suspensions, the PAD can achieve appreciable kills of oral bacteria, when they are embedded in a collagen gel or are present in carious teeth.
WILLIAMS et al., 2003 (20)	*Strep. mutans* (NCTC 10449)	Laser diode device (633nm)	PS: two sources of TBO PSC: 13± 3 mg/L PIT: 60s ET: 5 to 60s	The suspensions of *Strep. mutans* were exposed to a range of energy doses produced by varying power and time. A delivery system guided the laser light to an 800µm diameter spherical tip (an isotropic tip) from which light radiated producing a uniform sphere of light within the colony or suspension.	Neither TBO nor light alone had a significant antibacterial effect. Energy doses of 1,8J or more killed 100% of the bacteria present. The energy dose was the most important factor in killing bacteria.

TABLE 6- Data from in vitro studies using PDT against cariogenic microorganisms. Unclear data reported with "?"

Author name/year	**Microorganism**	**Light source**	**PS, PSC, PIT, ET**	**Methods**	**Results**
WOOD et al., 1999 (21)	Seven-day oral plaque biofilms formed on natural enamel surfaces in vivo	white light from a 400W tungsten filament lamp; average intensity: 22.5mW/cm^2; wavelength range 600-700nm	PS: Cationic Zn (II) phthalocyanine PSC: 0,001mg/L PIT: ? ET: 30min	Nylon rings were attached on the natural enamel surface in eight human volunteers. They carried two devices (control and PDT) for seven days. After being removed, the devices were placed in sterile reduced transport fluid, washed and irradiated with the light source. Control samples were exposed to either light or drug alone. Samples were then either viewed immediately using a confocal microscope or processed for TEM.	Transmission electron microscopy (TEM) of the in vivo formed plaque biofilms reveals considerable damage to bacteria in the biofilm, vacuolation of the cytoplasm and membrane damage being clearly visible after PDT. These results clearly demonstrate the potential value of PDT in the management of oral biofilms.
WILSON et al., 1996 (22)	*S. sanguis* (NCTC 10904)	GaAlAs diode laser (660nm; 11mW), in a pulsed mode (20kHz frequency)	PS: $AlPcS_2$ PSC: 100mg/L PIT:? ET:?	The lethal photosensitization of S. sanguis was evaluated when it is grown as a biofilm under conditions similar to those existing in the oral cavity, using hydroxyapatite as the substratum and artificial saliva as the nutrient source. The light doses vary from 0,8 to 12,2J	The decrease in viable count of S. sanguis was light-energy dose-related. No viable streptococci were detectable after irradiation of $AlPcS_2$-treated biofilms with 12,2J of GaAlAs laser light.
BURNS et al., 1995 (7)	*Strep. mutans* (NCTC 10449)	HeNe laser (633nm; 7,3mW) GaAlAs diode laser (660nm; 11mW), in a pulsed mode at a frequency of 20kHz	PS: TBO for HeNe laser and $AlPcS_2$ for diode laser PSC: 50mg/L for both dyes PIT: ET:120, 240 or 480s for dentine slices and 60 or 180s for collagen matrix	Desmineralized dentine slices were interposed between the laser light and the bacterial suspension which were then mixed to both dyes and irradiated. Lethal photosensitization was also done in the bacteria embedded in a collagen matrix. Survivors were enumerated by viables countings on TSB plates.	Significant killing of *Strep. mutans* (10^8 to 10^{10} CFU) is possible even when the bacteria were embedded in a collagen matrix or when the light was passed through des- mineralized dentine sections. In the presence of slices the number of bacteria killed was lower, but the increase in energy densities could overcome the light masking effect of the dentine. The HeNe was more effective than GaAlAs laser.

TABLE 7- Data from in vitro studies using PDT against cariogenic microorganisms. Unclear data reported with "?"

Author name/year	**Microorganism**	**Light source**	**PS, PSC, PIT and ET**	**Methods**	**Results**
WILSON et al., 1995 (23)	Samples of supra-gengival plaque obtained from 10 volunteers	HeNe laser (633nm and 7,3mW) and a GaAlAs diode laser (660nm; 11mW), in a pulsed mode at a frequency of 20kHz	PS: TBO for HeNe laser and $AlPcS_2$ for diode laser PSC: 100mg/L for TBO PIT: 0s ET: 60s for TBO and 180s for $AlPcS_2$	Plaque samples were treated with either TBO or aluminium disulphnated phthalocyanine (AlPcS2) and then exposed to light from a HeNe or GaAs (gallium arsenide laser) respectively. The bacterial suspension obtained was plated in duplicate onto the TSA (Tryptone soya agar) fotr the total viable counting; mitis-salivarius agar for streptococci and Rogosa agar for lactobacilli.	Greater kills were achieved by HeNe/TBO combination than by the GaAs/ $AlPcS_2$ combination, as the light energy dose delivered by the HeNe laser was lower than that from the GaAs laser. Substantial kills of bacteria in supragengival plaque samples can be achieved by both treatments.
BURNS et al., 1994 (24)	*Strep. mutans* NCTC 10449 *S. sobrinus* NCTC 10921 *L. casei* NCTC 10302 and *A. viscosus* NCTC 10951	GaAs diode laser (11mWpower output and 660nm wavelength)	PS:Aluminium disulphonated phthalocyanine ($AlPcS_2$) PSC: 100, 50 and 10mg/L PIT: 15 min ET:30, 60 or 90 s	Aliquots of each bacterial suspension were added to an equal volume of $AlPcS_2$ in TSB (Tryptone soya broth) in wells of a microtitre plate. Controls received only TSB. 4mm magnetic stirrer bars were added to the wells, the plate was placed on a magnetic stirrer and the suspensions exposed to light. Control samples were exposed to either light or drug alone. Survivors in each well were enumerated by viable counting on TSA.	The results have demonstrated that large numbers (10^6 UFC) of four cariogenic species of bacteria can be killed by GaAs laser in the presence of $AlPcS_2$, the laser light having no detectable effect on bacterial viability. Variation in susceptibility among the target organisms was observed, being the *Strep.mutans* the most susceptible.
DOBSON & WILSON, 1992 (25)	*S. sanguis* *P. gingivalis* *F. nucleatum* *Aa*	HeNe laser (633nm and 7,3mW power output)	PS: Crystal violet, methylene blue, TBO, arianor steel blue and phthalocyanine PSC: 0,005% for phthalocyanine; 0,001 and 0,1% for the others PIT:? ET: 10 and 60s.	Biofilms of each bacteria were prepared on agar plates and then exposed to light. 25mm diameter sterile nitrocellulose membranes were placed over those portions of the plates that have been exposed to the laser light and left in place for 5min. They were then removed, inverted and placed on the surfaces of fresh BHI plates. After incubation, the membranes were examined for bacterial growth.	TBO and MB enabled detectable killing of all four target organisms after exposure to HeNe light. The other PS tested were not effective against *Aa* or *F. nucleatum*. The successful use of low concentrations of PS, low doses of light and short exposure times in achieving a bactericidal effect implies that the use of this technique clinically is feasible.

Como conclusão pode-se notar a efetividade da PDT *in vitro* em relação ao *S. mutans*. Os trabalhos evidenciam a utilização de vários corantes associados a diferentes fontes de luz. Devia-se, entretanto, selecionar para os experimentos *in vitro* qual metodologia a ser utilizada.

O Departamento de Engenharia Mecânica da UFMG desenvolvia trabalhos nesta linha de pesquisa já tendo gerado teses de Doutorado dos Professores Gerdal Roberto de Sousa, Marcus Vinicius Lucas Ferreira e Lívio de Barros Silveira. A decisão foi de se utilizar a experiência adquirida nestes experimentos, embora os trabalhos objetivassem bactérias periodontopatogênicas com a utilização de laser ou LED vermelho, em baixa potência.

Desta forma, o trabalho *in vitro* foi realizado utilizando-se os corantes azul de metileno e azul de toluidina nas concentrações de 5, 10 e 25mg/l focando-se, entretanto, em *S. mutans*. Os experimentos piloto foram realizados utilizando-se as fontes de luz LED e laser vermelho. Entretanto, para a padronização da densidade de energia, o LED deveria ser utilizado por cinco minutos obtendo-se 60 J/cm^2 ao passo que o laser seria utilizado por apenas um minuto para a mesma densidade. Analisando-se que três minutos não seria um tempo clínico factível de ser utilizado em prováveis experimentos *in vivo* resolveu-se abandonar experimentos com LED embora os resultados tenham sido semelhantes em eficácia de redução de ufcs.

A metodologia dos procedimentos *in vitro* baseou-se no trabalho de tese do Professor Gerdal conforme resumo abaixo apresentado.

Sousa (2007) avaliou o efeito da fotossensibilização letal de bactérias periodontopatogênicas *in vitro* associando dois lasers e um LED ao azul de toluidina a 0,01%. Avaliou-se também a linearidade de absorção do TBO, verde de malaquita e azul de metileno pelas fontes de luz utilizadas. Estes

agentes fotossensibilizantes foram colocados em cubas acrílicas e irradiados, avaliando-se a quantidade de luz absorvida pelo corante através da luz residual transmitida ao medidor de potência (NOVA-Ophir Optronics, Jerusalém, Israel). Amostras de referência de *Actinobacillus actinomycetemcomitans* (ATCC 29525)*, Fusobacterium nucleatum* (ATCC 25586) ou *Prevotella intermedia* (ATCC 25611) foram utilizadas para preparo dos inóculos, que foram avaliados por espectroscopia. As suspensões bacterianas foram colocadas em contato com o TBO por cinco minutos, antes de serem irradiadas por três minutos pelos aparelhos emitindo no vermelho do espectro eletromagnético. Após aplicação da PDT, foram realizadas diluições seriadas e plaqueamento, para posterior contagem do número de ufcs. Os resultados foram comparados a um grupo controle, que não recebeu nenhum tratamento. O efeito do TBO e das fontes de luz, isoladamente, também foi avaliado. Observou-se uma significativa redução no número de ufcs viáveis apenas nos grupos submetidos à PDT. O laser 1, com potência de 100 mW e o LED (100 mW) foram mais eficientes na redução bacteriana que o laser 2 (40 mW), confirmando a influência da potencia do aparelho na absorção de luz pelo corante. As espectroscopias e as medidas da potência dos aparelhos mostraram a ressonância destes com os corantes avaliados. Baseado nos resultados satisfatórios encontrados *in vitro*, a PDT foi considerada uma técnica de baixo custo com potencial para ser utilizada no tratamento de patologias e infecções orais.

Os resultados dos experimentos *in vitro* foram formatados em um trabalho enviado para a Revista *Acta Odontologica Latinoamericana* que será apresentado em seguida de acordo com a formatação requerida. Este trabalho recebeu o aceite conforme Anexo 2.

Artigo 2

IN VITRO LETHAL PHOTOSENSITIZATION OF *S. MUTANS* USING METHYLENE BLUE AND TOLUIDINE BLUE O AS PHOTOSENSITIZERS

Abstract

The purpose of this *in vitro* study was to evaluate the antimicrobial effect of photodynamic therapy on *Streptococcus mutans* (ATCC 25175) suspensions by culture method, using a red laser for one minute in combination with toluidine blue O (TBO) or methylene blue (MB). Both photosensitizers were used in three concentrations (25, 10 and 5 mg/L). The activity of photosensitizers and laser irradiation were tested separately on the bacteria, as well as the irradiation of this light source in the presence of the TBO or MB. These groups were compared to a control group, in which the microorganism did not receive any treatment. The activity of both TBO and MB or laser irradiation, alone, were not able to reduce the number of *S.mutans*. In the groups of lethal photosensitization, a bacterial reduction of 70% for TBO and 73% for MB was observed when these photosensitizers were used at 25 mg/L and a reduction of 48% was observed for MB at 5 mg/L. In other concentrations there were no significant differences in comparison to the control group. Both the TBO and the MB at 25 mg/L associated with a red laser had an excellent potential for use in PDT in lethal sensitization of *S. mutans*.

Keywords: photodynamic therapy, S. mutans, lasers, photosensitizer

Estudo *in vitro* do efeito da terapia fotodinâmica sobre o *S. mutans* utilizando-se azul de toluidina ou azul de metileno como agentes fotossensibilizantes

Resumo

O objetivo deste estudo *in vitro* foi avaliar o efeito antimicrobiano da terapia fotodinâmica em suspensões de *Streptococcus mutans* (ATCC 25175), utilizando um laser vermelho durante um minuto associado a dois agentes fotossensibilizantes: azul de toluidina (TBO) ou azul de metileno (MB). Os agentes fotossensibilizantes foram utilizados em três diferentes concentrações (25, 10 and 5 mg/L). A atividade destes agentes e da fonte de luz foi testada separadamente sobre a suspensão bacteriana, assim como a irradiação desta fonte de luz na presença de TBO ou MB (terapia fotodinâmica). Estes grupos foram comparados a um grupo controle, onde nenhum tratamento foi realizado. A aplicação dos dois fotossensibilizantes (TBO ou MB) e da fonte de luz, separadamente, não foi capaz de reduzir o numero de colônias viáveis do *S.mutans*. Nos grupos onde a terapia fotodinâmica foi aplicada, uma redução bacteriana de 70% foi observada para o TBO e de 73% para o MB, quando estes agentes foram utilizados na concentração de 25 mg/L. O uso do MB a 5mg/L causou uma redução de 48%. Para as outras concentrações testadas não se observou nenhuma redução em relação ao grupo controle. Pode-se concluir que tanto o TBO quanto o MB a 25mg/L associados ao laser vermelho demonstraram um excelente potencial para promover a fotossensibilização letal do *S. mutans*.

Palavras Chave: terapia fotodinâmica, S. *mutans*, lasers, fotossensibilizantes

Introduction

Dental caries is a disease which, after demineralization of the enamel has occurred, progresses slowly down into the dentine. The lesion consists of an advancing zone of demineralization behind which is a zone of partially demineralized dentine infected with bacteria[1]. The difficulties in determining the amount of tissue removal necessary clinically and the inadequacies of most restorative materials currently available in effectively achieving a long-term seal means that an effective means of disinfecting both the infected and affected tissue is highly desirable before completion of treatment. If bacteria in infected but only partly demineralized tissue could be killed, even more tissue could be retained[2].

It is well known that the accumulation of bacterial biofilms on tooth surfaces results in some of the most prevalent bacterial-induced human diseases, caries and inflammatory periodontal diseases[3]. The prevention of caries (primary prevention), and the control of disease progression (secondary prevention), focus mainly on mechanical and/or chemical biofilm reduction[4] such as the use of antiseptics and antibiotics[3]. Antibacterial agents are widely used in the treatment of oral diseases, but problems of development of bacterial resistance mean alternative strategies are required to control bacterial plaque biofilm and treat caries, gingivitis and periodontal disease[5].

With ever-increasing levels of antibiotic resistance, light-activated antimicrobial agents (photosensitizers or PS) are becoming an attractive alternative to conventional antibiotics[6]. Photodynamic therapy (PDT) is an established treatment for localized tumors, involving the application and retention of an applied photosensitizing agent in malignant tissues and a substantial body of work has shown that this photodynamic approach can also be used to kill bacteria[7].

