UNESP - Universidade Estadual Paulista

"Júlio de Mesquita Filho"

Faculdade de Odontologia de Araraquara

EMANUELLE TEIXEIRA CARRERA

MODIFICAÇÃO EM FOTOSSENSIBILIZADOR E SUA APLICAÇÃO NA TERAPIA FOTODINÂMICA ANTIMICROBIANA EM SUBSTRATO DENTINÁRIO

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Dissertação apresentada ao Programa de Pós Graduação em Ciências Odontológicas – Área de Concentração em Dentística Restauradora, da Faculdade de Odontologia de Araraquara, da Universidade Estadual Paulista – UNESP, como parte dos requisitos para obtenção do título de Mestre em Ciências Odontológicas.

Orientadora: Profª. Drª. Alessandra Nara de Souza Rastelli

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Dissertação para obtenção do grau de Mestre

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Carrera ET. Modificação em fotossensibilizador e sua aplicação na Terapia Fotodinâmica Antimicrobiana em substrato dentinário. [Dissertação de Mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2016.

RESUMO

O objetivo deste trabalho, dividido em dois estudos, foi avaliar: (1) *in vitro* o efeito da terapia fotodinâmica na viabilidade de cultura planctônica de *Streptococcus mutans* mediada por azul de metileno associado ou não por nitrato de sódio em combinação com Laser; e (2) avaliar *in vitro* o efeito da terapia fotodinâmica na viabilidade do biofilme de *Streptococcus mutans* mediado por azul de metileno associado ou não por nitrato de sódio, ambos em associação com Laser em comprimento de onda vermelho (660nm). No primeiro estudo, a suspensão bacteriana contendo *Streptococcus mutans* $1x10^8$ UFC/mL foi preparada e 250µL foi pipetada de acordo com os grupos: L-D- (controle negativo), L-D+ (fotossensibilizador associado ou não em 6 concentrações: 0,625; 1,25; 2,5; 5; 10 e 20µM), L+D- (Laser 30J/cm^2), clorexidina (controle positivo), nitrato de sódio e o grupo L+D+ (azul de metileno associado ou não ao nitrato de sódio e irradiado com Laser). Para citotoxicidade no escuro, foram acrescidos 250µL do azul de metileno associado ou não, incubado no escuro por 5 minutos. Os grupos da terapia fotodinâmica foram incubados no escuro por 5min e irradiados por Laser 30 segundos. Alíquotas de cada diluição foram cultivadas em Placa de Petri com BHI ágar e incubadas a 37° C em estufa por 48h para posterior contagem das UFC/mL. Os resultados obtidos demonstraram que não houve citotoxicidade no escuro para as diferentes concentrações utilizadas do azul de metileno associado ou não. Observou-se redução completa na viabilidade do *Streptococcus mutans* em fase planctônica nas concentrações entre 0,625 e 5µM para azul de metileno associado. No segundo estudo induziu-se os biofilmes sobre fragmentos de dentina bovina (3x3x2mm) e foram divididos em grupos: L-D- (controle negativo), L-D+ (fotossensibilizador associado ou não em 6 concentrações: 0,625; 1,2; 2,5; 5; 10 e 20µM), L+D- (Laser 30J/cm^2), clorexidina (controle positivo), nitrato de sódio e grupo L+D+ (azul de metileno associado ou não e