PDT is a therapy modality which employs the combination of visible light, a drug (called photosensitizer or dye) and molecular oxygen usually present in the tissue. This photosensitive agent can be a molecule normally present in cells and tissues, but in the specific case of PDT its administration is generally the first step in the treatment process. In the second step, the targeted tissue is exposed to visible light at a wavelength specific for each dye, which is absorbed by the photosensitive agent. The combination of the two agents in the presence of oxygen leads to the production of different reactive oxygen species such as singlet oxygen ($^{1}O_2$), which will lead to a sequence of biological events resulting in the apoptosis of the cells or death of the microorganisms[8].

PDT has two main advantages over conventional antibiotic treatments. First, the bactericidal activity is confined to areas which have been treated using the photosensitizer and light—avoiding disruption of the indigenous microbiota at sites distant from the infected area. Second, the development of resistance to $^{-1}O_2$ by bacteria is unlikely due to its non-specific mode of action[6].

According to Wilson[9], several species of oral bacteria, in the presence of an appropriate photosensitizing agent, can be killed by light from a low-power laser. Susceptible species include the plaque-forming and cariogenic-species *S. sanguis, S. mutans, S sobrinus, L casei* and *A. viscosus*.

The aim of this *in vitro* study was to evaluate the antimicrobial effect of PDT on *S. mutans* suspensions, using a red laser in combination with TBO or MB as photosensitizers.

Material and Methods

Photosensitizers and light sources

TBO was dissolved in distillated water and stored in the dark at 250 mg/L and methylene blue (100mg/L) was obtained from Chimiolux® (Aptivalux, Brazil). Both photosensitizers were used at three concentrations: 25, 10 and 5mg/L. To achieve these concentrations, both photossensitizers were diluted in sterile distillate water.

The light source used was a red laser (TwinFlex®, MM Optics, Brazil) in the wavelength of 660 nm, a power output of 40mW and a light intensity of 1000 mW/cm^2. To reach a 60 J/cm^2 energy density, red laser was used for one minut.

Bacterial culture

The microorganism used in this study was *S. mutans* (ATCC 25195); it was maintained by subculture on *mitis salivarius* agar (Acumedia Manufacturers®, Inc. Lansing, Michigan) and 24 hours before the experiment it was placed to grow on brain heart infusion broth (BHI).

Standardized suspensions of *S. mutans* were prepared, adjusting the initial turbidity of the bacteria culture to A600nm = 0.5 (~10^9 cells/ml), as described by Paulino et al.[8] using a spectrophotometer (SpectrumLab 22PC®). The BHI broth containing the overnight culture of *S. mutans* was added to a sterile peptone saline solution until it reached the desired concentration (~10^9 cells/ml), which was confirmed by the spectrophotometer measurement. The entire experiment was performed under aseptic conditions in laminar air flow chamber.

Photodynamic therapy

Ten groups with three samples each were tested (n=3). The control group (G1) contained 1 ml of the standardized suspension (inoculum), prepared

as described above. To evaluate the light toxicity *per se* without any dye, 1ml of the standardized suspension was irradiated by red laser light for 1min (G2). To evaluate the dye toxicity *per se*, the PS in their higher concentrations were left in contact with bacterial suspensions for 5 minutes in the dark, using MB on group G3 and TBO on group G4. Groups 5 to 10 were submitted to PDT, varying the PS concentration. In groups 5, 6 and 7, MB was used at concentrations of 25, 10 and 5 mg/L, respectively. For groups 8, 9 and 10, the PS was TBO, used at the same concentrations as mentioned above. For all PDT groups, exposure time was 1 min and before irradiation all these groups were maintained in contact with the PS for 5 min in the dark (pre-irradiation time).

Serial dilutions of 10^4 UFC were obtained and aliquots of 100 µl were plated in triplicate on *mitis salivarius* agar. After incubation in a candle jar, in a microaerophilic atmosphere at 37^{o}C for 36 hours, the number of viable colony forming units (CFU) was obtained by visual counting. The dependent variables were type and concentration of photosensitizer. To determine the significance of the irradiation alone, the presence of sensitizer alone and the combination of sensitizer and light, the data were analyzed by a variance analysis (ANOVA) model using the factorial (2x2) design. The Tukey test was chosen for evaluating the significance of all pairwise comparisons with a significance limit of 5%.

Results

Neither irradiation of the suspensions in the absence of photosensitizer, nor incubation with dye alone had significant effects on the viabilities of the streptococcal suspensions ($p < 0.05$). The control group (G1) and the groups without PDT (G 2, 3 and 4) showed no statistically significant difference, although G2 (red laser alone) showed a small decrease in the number of viable microorganisms (13%). The results are shown in Fig. 1.

Both photosensitizers caused a decrease in the number of colony forming units, when used at a concentration of 25 mg/L, demonstrating the bactericidal effect of PDT after one single application of red laser for 60 seconds. For that concentration, there was no significant difference between MB and TBO. The combination of MB at 25 mg/L and red laser (G5) produced a 73% reduction in the number of viable CFU. For TBO, in the same concentration and in the presence of light (G8), the reduction was 70% when the groups were compared to the control group.

When smaller concentrations were tested, the association of MB at 5 mg/L and red laser (G7) showed a percentage reduction of 48%, which was not as effective as the same photosensitizer used at 25 mg/L.

No significant reduction in the viability of *S. mutans* was presented either for MB at 10 mg/L nor for TBO (10 or 5 mg/L), when these PS were used together with red light. Fig. 2 shows the percentage reduction in the number of viable CFU for varying PS concentration.

Discussion

This research found an effective reduction in the viable numbers of *S. mutans* after photosensitization, which is significant, as this species is amongst those reported to be most highly associated with caries in humans[10]. It was observed that both variables evaluated, type and concentration of the photosensitizer, could affect the results of PDT.

TBO is a cell membrane active photosensitizer[10], which can absorb red laser light and is bactericidal for multiple species, including organisms such as streptococci that are implicated in dental caries[1,11]. In other studies[3,12] this photosensitizer was found to be effective *in vitro*, even in the presence of demineralized dentine and collagen[13]. It has been shown that the light dose and the PS concentration required to kill bacteria

treated with TBO is far lower than that causing toxicity in cultured human keratinocytes and fibroblasts[3] .

Methylene blue has been widely used by several European transfusion services in the photodecontamination of blood plasma and has been shown to be particularly effective in the inactivation of viruses[10]. According to Ivanov *et al.* [14] the concentration of photosensitizers as MB and TBO could not be higher than 0,1% (1000 mg/L) because above this concentration they demonstrate toxicity to cells and could stain the dentine, making the treatment esthetically unviable.

In others studies, it was demonstrated that significant killing of the cariogenic organism *S. mutans* by PDT was possible *in vitro*: Zanin *et al.*[3] demonstrated a 95% reduction in the viability of *S. mutans* biofilms after lethal photosensitization using a light-emission diode combined with TBO. In a study analyzing bacteria in supragingival plaque scrapings, Wilson *et al*[15] found that substantial kills (97%) could be achieved by a helium/neon laser (HeNe) in the presence of TBO. It has also been shown previously that the viability of *S. mutans* biofilms can be reduced (99%) by TBO associated with a HeNe laser or a light emmiting-diode[12].

According to de Souza *et al.* [16] a great number of variables may influence the number of microorganisms affected by photodynamic therapy, including the type and concentration of the PS, the microorganism's physiological stage, photosensitizer incubation period before the irradiation, pre irradiation time, light exposure period and density of laser energy. Compared to the data in literature, this study found a smaller percentage reduction of *S. mutans* for both photosensitizers tested. It could be explained by the fact that we used shorter pre-irradiation and exposure times than those used in other studies described in the literature, because we tried to test the efficacy of an antimicrobial therapy that could be reproduced *in vivo*. Moreover, we used a low-power red

laser – a light source commonly used in dentistry – thus making PDT more easily accessible to dentists.

As reported by Zanin *et al.*[3], dental caries may be a disease well suited to photosensitization therapy. Caries is often a localized infection, and so the sensitizer could be applied to the lesion by means of a syringe and the light could then be delivered via an optical fiber. If bacteria within carious lesions could be eradicated by photosensitization *in vivo*, there would be beneficial consequences for dental health. Infected or damaged dentine could be better preserved, thereby making patient treatment easier (for both dentist and patient) by enabling lesions to be restored with minimal tissue removal, and improving the long-term prognosis for the repaired tooth.

The potential advantage of this over conventional caries treatment would be the ability to kill the bacteria *in situ* and then restore the site without the removal of the softened and demineralized dentine. This is clinically attractive, as it would reduce the amount of tooth tissue required to be removed [13].

The facility for killing the bacteria within the lesion would allow more objective decisions as to the amount of dentine which should be removed. The practitioners could apply the dye to the carious lesion within the cavity and then irradiate the site, after which a protective bacteriostatic lining could be placed. This would reduce the risk of recurrent caries while providing the clinician with a more objective means for removing those bacteria which are known to contribute to spread of caries in dentine, at the same time maintaining the demineralized dentine structure. This in turn would reduce the amount of tissue to be removed during cavity preparation[1].

Further works are now being undertaken to determine the efficacy of PDT on carious dentin *in vivo*, because according to Ten Cate *et al.*[17], bacteria in a carious lesion have a physiology different from that of planktonic cells. Nevertheless, this *in vitro* study was useful to determine the best type and concentration of the PS, as well as the best pre-irradiation and exposure time to be used with a specific light source. The antimicrobial effect of PDT on *S. mutans* suspensions was demonstrated as the result of this study and it will be used to define a clinical protocol in order to apply the photodynamic therapy *in vivo*.

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Figures and Legends

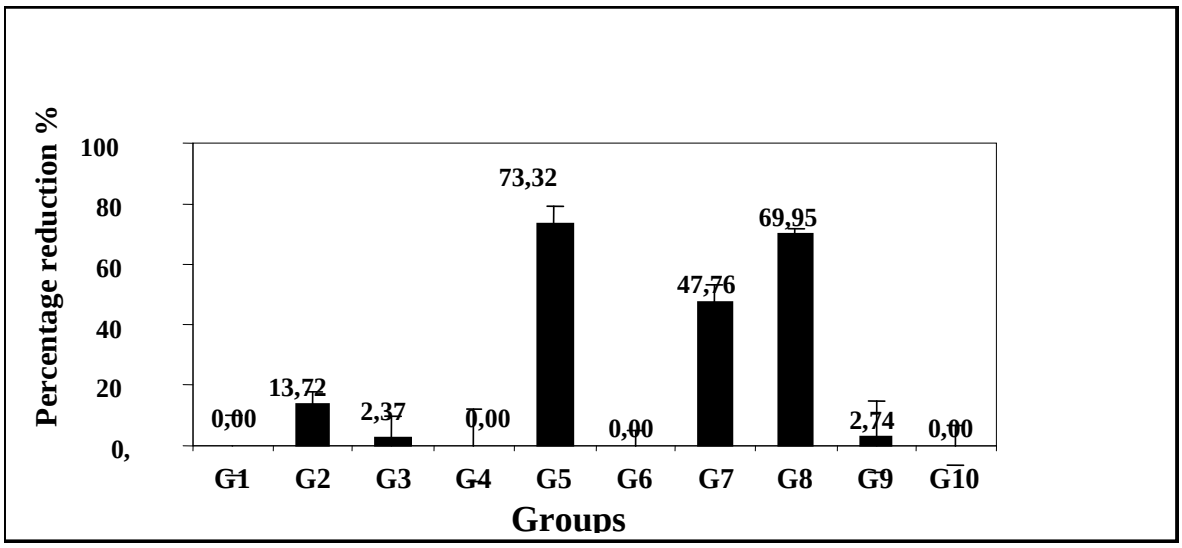

Figure 1- Percentage reduction of the number of viable CFU in relation to control group (G1-inoculum). G2 (red laser alone); G3 (MB alone); G4 (TBO alone); G5, G6 and G7 (PDT: red laser + MB 25, 10 and 5 mg/L, respectively); G8, G9 and G10 (PDT: red laser + TBO 25, 10 and 5 mg/L, respectively).

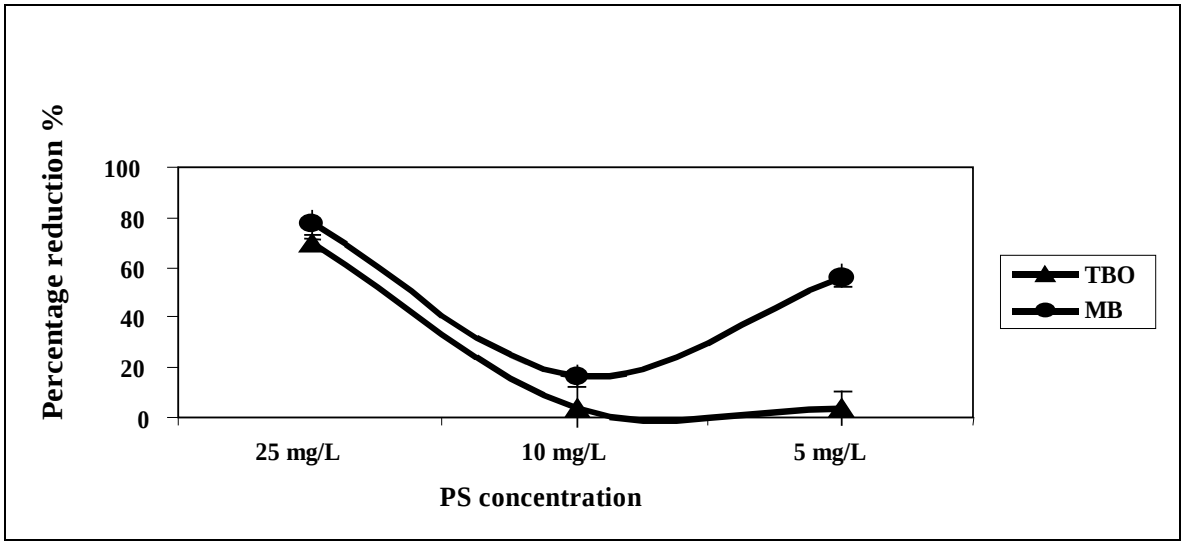

Figure 2 - Percentage reduction of the number of viable CFU with varying PS concentration. MB is represented by circles and TBO by triangles.

Paralelamente aos experimentos utilizando-se a fonte de luz laser questionou-se a necessidade dos profissionais em adquirir mais um equipamento para suas atividades clínicas. Embora este equipamento possa desempenhar outros procedimentos terapêuticos questionou-se a utilização da luz branca através da adaptação dos aparelhos fotoiniciadores que já fazem parte do arsenal dos consultórios odontológicos. Trabalho de Wood *et al.* (2006) utilizou lâmpada de filamento de tungstênio associado a diferentes corantes na fotossensibilização letal de biofilmes de *S. mutans*. Já Paulino *et al.* (2005) utilizaram aparelho fotoiniciador da empresa Dabi Atlante com comprimento de onda de 400 a 500 nm associados ao corante *Rose Bengal* em suspensões bacterianas de *S. mutans* e fibroblastos. Os resultados evidenciaram a eficácia do efeito antimicrobiano sobre o *S. mutans* sem alterar a viabilidade dos fibroblastos. Comenta-se ainda da possibilidade da mudança do espectro de emissão de luz pela troca do filtro do aparelho aumentando-se a aplicabilidade da terapia a uma ampla variedade de agentes fotossensibilizantes.

Mantendo-se a mesma metodologia empregada para o emprego da fonte de luz laser iniciou-se o experimento com luz halógena fornecida pelo aparelho Curing Light 3M Espe® (3M Espe, USA). Para estes experimentos foi utilizada a concentração de 25 mg/L tanto para o azul de metileno quanto para azul de toluidina. O tempo de exposição determinado foi de um minuto resultando em uma densidade de energia de 31 J/cm^2 com tempo pré-irradiação de cinco minutos. Os dados deste experimento estão sendo tabulados em um artigo a ser enviado para publicação.

Os resultados apresentados no gráfico 1 (página 67) evidenciam a eficácia do azul de metileno em relação ao azul de toluidina. Estes achados determinaram o abandono da utilização deste último. Testes realizados para a determinação do espectro de absorção do corante e o espectro de

emissão da fonte de luz halógena apresentados na figura 2 do artigo 4 (página 100) comprovam a ressonância obtida.

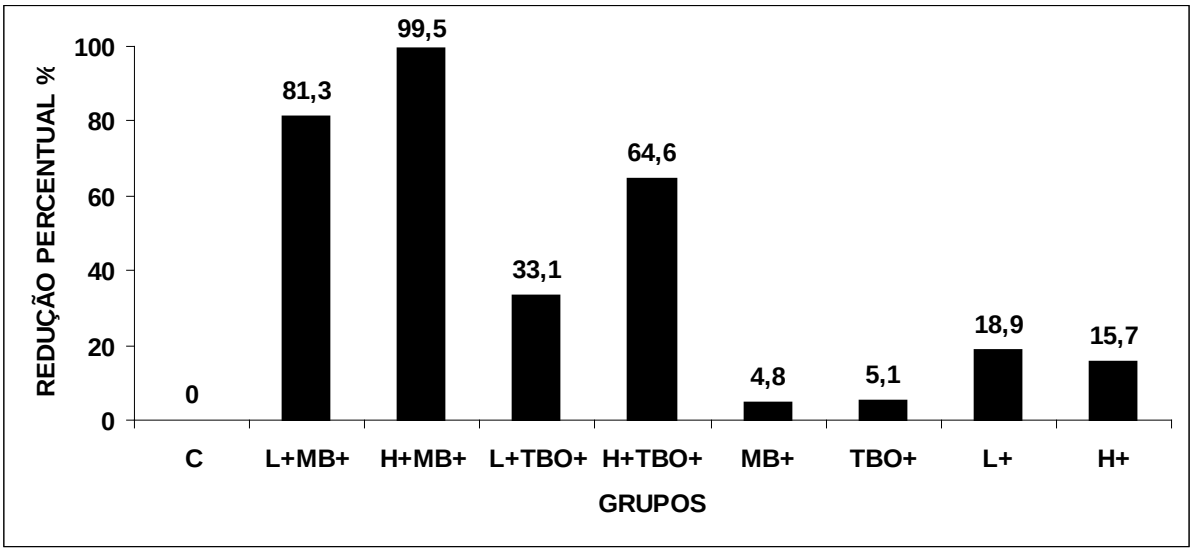

Gráfico 1- Redução percentual do número de ufcs viáveis em relação ao grupo controle.

Voltando-se ao gráfico 1 pode-se notar que a redução percentual obtida foi de 99,5% para a associação azul de metileno/luz halógena e 81,3% para laser/azul de metileno. Traduzindo-se estes percentuais para uma nomenclatura microbiológica pode-se afirmar a redução de 2,3 e 0,72 logs, respectivamente. A discussão destes resultados será apresentada nas considerações finais deste trabalho.

O protocolo para aplicação clínica da PDT baseou-se nos achados dos experimentos *in vitro* e no protocolo clínico da tese da Professora Maria de Lourdes de Andrade Massara.

A submissão ao Comitê de Ética e Pesquisa da UFMG para trabalhos *in vivo* foi aprovada pelo parecer 501/06 emitido em 28 de março de 2007.

Os testes foram realizados na clínica de Odontopediatria da FO-UFMG apenas nas crianças que obtiveram a permissão dos pais/responsáveis por

escrito, através da assinatura do Termo de Consentimento. Todas as crianças receberam atenção odontológica integral. O parecer do COEP-UFMG e o Termo de Consentimento Livre e Esclarecido se encontram no Anexo 3.

Os resultados deste experimento foram formatados em dois artigos apresentados a seguir. O primeiro, enfocando-se os resultados da fonte de luz laser, foi enviado em março de 2009 para a revista *Archives of Oral Biology* e está aguardando parecer dos revisores. O segundo encontra-se ainda em fase final de formatação para submissão à revista *Caries Research*.

Pode-se notar da leitura deste segundo artigo a inclusão dos resultados obtidos utilizando-se PCR em tempo real.

Estes experimentos foram realizados como etapa final, pois se aguardava a possibilidade de sua execução no Laboratório de Patologia da FO-UFMG. A não inclusão no primeiro artigo enviado para publicação deveu-se ao tempo de sua realização.

Artigo 3

IN VIVO ANTIMICROBIAL EFFECT OF PHOTODYNAMIC THERAPY IN CARIOUS LESIONS

Abstract:

Objectives: The aim of this study was to evaluate the effect of photodynamic therapy (PDT) on carious lesions in vivo.

Methods: Eight children aging from three to nine were selected, having molars with deep active carious lesions. In total, ten teeth comprised the sample. For each tooth, five portions of carious dentin were removed. Two increments were used as control, one to represent the infected superficial dentin and other to represent the disorganized affected deep dentin. The photosensitizer methylene blue at 25 µg/ml was placed in contact with the cavity and left for five minutes. Next, the tooth was irradiated by a red laser for one minute. After PDT, a portion of deep dentin was removed from the right half of the cavity having being directly irradiated. Other two portions, corresponding to the left half of the cavity were also removed, representing the superficial dentin and deep dentin after PDT. The cavities were restored with glass ionomer cement. The samples were immediately processed in laboratory and after incubation the number of viable CFU was obtained by visual counting.

Results: The results of the control groups were compared to those of test groups and demonstrated that PDT, in conditions used in this experiment, was not effective on carious lesions, regardless of the depth cavity evaluated.

Conclusions: Clinical problems as the reduced size from the laser's tips and the penetration of the photosensitizer through dentin may have contributed for the results. Other studies are needed to establish an effective clinical protocol for this therapy.

Keywords: Photodynamic therapy, carious lesions, in vivo, methylene blue, laser

Introduction

Dental caries is a major oral health problem that affects 60-90% of school children and the vast majority of adults. Etiologically, it represents complex interactions among the oral microbiota, diet, dentition, and the oral environment. Bacteria are crucial for the initiation and progression of carious lesions. They function as a microbial community called dental plaque, which reflects a typical example of a microbial biofilm[1].

The prevention of caries (primary prevention), and the control of disease progression (secondary prevention), focus mainly on mechanical and/or chemical biofilm reduction [1]. Antibacterial agents are widely used in the treatment of oral diseases, but problems of development of bacterial resistance mean that alternative strategies are required to control bacterial plaque biofilm and to treat caries and gingivitis [2].

According to Metcalf et al. [2], photodynamic therapy (PDT) is a promising antibacterial treatment. Upon irradiation with light corresponding to an absorption maximum of a photosensitizer, cytotoxic reactive oxygen species are produced which can cause rapid oxidation of cellular constituents and cell death.

Dental caries may be a disease well suited to photosensitization therapy. Caries is often a localized infection, and so the sensitizer could be applied to the lesion by means of a syringe and the light could then be delivered via an optical fibre [3]. The potential advantage of this over conventional caries treatment would be the ability to kill the bacteria in situ and then restoring the site without the removal of the softened and desmineralized

dentine. This is clinically attractive, as it would reduce the amount of tooth tissue required to be removed [4].

There is a large number of laboratory studies evaluating the PDT in different bacterial strains, serving as an indication of the effectiveness of this therapy. Wood et al.[5] evaluated the efficacy of PDT mediated by erythrosine in in-vitro biofilms of *Streptococcus mutans*, irradiated for 15min with a tungsten filament lamp. The PDT has proven to be effective in promoting the death of *S. mutans*, revealing a great potential of this therapy in the treatment of dental plaque.

Zanin et al. [3] investigated the antimicrobial effect of toluidine blue-O (TBO) associated with a red light emitting diode (LED) on the viabilities of in-vitro biofilms in enamel slabs. Neither irradiation of the biofilms in the absence of TBO, nor incubation with TBO alone, had significant effects on the viabilities of the streptococcal biofilms. The reduction in viability was of 95% for *S. mutans* and *S. sobrinus* biofilms, and of 99.9% for *S. sanguinis* biofilms.

Williams et al [6] determined the susceptibility to PDT of *S. mutans* when the organism was embedded in a collagen matrix and also evaluated the susceptibility to PDT of bacteria present in extracted carious human teeth. There was a reduction of 81% in number of viable microorganisms after 60s of irradiation using a red laser combined with TBO at 10µg/ml.

Since numerous in vitro studies have shown the success of photodynamic therapy against cariogenic microorganisms, the aim of this study was to evaluate the effect of PDT on carious lesion in vivo, testing this procedure as an alternative to the treatment of current caries, in order to confirm the results found in laboratory.

Material and Methods

Patients

Eight children of both sexes, aging from three to nine were selected, having molars with deep active carious lesions (diagnosed according to criteria of Hojo et al [7]), and without signals of pulpal or periodontal disease. The selected patients were children with no systemic disease and behavioral adaptation to receive restorative treatment.

Teeth with proximal carious lesions, pulpal involvement and insufficient clinical crown to allow restorative treatment or with physiological mobility were excluded. The patient's selection were made according to criteria described by Massara *et al*.[8]

Pretreatment radiographies were used to classify lesion depth and exclude pulpal and apical pathology. In all ten teeth that comprised the sample, lesion depth was more than 2/3 of the entire dentine thickness, involving only occlusal surface.

Parents were told about the objectives, importance and relevance of the study, as well as the procedures to be carried out on their children. Written authorized consents were obtained for the participation of the children. The study had the approval of the Ethics Committee of the Federal University of Minas Gerais (protocol 501/06 emitted in March 28th, 2007).

Clinical procedures

After rubber dam isolation of the tooth, the asepsis of the operative field was made with 70% alcohol for one minute. For each tooth, five portions of carious dentin were removed with a sterile regular dentin spoon n^{o} 17. For each increment, one different spoon was used. Two increments were

used as control and were removed from different depths, one to represent the infected superficial dentin and the other to represent the affected disorganized deep dentin. The other three increments were removed after the application of PDT.

The cavity was divided in the middle. Then, a portion of softened dentin, which is little resistant to instrumentation was removed (control group – superficial dentin: SC). A second portion was removed when the dentin became more resistant to the instrumentation, getting out in scales or flakes (control group- deep dentin: DC). Both portions corresponded to the right half of the cavity, being used as control groups.

At this moment, the photosensitizer methylene blue at 25 µg/ml (Chimiolux, Aptivalux, Brazil) was placed in contact with the cavity and left for 5 min (pre-irradiation time). Next, the tooth was irradiated by TWIN FLEX II® laser (MM Optics Ltda, São Carlos, Brazil) (wavelength of 660 nm and 40 mW of power) for one minute, which resulted in a energy density of 60 J/cm^2. After the application of PDT, a portion of deep dentin was removed from the right half of the cavity having being directly irradiated (PDT group- deep dentin directly irradiated: DD-PDT). Other two portions, corresponding to the left half of the cavity were also removed, representing the superficial dentin after PDT (group SPDT) and deep dentin after PDT (group DPDT). The absorption spectra of methylene blue and emission spectra of red laser were measured to attest if they were resonant.

For the restorative treatment of cavities the technique of modified ART was used[8] using a glass-ionomer cement, Ketac Fil Plus ® (3M Espe, USA), which was handled according to the manufacturer's instructions.

The five samples of dentine removed were immediately placed in eppendorfs containing 1 ml of sterile distilled water, vortexed (3 x 15 s)

and diluted up to 10^{-2}. Aliquots of 50 µl were plated in triplicate on BHI agar, to obtain the total viable colonies, mitis salivarius agar, selective for *Streptococcus* and Rogosa agar, selective for *Lactobacillus*. After incubation in a candel jar, in a microaerophilic atmosphere at 37°C for 48 h, the number of viable cfu was obtained by visual counting. Means and standard deviations were calculated for each group and logarithmic transformations were made to standardize the data before submitting them to statistical analysis (Wilcoxon test).

Results

Fig. 1 shows the characteristic absorption spectra of methylene blue and the MB structure and Fig. 2 shows the emission spectra of the red laser used in this experiment.

The results of the control groups were compared to the test groups as follows: SC with SPDT and DC with DDTPD and DPDT, considering the dentin depth.

The results showed that PDT, using methylene blue combined with a red laser was not effective on carious lesions, regardless of the depth cavity evaluated. In relation to the number of viable cfu available in BHI agar, the mean reduction in $\log_{10}$ (log kill) was 0.6 ± 0.5 for the superficial dentin (SC-SPDT), 0.5 ± 1.1 to deep dentin directly irradiated (DC-DDPDT) and 0.8 ± 0.7 (DC-DPDT) for deep dentin below the superficial dentin. The log kill for streptococci (mitis salivarius agar) was 0.9 ± 0.6, 0.6 ± 0.9 and 0.8 ± 0.4 and for lactobacilli (rogosa agar) was 0.3 ± 0.2, 0.4 ± 0.6 and 0.5 ± 0.4 respectively.

Although the statistical analyses (Wilcoxon test, $p<0,05$) has shown statistically significance differences between the groups, PDT used in this experiment could not be considered a antimicrobial treatment, because

according to Concannon et al. [9], the bactericidal activity of a material could be expressed as the 99% effective dose (ED99), which is the concentration of an antimicrobial at which there is a 2-log or more reduction in cfu.

Discussion

Photodynamic killing of bacteria utilizes light in combination with a photosensitizer to induce a phototoxic reaction, identical to the use of photodynamic therapy for skin cancer [10].

Previous work has shown that PDT is capable of killing oral bacteria in planktonic cultures, in plaque scrapings, and on mono-species biofilms in vitro [1-3,5,11,12]. Experimental laboratory conditions do not reproduce those found in vivo. Most in vitro studies use standard strains of isolated bacteria, not reproducing the bacterial ecological diversity in the microenvironment of a carious lesion. In vivo, different species interact surrounded by an extracellular matrix protein. Moreover, the photosensitizer's behavior regarding penetration and diffusion is different in laboratory and clinical conditions.

According to de Souza [13], a great number of variables may influence the number of microorganisms affected by PDT including: type and concentration of the photosensitizer, microorganism's physiologic stage, photosensitizer incubation period before the irradiation, exposure period and density of laser energy.

In this study, methylene blue was used in combination with a red laser because in a previous in vitro study this association demonstrated better results [14]. According to Ivanov *et al.* [15] the concentration of photosensitizers as MB and TBO could not be higher than 0,1% because

above this concentration they demonstrate toxicity to cells and could stain the dentine, making the treatment esthetically unviable.

The pre-irradiation time and exposure time were smaller than in vitro studies [1-3,5,11,12] , to achieve clinically acceptable treatment times. We used a low power red laser, a light source commonly used in dentistry. According to Walsh [16], the PDT can be undertaken with a range of visible red and near infrared lasers, and systems using low power visible red semiconductor diode lasers.

The laser shows specific features which make it different from other light sources. Some of these features are the monocromaticity and the coherence, characterized by the emission of photons with only one color and in the same wavelength, besides the unidirectionality, defined by the capacity of the light to propagate in only one direction [17]. Even though in vitro studies have shown the efficacy of PDT by using the red laser as the light source and pointing such characteristics above mentioned as advantages of the laser in relation to the other light sources, some problems were observed clinically.

According to the manufacturer (MM Optics®, São Carlos, SP- Brazil) the area of the laser beam at the end of the pen used in this experiment is of 4 mm^2, which limited the irradiated area. Of course this fact contributed to our findings, since the laser beam was smaller than the cavities and, therefore, unable to irradiate the whole area covered by the photosensitizer. Due to its straight characteristic, the laser beam was applied only on one section of the carious lesion. The emission spectra band of the red laser is very short when compared to the absorption spectra of the photosensitizer, as could be seen in Figs 1 and 2. This certainly could be contributed for the poor results found in this experiment.

Another variable which should be considered is the effect of the laser light on varying thicknesses of partially demineralized dentine, the structure which will reduce and dissipate the laser light [18].

Zanin et al. [3] related that the photosensitization process in biofilms containing high concentrations of extracellular polysaccharide in vitro may affect penetration of the photosensitiser, as well as reduce the quantity of light reaching the bacteria, thereby decreasing the effectiveness of the photosensitizing process.

Williams et al. [6] showed that contact between bacteria and TBO solution is a critical factor and sufficient time must be allowed for the solution to permeate the structure in which the bacteria are situated.

Burns, Wilson and Pearson [4] showed that dentine reduced the quantity of light reaching the bacteria, but increased energies densities applied to the sensitized bacteria could overcome the light-masking effect of the dentine. One alternative to increasing the exposure time to overcome the masking by the dentine would be to use a more powerful laser.

The present in vivo study failed to demonstrate any effective reduction of microbiota in carious lesions by photodynamic therapy using methylene blue associated with a red laser after one single application of 60 s. In carious tissue bacteria will be protected by the dentinal structure which may also limit the penetration of photosensitizer. Bacterial penetration of the dentine may also vary, depending on the species [6].

Despite this fact, the idea of eliminating bacteria in situ seems to be a promising alternative to the usual treatment of caries. Infected or damaged dentine could be better preserved, thereby making patient treatment easier (for both dentist and patient) by enabling lesions to be restored with minimal tissue removal, and improving the long-term

prognosis for the repaired tooth [3]. The use of photodynamic therapy against microbial pathogens in situ needs a thorough knowledge of the light-absorption characteristics of the proposed photosensitizer and of its target environment. Other studies are, therefore, needed to establish an effective clinical protocol for this therapy.

Based on the obtained data we can conclude that the PDT, in conditions used in this work, was not effective to reduce the number of viable microorganisms present in carious lesions. Clinical problems as the reduced size of the laser tips and the penetration of the photosensitizer through dentin may have contributed for the results. Other studies are needed to establish an effective clinical protocol for this therapy.

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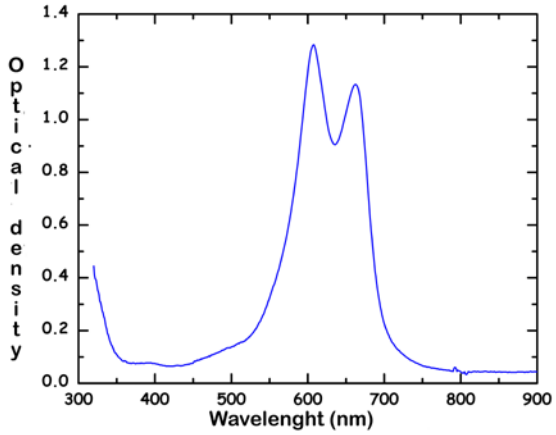
Figures:



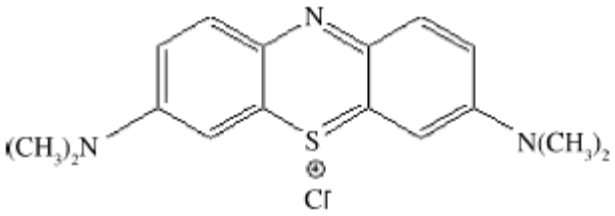


Figure 1

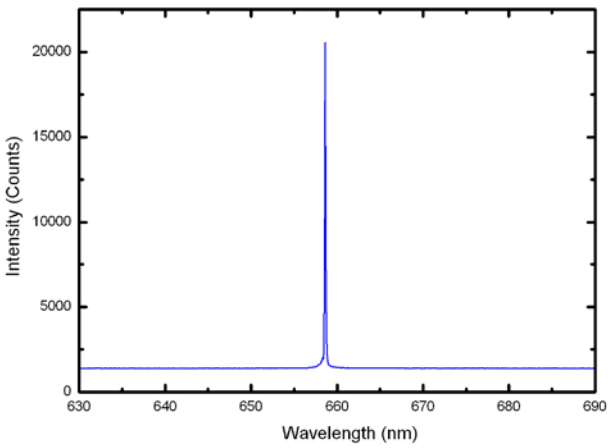

Intensity (Counts)
20000
15000
10000
5000
0
630
640
650
660
670
680
690
Wavelength (nm)

Figure 2

Captions:

Fig.1- Absorption spectra of methylene blue and methylene blue structure.

Fig.2- Emission spectra of red laser (Twin Flex II® MM Optics, Sao Paulo, Brazil).

Artigo 4

ANTIMICROBIAL EFFECT OF PDT IN CARIOUS LESIONS *IN VIVO*, USING CULTURE AND REAL TIME PCR METHODS

Abstract:

The aim of this study was to evaluate the antimicrobial effect of photodynamic therapy (PDT) in carious lesions *in vivo* by culture and real-time PCR methods. Ten teeth with deep active carious lesions were selected and five portions of carious dentin were removed for each tooth. Two increments were used as control, to represent the superficial and deep dentin, respectively. Methylene blue at 25 mg/L was placed in contact with the cavity for five minutes, before being irradiated with a halogen light source for one minute. Then, after PDT, other three portions were removed. The samples were processed in laboratory and the number of viable cfu was obtained. The real time PCR analyses were performed in two increments of carious dentin, removed before and after PDT. The *Streptococcus mutans* DNA was isolated from carious dentin samples and amplification and detection of DNA were performed with Real-Time PCR. The cavities were then restored with glass ionomer cement. Using conventional culture methods, the results demonstrated that viable bacteria were significantly reduced in all of the agar plates following photosensitization. No difference was found between both groups regarding *S. mutans* DNA quantification by real-time PCR. Although PDT may not affect the number of *S. mutans* DNA copies immediately after the treatment, clear reduction of the number of cfu was found. Despite of its promising use for eliminating bacteria in dental caries treatment, further studies are necessary to establish an effective clinical protocol for the PDT.

Key words: photodynamic therapy, quantitative PCR, caries, *Streptococcus mutans*.

Introduction

Although subsequently used for a variety of different applications, the concept of photodynamic therapy (PDT) was initiated in 1900 when Raab [Kessel, 1900] described the antimicrobial action of acridine and light on *Paramecium* species. Descriptions in the literature of the use of dye and light combinations to kill bacteria date back more than a century ago, even though these treatments have not been comprehensively investigated in animal studies or in clinical trials [O'riordan et al., 2005].

Photosensitization mechanisms are initiated by the absorption of light by a given photosensitizer. After absorption of light, part of the energy is transferred to the triplet state in the photosensitizer molecule. Either charge (type I reaction) or energy (type II reaction) is transferred to a substrate or to molecular oxygen to generate reactive oxygen species. In photodynamic action, singlet oxygen ($^{-1}O_2$) is considered to play a major role. This highly reactive oxygen initiates further oxidative reactions in the proximate environment, such as the bacterial cell wall, lipid membranes, enzymes, or nucleic acids [Maisch et al., 2007].

According to Konopla and Glosklinski [2007], pre-clinical work has shown that photosensitizers are more toxic against microbial species than against mammalian cells, and that the illumination-based toxicity occurs much earlier in prokaryotic than in eukaryotic cells. PDT appears to be more efficient for treatment of localized and superficial infections, like oral infections such as caries [Burns et al., 1994], periodontal diseases [komeric et al., 2003] and root canal infections [Martin, 1991].

The advantages of photodynamic therapy over conventional antimicrobial agents are, among others, the rapid killing of target organism depending mainly on the light energy dose delivered and therefore the power output of the light source used. Finally, antimicrobial effects can be confined to

the site of the lesion by careful topical application of photosensitizer and the area of irradiation can be restricted further by using an optical fibre [Zanin et al., 2005].

The use of PDT in dental research has received considerable investigation. The organisms in supragingival plaque, considered to be the main etiological agents of caries, include predominantly Gram-positive species such as *Streptococcus mutans*, *Streptococcus sobrinus*, various lactobacilli and *Actinomyces viscosus* [Wilson, 2004]. These species do appear to be susceptible to various PDT treatments in vitro [Wilson, 2004], in human dental plaque samples [Wood et al., 1999], in a collagen matrix that resembled demineralized dentine [Burns et al., 1995], and in extracted carious teeth [Williams et al., 2004].

Although PDT has previously been used to kill pathogenic microorganisms *in vitro*, only few studies have reported its use to treat infections in animal models or patients [Hamblin and Hasan, 2004]. Despite the positive results found with PDT *in vitro*, further *in vivo* studies should be carried out in order to confirm its efficacy, and to make practical its use in clinical practice. Thus, the purpose of this study was to evaluate the antimicrobial effect of photodynamic therapy using a halogen light in association with methylene blue in carious lesions *in vivo* by culture and real-time PCR methods.

Material and Methods

Photosensitizers and light sources

Methylene blue (MB) at 25 mg/L (Chimiolux®, Aptivalux, Brazil) was used as photosensitizer. The absorption spectrum was measured by a spectrophotometer.

The light source used was a halogen light curing unit (Curing Light 3M Espe®, 3M Espe, USA), without the wavelength filter, emitting a white light with a large emission spectra band. The light emission pattern of the light source was evaluated, measuring the emission spectra with a spectrometer and the power output with an optical power meter.

The photosensitizer was put in contact with the teeth for five minutes (pre-irradiation time) before irradiation. Pre-irradiation time is important to help achieve PDT's antibacterial effect, as it helps to keep the photosensitizer inside the bacteria, allowing more light absorption [Bevilacqua et al., 2007]. The teeth were then irradiated for one minute.

Patients

This study was approved by the Ethics Committee of the Federal University of Minas Gerais (protocol 501/06 acrescentar numero SISNEP). Parents were told about the objectives, importance and relevance of the study, as well as the procedures to be carried out on their children and written authorized consents were obtained for the participation of the children. Ten molars with deep active carious lesions (diagnosed according to the criteria of Hojo et al. [1994]) were selected from children of both genders aged between three years and nine years who presented behavioral adaptation to receive restorative treatment and no systemic disease. Teeth with proximal carious lesions, with signals of pulpal or periodontal disease and insufficient clinical crown to allow restorative treatment or with physiological mobility were excluded. Pretreatment radiographies were used to classify lesion depth and exclude pulpal and apical pathology. In all ten teeth that comprised the sample, lesion depth was more than 2/3 of the entire dentine thickness, involving only occlusal surface [Massara et al., 2002].

Clinical procedures

The clinical procedures were performed according to Araujo et al. [2009, unpublished results][1]. The asepsis of the operative field was made with 70% alcohol for one minute after rubber dam isolation of the tooth. For each tooth, five portions of carious dentin were removed with a sterile regular dentin spoon n^{o} 17. For each increment, one different spoon was used. Two increments were used as control and were removed from different depths, one to represent the infected superficial dentin (control group –superficial dentin: SC) and the other to represent the disorganized deep dentin (control group- deep dentin: DC). Both portions corresponded to the right half of the cavity. The other three increments were removed after the application of PDT (see Figure 1).

Then, MB blue at 25 mg/L was placed in contact with the cavity and left for 5min in dark (pre-irradiation time). Next, the tooth was irradiated by halogen light curing unit for one minute, which resulted in an energy density of 31 J/cm^{2}. In order to dissipate the heat generated by the light source, the light dose was fractionated with an interval of 20 s between two applications of 30 s. After the application of PDT, a portion of deep dentin was removed from the right half of the cavity having being directly irradiated (PDT group- deep dentin directly irradiated: DD-PDT). Other two portions, corresponding to the left half of the cavity were also removed, representing the superficial dentin after PDT (group SPDT) and deep dentin after PDT (group DPDT).

The cavities were restored with a glass-ionomer cement, Ketac Fil Plus ® (3M Espe, USA), which was handled according to the manufacturer's instructions. For the restorative treatment of cavities the technique of modified ART (atraumatic restorative treatment) was used, as described by Massara et al. [2002].

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Microbiological Analyses

The five samples of dentine removed were immediately placed in eppendorfs containing 1ml of sterile distilled water, vortexed (3 x 15 s) and diluted up to 10^{-2}. Aliquots of 50 µl were plated in triplicate on brain heart infusion agar (BHI), to obtain the total viable colonies, *mitis salivarius* agar, selective for *Streptococcus* and Rogosa agar, selective for *Lactobacillus*. After incubation in a candel jar, in a microaerophilic atmosphere at 37°C for 48 h, the number of viable cfu was obtained by visual counting.

Real time PCR

Using sterile regular dentin spoon n° 17, two increments of the superficial carious dentine were collected before (control PCR) and after the PDT (PDT PCR). The increments were immediately placed in eppendorfs and stored at -80°C until used. The real time PCR analyses were performed in dentin samples of six patients.

DNA was isolated from carious dentin samples by using the QIAamp DNA Blood Mini Kit (Quiagen, Valencia, CA, USA), according to the manufacturer's instructions. Once isolated, the extracted DNA was stored at -80°C until used. The nucleic acid concentration was determined spectrophotometrically.

Genomic DNA from *S. mutans* ATCC 25175, used to build the standard curve, was isolated and purified from an overnight culture of (BHI) as described previously [Bowden et al., 1993].

Primers specific for the *S. mutans* were designed from regions of identity within the 16S ribosomal DNA (rDNA) sequence as reported by Kozarov et al. [2006]. A quantification standard curve was achieved by using eight 10-fold serial dilutions of 16S ribosomal DNA sequence included in each run. The concentrations of the dilutions ranged of 10^3 to 10^{10} copies/well.

The standard curve and test samples were assayed in duplicate, with the final data presented as number of copies/μl and *S. mutans* DNA copies / 100 ng of DNA, respectively. The negative control contained reagents only.

Quantitative real time PCRs were performed in a reaction volume of 25 μl containing SYBR Green PCR Core Reagents (PE Applied Biosystems, Warrington, UK). Each reaction mixture contained 5 μM dATP, 5 μM dCTP, 5 μM dGTP, and 10 μM dUTP, 2.5 μl of 10X SYBR Green PCR Buffer, 6,25 μM $MgCl_2$ and 0,5U AmpliTaq Gold DNA Polymerase, 5 μM of each *S. mutans* primer and 100 ng of the extracted nucleic acid and then distilled water was added to a total volume of 25 μl. Amplification and detection of DNA were performed with the Step One™ Real-Time PCR System (Applied Biosystems, UK) with optical grade 48-well PCR plates and optical caps. The reaction conditions were 94°C for 10min, followed by 40 cycles of 94°C for 15 s and 60°C for one minute [Kozarov et al., 2006].

Statistical analyses

For culture methods, means and standard deviations for the number of cfus were calculated for each group and logarithmic transformations were made to standardize the data before submitting them to statistical analysis (Wilcoxon test, $p<0,05$).

For real time PCR results, a parametric Student t test was used. Statistical analyses were performed using SPSS 12.1 software (SPSS Inc., Chicago, IL) and p value ≤ 0.05 were considered statistically significant.

Results

Photosensitizers and light source

Figure 2 shows the absorption spectra of methylene blue and the emission spectra of the light source used in this experiment. The power output of

the halogen light source was 260 mW. The MB presented an absorption band in the range of 550-700 nm, which is coincident with the emission spectra of halogen light source (500-800 nm), suggesting that it can be used to photoactivate the dye.

Microbiological analyses

The results of the control groups were compared to the test groups as follows: untreated superficial dentin versus PDT-treated superficial dentin and untreated deep dentin versus PDT-treated deep dentin directly irradiated and versus PDT-treated deep dentin not directly irradiated.

The log kill was obtained by the $\log_{10}$ of the control group minus the $\log_{10}$ of the test groups. According to Concannon et al. [2003], the bactericidal activity of an agent could be defined as a reduction in viable bacteria of at least 2 $\log_{10}$ cfu/ml. In the BHI agar, PDT using MB and one minute irradiation with a halogen light resulted in mean $\log_{10}$ reductions in cfu of between 2.5 ± 0.6 (for the superficial dentin), 1,9 ± 0.9 (for deep dentin directly irradiated), and 2,3 ± 0,8 (for deep not directly irradiated). In relation to the number of viable cfu available in *mitis salivarius* agar (selective for streptococci) the reduction in $\log_{10}$ (log kill) was 2,4 ± 0,8, 2,2 ± 0,9 and 2,2 ± 0,9 and for Rogosa agar (selective for lactobacilli) was 2,5 ± 0,7, 2,1 ± 1 and 2,0 ± 0,9 respectively. The results obtained with culture method could be seen in Fig 3.

The $\log_{10}$ found in the superficial dentin as well as in the deep directly irradiated and not directly irradiated dentin was statistically different from the control $\log_{10}$. The statistical analyses showed differences between the control groups and the test groups, regardless of the depth cavity and of the agar evaluated, supporting the efficacy of the therapy.

Real time PCR

A S. mutans standard curve was tested with expected values from 10^3 to 10^{10} copies/ml. The melt curve of the reaction is illustrated in Fig.4.

The real-time PCR analyses showed that *S. mutans* DNA was found in all carious dentine samples, and there was no significant difference between the groups evaluated (p= 0,636). The mean for control group were 1.48 ± 1.03 x 10^9copies/ml and for PDT group using halogen lamp were 2.36 ± 1.47 x 10^9copies/ml. These results were demonstrated in Fig.5.

Discussion

Dental caries continues to be a significant public health problem in many parts of the world. Although the bacteria responsible for caries initiation and early caries progression have been studied extensively, the microbiology of dentine caries has been reported to show considerable diversity and has not yet been fully characterized. Dissolution by acid of the surface enamel exposes the underlying avascular mineralized connective tissue matrix of dentine, which is prone to invasion. This occurs by migration of bacteria into the network of tubules occupied by processes of the pulpal odontoblasts. There is evidence that interspecies cooperation enhances the migration of the mixed bacterial flora through the dentinal tubules [Byun et al., 2004].

Lethal photosensitisation of a wide range of bacteria responsible for caries [Burns et al., 1994], periodontal diseases [komeric et al., 2003] and root canal infections [Martin, 1991] has been demonstrated. With the recognition that each of these diseases is associated with a specific organism, or group of organisms, increasing interest is being shown in the use of antimicrobial agents to supplement these rather crude mechanical procedures. In all of the above-mentioned oral infections, the topical application of antimicrobial agents is an important part of disease prevention and/or treatment. PDT could provide an alternative to such

agents as a means of killing bacteria in these situations, as access of the photosensitiser and light to the disease lesion presents no great difficulty in any of the three diseases [Wilson, 2004].

The advantages of this approach are that bacteria can be eradicated in very short periods of time (seconds or minutes), resistance development in the target bacteria is unlikely and damage to adjacent host tissues and disruption of the normal microflora can be avoided [Wilson, 2004].

Although some *in vitro* studies simulated the effect of antimicrobial PDT on caries lesions using demineralised slices of dentine [Burns et al., 1995] bacteria embedded in a collagen matrix [Burns et al., 1995; Williams et al., 2004], bovine enamel [Zanin et al., 2006] and *ex vivo* carious dentine [Williams et al., 2004] the results from these studies need to be confirmed by *in vivo* assays.

Using conventional culture methods, our results demonstrated that viable bacteria were significantly reduced in all of in the agar plates following photosensitization using a halogen light in combination with MB. Once a similar log reduction was observed, regardless of the culture means used, we believe that PDT was able to affect different species of microorganisms. The lack of both collimation and coherence of halogen light sources, which result in wider bands of emission, provide light emission throughout the entire absorption spectrum of photosensitizer, which may promote optimisation of photodynamic processes. The relatively non–specific bactericidal effect of a light source in combination with a photosensitizer is of clinical significance since the causative microorganisms of carious lesions are invariably unknown. Targeting of specific bacterial strains, while elegant, could in the case of caries lesions be less satisfactory and may result in limited effect clinically [Burns et al., 1994].

According to Paulino et al. [2005], some studies clearly indicate that the use of the visible-light isolated from a hand held photopolimerizer (HHP) unit does not induce any thermal or photochemical damage to the retina. In addition to that, the cost of use of HHP is lower than that of a laser light. Also, with the use of a halogen light, it is possible to have an efficient bactericide treatment, especially if we consider the applicability of therapy to a bigger variety of photosensitive dyes, due to the broad spectra emission band.

Although the conventional culture method is considered to be the 'gold standard' for detecting the antimicrobial effect of PDT, polymerase chain reaction (PCR) offers a more highly sensitive and specific detection method for bacteria than conventional culture methods. Moreover, molecular techniques have the potential to produce a reliable means of quantifying bacterial DNA and therefore bacterial numbers [Hata et al., 2006].

When the real-time PCR results were compared with data previously obtained by culture methods, it could be observed that *S. mutans* DNA was found in all carious dentin samples evaluated, for both control and test groups, opposing preliminary results found by culture method. Chen et al. [2007] reported, in a previous work, that 64.3% of the children who were *S. mutans* negative by the culture method were *S. mutans* positive by real-time PCR, which could confirm the higher sensitivity for the real time PCR method.

The fact that we found different results comparing the data obtained with culture method and real time PCR method, leads us to believe that more studies are needed to be performed concerning the clinical application of PDT in dentistry. One important caveat in our study is about the real importance of *S. mutans* in the progression of deep dental caries. As we have not investigated other bacteria by real time PCR, we can not rule out

that the effects of PDT over other relevant microorganisms. The idea of eliminating bacteria in situ seems to be a promising alternative to the usual treatment of caries, but further studies are necessary to establish, among other things, the real mechanism of action of PDT, in an attempt to explain the results described above.

According to Hamblin and Hasan [2004], there are two basic mechanisms that have been proposed to account for the lethal damage caused to bacteria by PDT: (a) DNA damage and (b) damage to the cytoplasmic membrane, allowing leakage of cellular contents or inactivation of membrane transport system and enzymes. According to Wood et al. [2006], *S. mutans* is photoinactivated mainly by membrane damage due to lipid peroxidation. This could be an explanation for the distinct results encountered by the two methods evaluated. Another point to be assessed would be the time required to degrade DNA. As the samples were collected immediately after the application of PDT, there could not have given sufficient time to occur any damage to nucleic acids.

As it can be seen, all the possible explanations above are assumptions that need specific answers before PDT clinical use in dentistry. More studies need to be made to bring new information on the application of PDT in the treatment of caries disease in order to improve the currently used conventional treatment, enabling one more way to avoid the cavity to be restored to be at a risk of suffering further recurrence of caries through reducing the number of viable microorganisms in the restoration.

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Figures and Legends

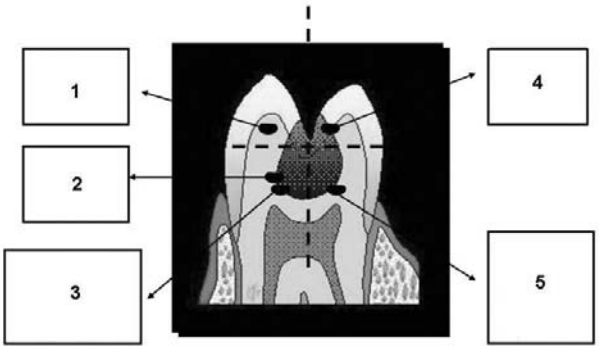

Figure 1: Schematic view of the dentin samples removed during the clinical procedures. The increments 1 and 2 were used as control, representing the untreated superficial and untreated deep dentin respectively. The increments 3, 4 and 5 were removed after the PDT (3- PDT-treated deep dentin directly irradiated, 4- PDT-treated superficial dentin and 5- PDT-treated deep dentin not directly irradiated).

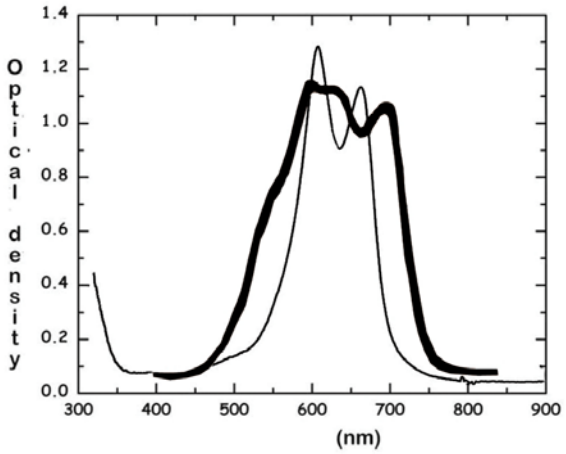

Figure 2 - Absorption spectra of MB (thin line) and emission spectra of the halogen light source used (thick line), showing that they are resonant.

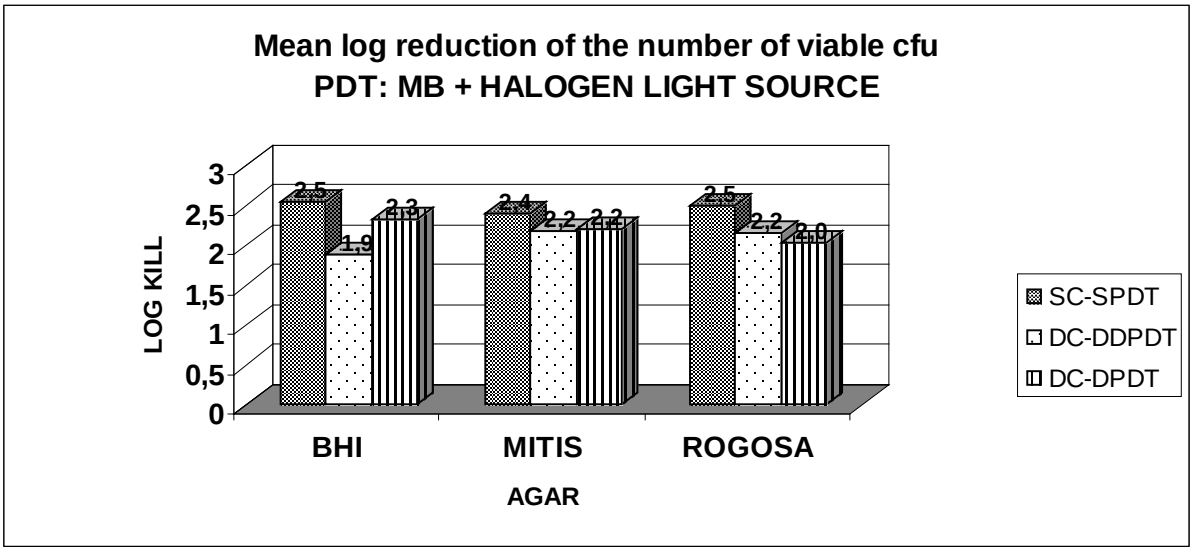

Figure 3 – Mean log reductions obtained with the culture method. The control group were compared to the test groups as follows: untreated superficial dentin minus PDT-treated superficial dentin (SC-SPDT); untreated deep dentin minus PDT-treated deep dentin directly irradiated (DC-DDPDT) and minus PDT-treated deep dentin not directly irradiated (DC-DPDT).

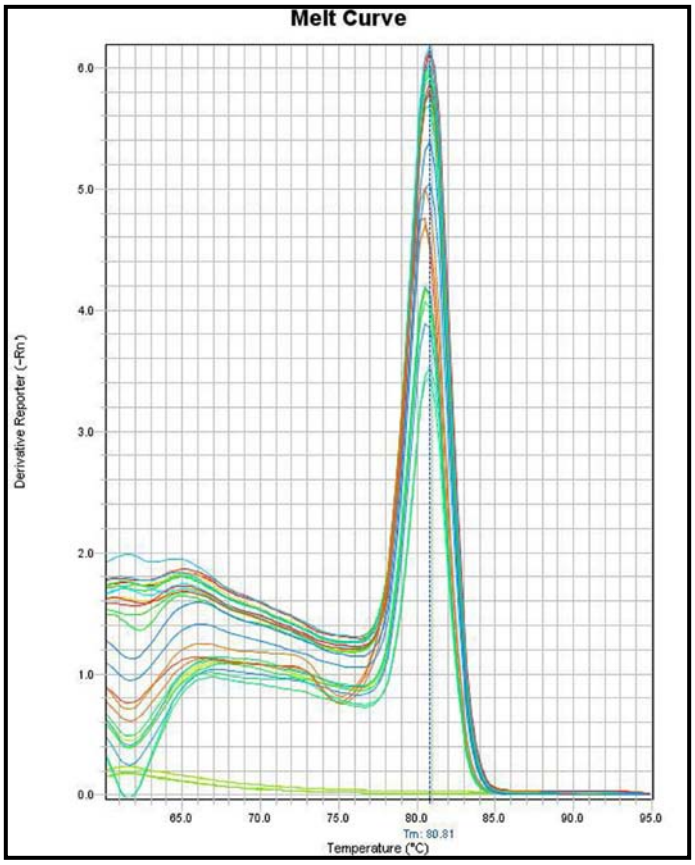

Figure 4. The melt curve of real time PCR reaction, showing the specificity of the reaction.

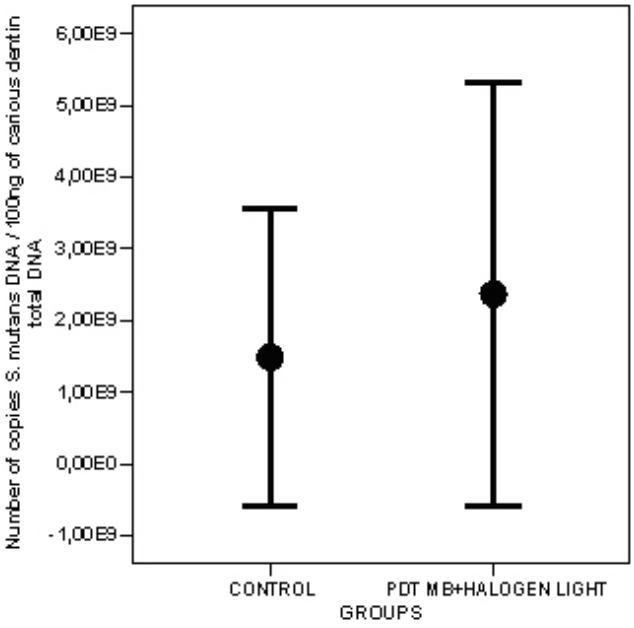

Figure 5. Real time PCR analyses showing the number of *S. mutans* DNA copies / 100ng of carious dentin total DNA, comparing the control group and the PDT group.

5 CONSIDERAÇÕES FINAIS

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A discussão apresentada em cada artigo separadamente já representa o pensamento do conhecimento adquirido. Entretanto, inúmeras questões emergem deste estágio de compreensão do mecanismo de redução bacteriana e da ecologia resultante das lesões seladas após a aplicação desta terapia. Seguindo-se a mesma formatação de apresentações *in vitro* e *in vivo*, iniciar-se-á estas considerações pelos trabalhos *in vitro*.

Sabe-se que a concentração dos agentes fotossensibilizantes interfere na efetividade da PDT, não devendo, entretanto, ser superior a 0,1% devido à toxicidade celular ou mesmo a uma pigmentação inadequada da dentina para utilizações *in vivo* (IVANONV *et al*, 2000). Porém, continua inexplicada a redução demonstrada nas concentrações de 5, 10 e 25 mg/L. É sabido que a efetividade da PDT é dose dependente e, desta forma, resultados semelhantes para TBO e MB seriam esperados. No entanto, isto não ocorreu. O tempo de fotossensibilização foi o mesmo para todas as concentrações. Portanto, continua o questionamento se poderia haver modificação nos resultados se o tempo de fotossensibilização também variasse conforme a concentração. Estes resultados também levam a questionamentos sobre o mecanismo de ação de morte celular.

Na impossibilidade de se conduzir vários ensaios ao mesmo tempo, apenas optou-se pela utilização da concentração de 25 mg/L para a luz halógena, pela sua maior efetividade dentro de um tempo considerado aceitável para ensaios clínicos. Justifica-se este procedimento pela utilização da concentração inibitória mínima observada. Todavia, estes questionamentos deverão ser mais bem compreendidos em pesquisas futuras. Pode-se, entretanto, testar-se o MB na concentração de 5mg/L. Na medida do possível pode-se testar o MB com outros tempos de fotossensibilização. Acredita-se, entretanto, que para aplicações clínicas

restauradoras, tempos maiores de fotossensibilização inviabilizariam sua utilização como procedimento usual.

Questionamento importante a ser realizado é em relação ao tipo de luz empregada. A literatura converge seus ensaios utilizando-se fontes de laser e LEDs. Por que não a luz halógena? Pode-se entender perfeitamente a necessidade de ressonância entre a fonte de luz e o agente fotossensibilizante e o tempo necessário para a interiorização do fotossensibilizante intracelularmente. Estudos anteriores têm trabalhado com a ressonância do laser e LED com o azul de metileno (SOUSA, 2007; ZANIN *et al*, 2006; ZANIN *et al*, 2005).

De forma ideal, dever-se-ía desenvolver um fotossensibilizante específico para a o comprimento de onda da luz halógena. Ainda, buscando-se o ideal, que este corante seja incolor para evitar-se, totalmente, qualquer pigmentação indesejada aos tecidos dentais. Isto ainda não foi conseguido. Nos experimentos realizados, testou-se o fotoativador sem o filtro de comprimento de onda mantendo-se, entretanto, o filtro para radiações infravermelho. Os resultados obtidos para esta ressonância, como demonstrados no quarto artigo, ofereceram perspectivas bastante favoráveis para que os testes pudessem ser realizados. Apesar do largo espectro de emissão deste tipo de fonte de luz, este teste socializa o emprego desta técnica, pois todo profissional já dispõe deste tipo de equipamento em seus consultórios.

Observando-se o gráfico da página 100, notam-se dois picos do espectro de emissão da luz halógena por volta de 550 nm e 730 nm. Sua área engloba quase a totalidade da área do espectro de absorção do MB. Por outro lado, os espectros de emissão para o laser e LED são restritos ao comprimento de 660 nm. Analisando-se os resultados do trabalho de Tardivo et al. (2005), em que considera-se os picos de ressonância com o tipo de mecanismo de ação, verifica-se que a luz halógena pode oferecer

vantagens que precisam ser melhor investigadas. De acordo com estes autores, dependendo de sua concentração, o MB pode se apresentar como monômero, cujo pico de absorção se dá em 664 nm ou como dímero, absorvendo a 590 nm. Monômeros e dímeros podem se envolver em diferentes tipos de reações fotoquímicas, as quais afetam o mecanismo e a eficiência de morte celular. A possibilidade da utilização das duas reações fotodinâmicas, tipo I e tipo II, ao mesmo tempo, parece interessante.

Os resultados dos testes *in vitro* corroboram este pensamento, com uma redução de 99,5% para a luz halógena. Embora não tenha havido diferenças estatisticamente significantes para as reduções apresentadas, ou seja, 99,5% para a luz halógena e 81,3 % para o laser, a redução em números absolutos é bastante grande reforçando a necessidade de mais estudos para esta fonte de luz.

Deve-se também salientar que a manutenção do filtro de calor não inibiu a sensação térmica desagradável gerada pelo emprego do tempo de irradiação em um minuto. Desta forma, nos testes *in vivo*, resolveu-se por duas irradiações de 30 s com um intervalo de 20 s. Este fracionamento das irradiações pode suprir a oxigenação celular e melhorar a efetividade da resposta conforme Maisch *et al.* (2007) e Metcalf *et al.* (2006). O que pode parecer um efeito limitante, na verdade, pode ser uma das chaves para o sucesso dessa terapia.

Baseado neste achados, desafio maior foi a realização do ensaio *in vivo*. Salienta-se que não há ainda na literatura trabalhos *in vivo* tratando de odontologia restauradora. O estabelecimento de um protocolo clínico deveria garantir a universalidade do atendimento e controle estrito dos pacientes. Baseou-se em ensaios clínicos já realizados na FO-UFMG. Os critérios de inclusão e exclusão foram os utilizados por Massara *et al.* (2002). Entretanto, o preparo asséptico do campo operatório foi realizado

com a aplicação de álcool 70% por um minuto após o isolamento absoluto. Todos os cuidados foram tomados para que não houvesse nenhuma interferência deste preparo de campo com o dente a ser tratado.

A remoção de sete fragmentos de dentina exigiu também cuidados especiais com a utilização de uma colher de dentina n^{o}17 estéril por fragmento. Cinco fragmentos foram utilizados para os testes microbiológicos de cultura e dois para o PCR em tempo real. Esta quantidade de fragmentos gerou pequenos volumes com maior ou menor dificuldade para serem obtidos devido à consistência do tecido cariado. Os fragmentos para PCR em tempo real foram armazenados em freezer a -80^{o}C e os fragmentos para cultura microbiológica foram processados imediatamente após a sua remoção. Todas as cavidades foram seladas com cimento de ionômero de vidro quimicamente ativado Ketac Fill® (3M Espe, USA).

Inicialmente é mister informar que as avaliações clínicas realizadas em todas as crianças até oito meses após o tratamento apresentaram viabilidade clínica e radiográfica de dentes sem sintomatologia e alterações periapicais. Apesar do pouco tempo de avaliação, o resultado sugere a obtenção de sucesso. Todas as crianças ainda estão sendo acompanhadas e o serão até a exfoliação dos dentes submetidos à terapia.

Este aparente sucesso clínico está sendo obtido para as duas fontes de luz, apesar da diferença na redução bacteriana mostrada nos testes laboratoriais dos fragmentos de dentina removidos. Principalmente para as lesões de cárie, talvez a associação de um corante com uma fonte de luz laser, com um espectro de emissão muito reduzido seja muito específica e insuficiente para abranger toda a microbiota presente nas cavidades. Estes achados são corroborados pelos resultados obtidos em

cultura específicos para *lactobacillus* (ágar rogosa) que apresentou baixa redução microbiana.

Também o diâmetro da ponta ativa bem inferior ao diâmetro da cavidade possa explicar a ausência de uniformidade de densidades de energia em toda a extensão da lesão cariosa. Ao contrário, com a luz halógena, o diâmetro da ponta ativa era compatível com a abertura da cavidade de cárie o que pode ter proporcionado distribuição mais equitativa de densidade de energia. Da mesma forma, o amplo espectro pode ter contribuído decisivamente para o resultado obtido de uma redução de 2 log. Pode-se notar na Figura 3 do quarto artigo (página 100) a similaridade de redução obtida para os três meios de cultura.

Não há na literatura consenso sobre a esterilidade de uma cavidade previamente ao seu selamento. O estado da arte nos dá a segurança de que a remoção da dentina infectada e o selamento permitem a remineralização da dentina afetada. Entretanto, a permanência desta dentina mais superficial, infectada, desorganizada e não passível de remineralização impediria a reorganização ou mesmo a remineralização das regiões mais profundas? Resta somente a certeza de muito caminho a se percorrer. Salienta-se que esta é apenas a visão bioquímica do processo.

Os trabalhos de ART e ARTm já apresentam resultados clínicos satisfatórios e estas formas de abordagem para o tratamento da cárie dental tem sido realizadas ao redor do mundo. Seria então a aplicação da PDT apenas um passo a mais?

E os aspectos biomecânicos? A restauração de um dente é devolver a sua forma e função. É possível manter a função oclusal de um dente com esmalte sem sustentação dentinária? Estas questões devem ser respondidas separadamente para a dentição decídua, permanente e

principalmente em relação à dentição mista. Não é este o momento para se fechar as questões, mas dever-se-ia pelo menos adiantar que pensa-se que em relação a cúspides de não contenção estas respostas seriam quase que certamente respondidas como sim, escrevendo-se o sim com letras maiúsculas.

A certeza é que os resultados apresentados utilizando-se cultura microbiológica e PCR devem ser apenas um ponto de partida para novos ensaios sem perder de vista a viabilidade clínica dos procedimentos.

O aparente antagonismo dos resultados apresentados pelo PCR em que não há evidência da redução do DNA bacteriano em relação à cultura só aumenta as perguntas a serem respondidas. Em realidade, não existe possibilidade de se comparar os dois testes e a "verdade" atual leva a considerar-se a cultura como padrão ouro. Em relação aos resultados do PCR em tempo real a afirmativa apresentada no último trabalho de que a PDT não afetou o número de cópias de DNA de *S. mutans* imediatamente após o tratamento é a única possível e verdadeira.

Seria o PCR em tempo real capaz de distinguir bactérias viáveis das bactérias não viáveis, quando o mecanismo de morte celular é conduzido por outra via que não envolve danos aos ácidos nucléicos? Wood *et al.* (1999) avaliaram biofilmes de placas bacterianas em microscopia eletrônica de transmissão e observaram vacuolação e condensação do citoplasma bacteriano, o qual se apresentava evidentemente lesado, acompanhado por uma retração das membranas e paredes celulares. Outros autores também afirmam que os danos causados pela PDT podem ocorrer tanto via ácidos nucléicos quanto via ruptura de membrana e parede celular, afetando a permeabilidade e ocasionando a morte do microorganismo. (WOOD *et al.*, 2006; HAMBLIN e HASAN, 2004).

Como o mecanismo de morte celular induzida pela PDT ainda não foi totalmente esclarecido, assim como o espaço de tempo necessário para a irreversibilidade do processo de morte, protocolos para novas pesquisas específicas para buscar respostas a estes questionamentos estão sendo preparados.

Embora os estudos sobre terapia fotodinâmica no tratamento da cárie e de outras doenças orais ainda estejam no inicio, há fortes indícios de que esta terapia se encaixa perfeitamente na filosofia de tratamento hoje preconizada, pois, a idéia de se eliminar os microorganismos dentro da lesão de cárie, sem remoção de tecido, preservando estrutura dental e propiciando ao organismo condições favoráveis para seu reparo nos parece bastante promissora.

6 REFERÊNCIAS

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7 ANEXOS

ANEXO 1: Normas de publicação das revistas

Artigo 1- *Journal of Laser Applications*

Submission Information: Send manuscripts by e-mail (pdf or MS Word) to:

Anja Selnau
Journal of Laser Applications®
c/o Laser Institute of America
13501 Ingenuity Drive, Ste 128
Orlando, FL 32826
Tel: 407-380-1553
Fax: 407-380-5588
Email: **aselnau@laserinstitute.org**

A cover letter should specify authors, title, Journal, and any special requests. It is strongly preferred to correspond directly with the author rather than through the reports division or through executives of the author's laboratory. Manuscripts returned to authors for revision should be returned within three months. A manuscript returned later than this will generally be regarded as newly submitted and will receive a new receipt date. Authors whose manuscripts have been accepted for publication will receive a notice informing them of the issue for which it is tentatively scheduled. Upon receipt of proof a*ll subsequent correspondence* about the paper should be addressed to: EditorialSupervisor: Journal of Laser Applications®

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Melville, NY 11747-4502
Tel: 516-576-2443
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Two types of manuscripts are acceptable: Full-Length and Communications. The latter are short contributions not exceeding in length three printed pages including allowances for illustrations, references, and tables. Abstracts are required for manuscripts of both types. The Manuscript, including the abstract, references, and captions, should be neatly typed in English, double-spaced, on one side of good letter-size 21.6x28-cm (8-1/2x11-in.) white paper with ample margins. It should be carefully proofread by the author.

The manuscript must be in good scientific American English; this is the authors' responsibility. Unclear or excessive handwritten insertions are not acceptable. *Number all pages in single sequence beginning with the title and abstract page.* Authors should submit clear copies or PDF/MS Word file of the manuscript including original illustrations. The Title should be concise but informative enough to facilitate information retrieval. The Abstract should be self-contained (contain no footnotes). It should be adequate as an index (giving all subjects, major and minor, about which new information is given), and as a summary (giving the conclusions and all results of general interest in the article). It should be about 5% of the length of the article, but less than 500 words for full-length articles and correspondingly shorter for *Communications*.

It must appear on its own sheets separate from the text. Keywords must be provided. "Part I," or simply "I," will not be included as part of the title of an article unless Part II has already been

submitted for publication in this Journal. Part III, IV,..., etc., are likewise unacceptable unless the prior parts have already been accepted or have appeared in this Journal, and are properly identified in the references. Author's **names** should preferably be written in a standard form for all publications to facilitate indexing and avoid ambiguities.

Equations: Equations should be punctuated and aligned to bring out their structure and numbered on the right. Mathematical operation signs indicating continuity of the expression should be placed at the left of the second and succeeding lines. Use x rather than a centered dot, except for scalar products of vectors. The solidus (/) should be used instead of built-up fractions in running text, and in display wherever clarity would not be jeopardized. Use "exp" for complicated exponents.

Notation: Notations must be legible, clear, compact, and consistent with standard usage. All unusual symbols whose identity may not be obvious must be identified the first time they appear, and at all subsequent times when confusion might arise. Superscripts are normally set directly over subscripts; authors should note where readability or the meaning requires a special order.

References and footnotes: References and footnotes are treated alike. They must be numbered consecutively in order of first appearance in the text and should be given in a separate double-spaced list at the end of the text material. Reference should be made to the full list of authors rather than to first author followed by an abbreviation such as *et al*. References within tables should be designated by lowercase Roman letter superscripts and given at the end of the table. For the proper form, see the *AIP Style Manual* and recent issues of this journal. The number of a grant or contract is meaningless to our readers and should be omitted unless its inclusion is required by the agency that supports the research.

Separate Tables: Separate Tables (with Roman numerals in the order of their appearance) should be used for all but the simplest tabular material; they should have captions that make the tables intelligible without references to the text. The structure should be clear, with simple column headings giving all units. Unaltered computer output and notation are generally unacceptable. Long tables should, if possible, be submitted in a form ready for direct photo-reproduction.

Additional Guidelines for Preparation of Electronic Graphics Files

- **Acceptable formats: PostScript** (.ps), **Encapsulated PostScript** (.eps, using either Arial or Times Roman fonts), or **Tagged Image File Format** (.tif, lzw compressed). Application files (e.g., Corel Draw, Microsoft Word) are not acceptable.
- When submitting your manuscript, **submit ALL illustrations** for your paper, including line art.
- Make sure there is **only ONE figure per file**. Each figure file should contain all parts of the figure. For example, if Figure 1 contains three parts (a, b, c), then all parts should be combined in a single file for Figure 1.
- **Set the correct orientation** for each graphics file.
- **Settings:** Set the graphic for **600 dpi** resolution for line art, **264 dpi** for halftones, and **600 dpi** for combinations (line art + halftone).
- **Save line art as black/white bitmap,** not grayscale.
- **Save halftones and combinations as grayscale,** not black/white bitmap.
- **Submit color files at 300 dpi TIFF, PS, or EPS format.** If selecting a file mode, use CMYK (cyan, magenta, yellow, black) or RGB (red, green, blue).

Use this checklist to avoid the most common mechanical errors in submitted manuscripts.

1. The manuscript must be double-spaced throughout.
2. Number all pages in sequence.
3. Type title and abstract on a separate first page.
4. Type list of references (including footnotes), list of figure captions, and tables on pages separate from each other and from the main text.
5. Type references in the style used by AIP journals.
6. Provide marginal notes to clarify symbols and expressions for the compositor.

Artigo 2- *Acta Odontológica Latinoamericana*

(http://www.actaodontologicalat.com/autores.html)
Instrucciones para autores

Modificadas de acuerdo a las recomendaciones del International Committe of Medical Journal Editors (www.icmje.org). Se solicita que el manuscrito, con sus tablas y figuras sean enviados por correo electrónico a **editor@actaodontologicalat.com** con copia a **mitoiz@odon.uba.ar**. Se aconseja a los autores que retengan una copia para mejor control.

Los autores serán responsables de todo lo manifestado en los artículos. Se entiende que el manuscrito no ha sido enviado a otra revista.

Debido a las consideraciones expresadas en la Política Editorial los trabajos se publicarán en inglés. AOL realizará, además de la revisión científica, una revisión técnica e idiomática. Para facilitar la labor de los autores, AOL ofrece un servicio de traducción de los manuscritos. Los autores que deseen utilizarlo, deberán manifestarlo en la carta de envío de su trabajo. La tarifa de este servicio es de 5 dólares cada 100 palabras de traducción completa. En el caso de trabajos enviados en inglés, AOL podrá realizar correcciones idiomáticas, las cuales serán facturadas a los autores según el grado de corrección que los manuscritos requieran. El servicio de traducción/corrección se realizará solo si el trabajo es aceptado para su publicación., después de la revisión científica.

El manuscrito será escrito a doble espacio, con el siguiente ordenamiento: título reducido para los encabezamientos de no más de 40 letras; título completo en inglés; autores, separados por comas indicando el primer nombre completo, iniciales de los demás nombres y apellido, lugar (o lugares) de trabajo (en inglés), indicando con supra-índices los correspondientes a cada autor; abstract de 150 a 300 palabras; key words (no más de seis, que deben figurar en el mesh de PubMed); titulo en castellano o portugués; resumen en castellano o portugués.y palabras clave en castellano o portugues. Si desea, el resumen en castellano o portugués podrá tener mayor extensión (hasta 500 palabras).

El texto deberá contener los siguientes subtítulos: introducción, materiales y métodos, resultados, discusión, agradecimientos, correspondencia (nombre y dirección postal y electrónica del autor a quien deba dirigirse), referencias y leyendas para las figuras.

Las referencias deberán indicarse en el manuscrito con supraíndices y numerarse consecutivamente según el orden de su aparición en el texto. La lista de referencias deberá mantener el formato y puntuación del siguiente ejemplo:

1. Ghiabi M, Gallagher GT, Wong DT. Eosinophils, tissue eosinophilia and eosinophil-derived transforming growth factor alpha in hamster oral carcinogenesis. Cancer Res 1992; 52:389-393.

Deben usarse las abreviaturas de los nombres de las revistas como figuran en MEDLINE/PubMed. En caso de citar resúmenes publicados agregar [Abstract], después del título. En el caso de los libros, deberá incluirse el título del libro y del capitulo si corresponde, autor/es del libro y del capítulo, año de edición y empresa editorial.

Las fotografías deberán enviarse en archivos separados, sin sus leyendas, en formato *.tiff ,*.jpg o *.epf, resolución 300dpi, tamaño 100 %, numerados consecutivamente según su aparición en el texto. Los esquemas y gráficos deberán guardar proporción como para ser legibles en el ancho de una o dos columnas de la revista impresa (7,5 ó 16,5 cm) y deben confeccionarse en programa Excel o Power Point. Las fotografías, esquemas y gráficos deben indicarse como Fig. (figuras) y ser numerados en arábigo, consecutivamente según su aparición en el texto. Las tablas serán numeradas en arábigo en series separadas. Las leyendas para las figuras deben incluirse al final del texto del manuscrito. Las figuras o esquemas en colores se publicarán solamente si el autor se hace cargo de su costo.

La publicación tendrá un costo de 15 dólares por página impresa. Los autores recibirán un archivo PDF de su trabajo y un ejemplar de la revista impresa.

Artigo 3 – *Archives of Oral Biology*

Guide for Authors

A Multidisciplinary Journal of Oral & Craniofacial Sciences

Submissions: Authors are requested to submit their original manuscript and figures online via Editorial Manager. Editorial Manager is a web-based submission and review system. Authors may submit manuscripts and track their progress through the system to publication. Reviewers can download manuscripts and submit their opinions to the editor. Editors can manage the whole submission/review/revise/publish process.
Please register at: http://ees.elsevier.com/aob
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Email: AOB@elsevier.com.

Submission of a paper implies that it has not been published previously, that it is not under consideration for publication elsewhere, and that if accepted it will not be published elsewhere in the same form, in English or in any other language, without the written consent of the publisher. Each manuscript must be accompanied by a statement signed by the corresponding author that the manuscript in its submitted form has been read and approved by all authors. Authors should supply details of related papers submitted or recently published elsewhere. If the manuscript reports experiments or observations using animals or human subjects a statement must be included in the letter of submission indicating that the protocol has been examined and approved by an institutional review board. Authors are invited to suggest upto three referees they consider suitable to review their submission. Full postal and Email addresses should be included. The editors may or may not, at their discretion, utilise these suggestions.

Scientific Standards: The aim of Editors and referees is to maintain a high standard of scientific communication. Normally papers are assessed by two referees selected by the Editor, and decisions regarding acceptance are based mainly upon the advice of the referees. Where appropriate, the referees' views are forwarded to the authors for their consideration. Authors may occasionally consider referees' suggestions to be ill-conceived but if their text is misunderstood by referees it is likely to be misunderstood by readers of the journal.

Types of Contributions: Original papers and review articles are welcomed. There will be no differentiation on the basis of length into full or short communications. All submissions will be refereed. Reviews may be submitted in outline prior to full submission.

Manuscript Preparation: Papers should be as concise as possible and, in view of the international character of the journal, English usages that may present difficulties to readers whose first language is not English should be avoided. The spellings used can be in English or American, but must be consistent within the manuscript. Authors should express their own findings in the past tense and use the present tense where reference is made to existing knowledge, or where the author is stating what is known or concluded. Original papers should follow the pattern of: Introduction, Materials and Methods, Results or Findings, Discussion.
Authors will gain much assistance by consulting: Edward J. Huth, Scientific Style and Format (Sixth Edition). The Council of Biology Editors Manual for Authors, Editors and Publishers, Cambridge.

Editors reserve the right to revise the wording of papers in the interest of the Journal's standards of clarity and conciseness.

General: Manuscripts must be word processed (preferably in Word format), double-spaced with wide margins and a font size of 12 or 10 pt. For hardcopy submissions, good quality printouts are required. The corresponding author should be identified (include a Fax number and E-mail address). Full postal addresses must be given for all co-authors. Please check the current style of the journal, particularly the reference style (Vancouver), and avoid excessive layout styling as most formatting codes will be removed or replaced during the processing of your article. In addition, do not use options such as automatic word breaking, justified layout, double columns or automatic paragraph numbering (especially for numbered references). The Editors reserve the right to adjust style to certain standards of uniformity. Authors should retain copies of all versions of their manuscript submitted to the journal. Authors are especially requested to be vigilant over the submission of the correct version of the manuscript at the various stages of the editorial process.

Text: Follow this order when typing manuscripts: Title, Authors, Affiliations, Abstract, Keywords, Main text, Acknowledgments, Appendix, References, Vitae, Figure Captions and then Tables. Do not import the Figures or Tables into your text. The corresponding author should be identified with an asterisk and footnote. All other footnotes (except for table footnotes) should be identified with superscript Arabic numbers.

Title page: As titles frequently stand alone in indexes, bibliographic journals etc., and indexing of papers is, to an increasing extent, becoming computerized from key words in the titles, it is important that titles should be as concise and informative as possible. Thus the animal species to which the observations refer should always be given and it is desirable to indicate the type of method on which the observations are based, e.g. chemical, bacteriological, electron-microscopic or histochemical etc. A "running title" with not more than 40 letters and spaces must also be supplied. A keyword index must be supplied for each paper.

Structured abstracts:The paper should be prefaced by an abstract aimed at giving the entire paper in miniature. Abstracts should be no longer than 250 words and should be structured as per the guidelines published in the Journal of the American Medical Association (JAMA 1995;273: 27-34). In brief, the abstract should be divided into sections including the following: (1) Objective; (2)Design -if clinical to include setting, selection of patients, details on the intervention, outcome measures, etc.; if laboratory research to include details on methods; (3) Results; (4) Conclusions.

Received/Accepted Dates:A received date will be added to all papers when they are received by the Accepting Editor. An accepted date will also be added when the papers are received at the publishing office.

Introduction: This should be a succinct statement of the problem investigated within the context of a brief review of the relevant literature. Literature directly relevant to any inferences or argument presented in the Discussion should in general be reserved for that section. The introduction may conclude with the reason for doing the work but should not state what was done nor the findings.

Materials and Methods: Enough detail must be given here so that another worker can repeat the procedures exactly. Where the materials and methods were exactly as in a previous paper, it is not necessary to repeat all the details but sufficient information must be given for the reader to comprehend what was done without having to consult the earlier work. Authors are requested to make plain that the conditions of animal experiments were humane; for instance, the mode of anaesthesia and of killing should be specified. In human experimentation, authors should state briefly that the subjects gave informed consent, and preferably that the work was approved by an appropriate ethics committee or review board.

Results or Findings: These should be given clearly and concisely. Care should be taken to avoid drawing inferences that belong to the Discussion. Data may be presented in various forms such as histograms or tables but, in view of pressure on space, presentation of the same data in more than one form is unacceptable. It is usually necessary to analyze numerical results statistically. A statement of the number, their mean value and some appropriate measure of their variability is usually sufficient. The method of analysis followed should be indicated. A statement that the difference between the mean values of two groups of data is statistically significant should give the probability level set as significant by the investigator and indicate the statistical test used. It is not sufficient to quote the use of a statistical package without naming the tests used.

Discussion: This section presents the inferences drawn from the Results: these should be recapitulated only sparingly, sufficient to make the argument clear.

Acknowledgments: as appropriate.

References: All manuscripts should use the 'Vancouver' style for references, which should be numbered consecutively in the order in which they are first cited in the text and listed at the end of the paper.

For journal references, all authors should be included when there are six or fewer (first six followed by 'et al.' when seven or more), followed by the title of article, name of journal abbreviated according to British Standard 4148: 1975 (or left in full), year, volume, and first and last pages. For example:

1. Dezan CC, Nicolau J, Souza DN, Walter LRF. Flow rate, amylase activity, and protein and sialic acid concentrations of saliva from children aged 18, 30 and 42 months attending a baby clinic. *Arch Oral Biol* 2002; **47**: 423?427.

For book references, the author(s)should be followed by the chapter title (if appropriate), editor(s) (if applicable), book title, place of publication, publisher, year and page numbers. For example:

2. Gorlin RJ, Pindborg JJ, Cohen MM Jr. Syndromes of the Head and Neck, 2nd Edition. New York: McGraw-Hill, 1976.

Papers in the course of publication should only be entered in the references if the paper has been accepted by a journal, and then given in the standard manner in the text and list of references but with the words "In press" following the name of the journal.

Units and Symbols: In general, *Archives of Oral Biology* will use the recommended SI (Systeme Internationale) units and symbols. The use of the litre, usually better written in full, in place of SI dm^3 and ml^3 in place of SI cm, will continue to be accepted. For details of the SI symbols, authors are referred to: Symbols, Signs and Abbreviations (1969) by the Royal Society of Metric and Decimal Systems in Council of Biology Editors Style Manual (1978) 4th edn, published by Council of Biology Editors Inc. Units of enzyme activity must be clearly defined, preferably using SI units. Centrifugal force should be stated in multiples of g, rather than as rev/min.

Units and abbreviations: As *Archives of Oral Biology* is a journal with a multidisciplinary readership, abbreviations, except those universally understood such as mm, g, min. u.v., w/v and those listed below should be avoided if possible. Examples of abbreviations which maybe used without definition: ADP, AMP, ATP, DEAE-cellulose, DNA, RNA, EDTA, EMG tris. Other abbreviations used to improve legibility should be listed as a footnote on the title page. Chemical symbols may be used for elements, groups and simple compounds, but excessive use should be avoided. Abbreviations other than the above should not be used in titles.

Bacterial nomenclature. Organisms should be referred to by their scientific names according to the binomial system. When first mentioned the name should be spelt in full and underlined to denote italics. Afterwards the genus should be abbreviated to its initial letter, e.g. '*S. aureus*' not '*Staph. aureus*'. If abbreviation is likely to cause confusion or render the intended meaning unclear the names of microbes should be spelt in full. Only those names which were included in the Approved List of Bacterial Names, *Int J Syst Bacteriol* 1980; 30: 225?420 and those which have been validly published in the *Int J Syst Bacteriol* since 1 January 1980 have standing in nomenclature. If there is good reason to use a name that does not have standing in nomenclature, the names should be enclosed in quotation marks and an appropriate statement concerning the nomenclatural status of the name should be made in the text (for an example see *Int J Syst Bacteriol* 1980; 30: 547?556). When the genus alone is used as a noun or adjective, use lower case roman not underlined, e.g.'organisms were staphylococci' and 'streptococcal infection'. If the genus is specifically referred to underline e.g. 'organisms of the genus *Staphylococcus*'. For genus in plural, use lower case roman e.g. 'salmonellae'; plurals may be anglicized e.g.'salmonellas'. For trivial names, use lower case roman e.g. 'meningococcus'.

Numbers, measurements and statistics. Numbers one to nine are spelled unless they are measurements (e.g.5mL). Numbers greater than nine are spelled out if they begin in a sentence, or when clarity requires it. Numbers above and including 10 000 have a space, not a comma. A decimal point is preceded by a number or cypher e.g. '0.5'.Decimal points in columns should be aligned vertically. Dates are usually provided in full: 14 April 1949. Measurements may be expressed in SI or non-metric units. Use 10 ml/h rather than -1 or per.

Abbreviations. Use capitals for: MIC, MBC, WBC, RBC, DNA, RNA, Group A, B etc. for antigenic or other groups, PHLS, CDSC, CDC, WHO, CSF, MSU, EMU, CSU. Use cfu, pfu, mm, m, min, h, in, ft, g, kg, mL, L, im, iv, iu, P(probability). Use sp. and spp. (species, singular and plural). Use Gram's stain and Gram-negative bacillus. Use in-vitro (adjective) but in vitro(adverb), post-mortem (adjective) but post mortem (adverb). Spelling. Use British spellings: Haemophilus, haematology, paediatrics, leucocyte, leukaemia, bacteraemia, sulphonamides, aetiology; but note neutropenia, fetal. Please note the journal uses UK 'z' spelling (e.g., colonizes).

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Artigo 4 – *Caries Research*

Aims and Scope
'Caries Research' is an international journal, the aim of which is to promote research in dental caries and related fields through publication of original research and critical evaluation of research findings. The journal will publish papers on the aetiology, pathogenesis, prevention and clinical control or management of dental caries. Papers on health outcomes related to dental caries are also of interest, as are papers on other disorders of dental hard tissues, such as dental erosion. Aspects of caries beyond the stage where the pulp ceases to be vital are outside the scope of the journal. The journal reviews papers dealing with natural products and other bacterial inhibitors against specific criteria, details of which are available from the Editor.

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Copies of any "in press" papers cited in the manuscript must accompany the submission. Manuscripts reporting on clinical trials must be accompanied by the CONSORT checklist (see below).

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Reviews can have a freer format but should nevertheless commence with a Title page, an Abstract and an Introduction defining the scope.
Current topics are concise articles that present critical discussion of a topic of current interest, or a fresh look at a problem, and should aim to stimulate discussion.
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Text should be one-and-a-half-spaced, with wide margins. All pages should be numbered, starting from the title page. A conventional font, such as Times New Roman or Arial, should be used, with a font size of 11 or 12. Avoid using italics except for Linnaean names of organisms and names of genes. Manuscripts should be prepared as a text file plus separate files for illustrations. The text file should contain the following sequence of sections: Title page; Declaration of interests; Abstract; Introduction; Materials and Methods; Results; Discussion; Acknowledgements; References; Legends; Tables. Each section should start on a new page, except for the body of the paper (Introduction to Acknowledgements), which should be continuous.
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Declaration of Interests: Potential conflicts of interest should be identified for each author or, if there are no such conflicts, this should be stated explicitly. Conflict of interest exists where an author has a personal or financial relationship that might introduce bias or affect their judgement. Examples of situations where conflicts of interest might arise are restrictive conditions in the funding of the research, or payment to an investigator from organisations with an interest in the study (including employment, consultancies, honoraria, ownership of shares). The fact that a study is conducted on behalf of a commercial body using funds supplied to the investigators' institution by the sponsor does not in itself involve a conflict of interest. Investigators should disclose potential conflicts to study participants and should state whether they have done so.
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Clinical trials should be reported according to the standardised protocol of the CONSORT Statement. The CONSORT checklist must be submitted together with papers reporting clinical trials.
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Unless the purpose of a paper is to compare specific systems or products, commercial names of clinical and scientific equipment or techniques should only be cited, as appropriate, in the 'Materials and Methods' or 'Acknowledgements' sections. Elsewhere in the manuscript generic terms should be used.
Results: Results should be presented without interpretation. The same data should not be presented in both tables and figures. The text should not repeat numerical data provided in tables or figures but should indicate the most important results and describe relevant trends and patterns.
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(c) Monographs: Matthews DE, Farewell VT: Using and Understanding Medical Statistics.

Basel, Karger, 1985.

(d) Edited books: DuBois RN: Cyclooxygenase-2 and colorectal cancer; in Dannenberg AJ, DuBois RN (eds): COX-2. Prog Exp Tum Res. Basel, Karger, 2003, vol 37, pp 124-137.

(e) Patents: Diggens AA, Ross JW: Determining ionic species electrochemically. UK Patent Application GB 2 064 131 A, 1980.

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Anexo 2: Carta de aceite

ACTA ODONTOLOGICA LATINOAMERICANA

Buenos Aires, 6 de octubre de 2008

Dra. Patricia Valente Araujo
Facultad de Odontologia da UFMG

Estimada Dra. Valente Araujo:

Tengo el agrado de dirigirme a Ud. A fin de remitirle el informe de referato del trabajo oportunamente enviado para su publicación en AOL (T 287). Para ser aceptado, le solicitamos tenga en cuenta que el revisor ha sugerido unas mímimas correcciones : describir el numero (n) de cada grupo.

Puede Ud. enviarnos su trabajo corregido por e-mail. Una vez aceptado en su contenido científico, el trabajo será revisado en cuanto a sus aspectos editoriales e idiomáticos. Adjuntamos una copia de las Instrucciones para Autores, que figuran en la Revista, para que Ud. considere los eventuales costos de corrección y de publicación.

Sin otro particular y agradeciendo su colaboración a AOL, la saludo atentamente,

Dr. Rómulo Luis Cabrini
Editor

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e-mails: postmast@cap.odon.uba.ar –fliacabrini@fibertel.com.ar

ANEXO 3: Parecer do COEP-UFMG

UFMG

Universidade Federal de Minas Gerais
Comitê de Ética em Pesquisa da UFMG - COEP

Parecer nº. ETIC 501/06

Interessado (a): Prof. Luiz Thadeu de Abreu Poletto
Depto. Odontologia Restauradora
Faculdade de Odontologia - UFMG

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP, aprovou, no dia 28 de março de 2007, após atendidas as solicitações de diligência, o projeto de pesquisa intitulado **"Aplicação da terapia fotodinâmica na Odontologia: associação de agentes fotossensibilizantes e fontes de luz na redução de patógenos cariogênicos"** bem como o Termo de Consentimento Livre e Esclarecido do referido projeto.

O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.

Profa. Dra. Maria Elena de Lima Perez Garcia
Presidente do COEP/UFMG

Av. Presidente Antonio Carlos, 6627 – Unidade Adminstrativa II - 2º Andar – Sala 2005 - Cep 31270-901 – Belo Horizonte-MG
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8 APÊNDICE

APÊNDICE 1: Termo de consentimento Livre e Esclarecido

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO PÓS-INFORMAÇÃO, PARA PARTICIPAÇÃO EM PESQUISA, CONFORME RESOLUÇÃO N° 196 DE 10/10/96, DO CONSELHO NACIONAL DE SAÚDE

Título da Pesquisa: **Aplicação da terapia fotodinâmica na odontologia: associação de agentes fotossensibilizantes e fontes de luz na redução de patógenos cariogênicos**

Coordenador do Projeto: Prof. Dr. Luiz Thadeu de Abreu Poletto
Pesquisadora Responsável: Profa. Patrícia Valente Araújo

Introdução: Convidamos você a ler atentamente as informações contidas neste documento, antes de consentir que sua criança participe como voluntário colaborador desta pesquisa. Aqui estão os esclarecimentos sobre os objetivos, os benefícios, os riscos, os desconfortos e os procedimentos deste estudo. Esclarece-se, também, o seu direito de interromper a participação de sua criança no estudo a qualquer momento.

Resumo: A cárie dentária é uma doença que afeta os dentes, tanto de leite quanto os definitivos. Ela começa a ser percebida quando surge uma mancha branca na superfície do dente, por causa da descalcificação que vai acontecendo, devido à presença de bactérias. Se essa descalcificação continua, aos poucos o dente vai sendo corroído e mais tarde surge uma cavidade. Essa cavidade pode ir aumentando, atingindo as partes mais internas dos dentes. Nessa fase da doença, uma das formas de paralisar o aumento da cavidade é fazer uma restauração no dente, ou seja, fechar a cavidade com um material apropriado. Porém, algumas bactérias podem permanecer nas paredes da cavidade, dificultando o processo de cura. Os pesquisadores já sabem que a terapia fotodinâmica, que é a proposta de nosso estudo, promove a morte de bactérias em diversas situações e nós queremos observar se esta terapia pode ser aplicada no tecido cariado, matando as bactérias presentes e criando melhores condições para a cura da doença naquele dente onde ela foi aplicada. Na terapia fotodinâmica, um produto é aplicado sobre a dentina cariada e é iluminado com uma fonte de luz, antes da restauração da cavidade. Tanto o produto aplicado quanto a fonte de luz não causam nenhum dano aos tecidos.

Para restaurar os dentes cavitados pela cárie, usaremos apenas instrumentos manuais que raspam as paredes da cavidade para retirar o tecido cariado sem a necessidade de motores. Como essa raspagem retira apenas o material do dente que está bem amolecido, ou seja cariado, não dói e por isso a pessoa não precisa ser anestesiada.

Objetivos: Verificar se há diferença entre o tecido dentinário removido antes da aplicação da terapia fotodinâmica e o tecido dentinário removido após a aplicação desta, em relação ao número de microorganismos presentes.

Procedimentos: Nós selecionamos crianças sem outros problemas de saúde, mas que tem cavidades de cárie profundas e que estão aumentando. Sua criança é uma delas. Vamos fazer o tratamento restaurador, usando instrumentos manuais para retirar o tecido cariado amolecido dessas cavidades, sem

anestesia. Sua criança não vai sentir dor. Esse tecido cariado, que normalmente é jogado fora, será guardado e analisado. O dente será restaurado com um material próprio, já usado normalmente nos tratamentos odontológicos. Sua criança será acompanhada por tempo indeterminado, pois receberá todo o tratamento odontológico que precisar, entrando num programa odontológico permanente de saúde. Receberá orientações sobre higiene bucal, sobre a importância de controlar o consumo de açúcar, aplicações periódicas de flúor e controle radiográfico, de acordo com a necessidade dela. Não haverá qualquer custo por sua criança fazer parte deste estudo. Você não pagará nada e não receberá qualquer remuneração por essa participação.

Desconforto: Como escrito anteriormente, os procedimentos realizados na boca das crianças não trarão qualquer desconforto para elas.

Riscos: Em princípio, não existem riscos para a sua criança. No entanto, pode ser que ela queixe um pouco de sensibilidade no dente nas primeiras horas após o tratamento. Se isso ocorrer, entre imediatamente em contato com o pesquisador principal mencionado ao final deste documento. Faça o mesmo se ocorrerem outras alterações em qualquer momento.

Benefícios: Além de contribuir para que ocorra a paralisação do aumento da cavidade de cárie na sua criança, ela terá a oportunidade de receber um tratamento menos invasivo, mais agradável, menos estressante, o que poderá contribuir para uma maior adaptação comportamental. O estudo poderá também contribuir para que os dentistas tenham conhecimento dos efeitos da terapia fotodinâmica nas bactérias da cárie, buscando sempre um tratamento restaurador mais simples tanto para o dentista quanto para o paciente, permitindo que as lesões sejam restauradas com remoção mínima de tecido e com um prognóstico a longo prazo mais favorável.

Confidencialidade: As informações fornecidas sobre sua criança serão acessíveis apenas aos pesquisadores. Dentro dos limites da lei, os dados serão mantidos em sigilo.

Contato com o pesquisador: Poderá ser feito com a Profa. Patrícia Valente Araújo no telefone 32818881 ou 99264577. Em caso de dúvidas em relação aos seus direitos como participante de pesquisa, você deverá ligar para o presidente do Comitê de Ética em Pesquisa da UFMG, através do número 3248-9364.

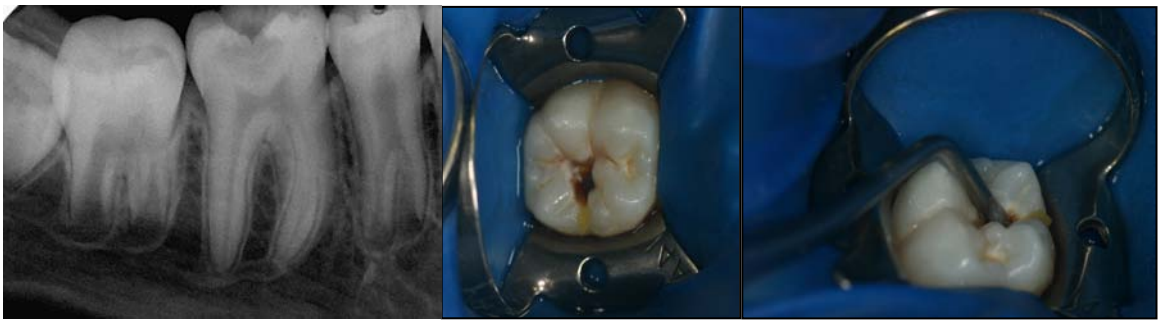
Consentimento: Li e entendi as informações contidas neste documento. Tive a oportunidade de fazer perguntas e todas as minhas dúvidas foram respondidas satisfatoriamente. Minha criança está participando desta pesquisa por minha vontade, até que eu decida o contrário.

Belo Horizonte, ____ de _____ de _____

_____

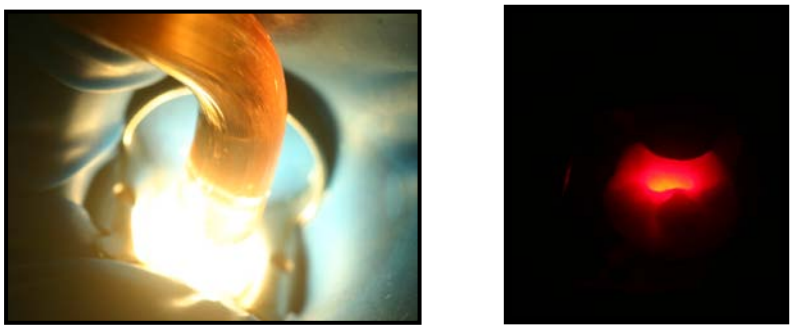
Nome do Responsável:
Idade:
Endereço:

APÊNDICE 2:Sequência clínica dos procedimentos *in vivo*



Radiografia inicial, aspecto inicial da lesão e remoção dos fragmentos controle

Aplicação do agente fotossensibilizante Chimiolux® durante cinco minutos (tempo pré-irradiação)



Aplicação da fonte de luz (halógena ou laser vermelho) por um minuto

Remoção dos fragmentos após a PDT e restauração com cimento de ionômero de vidro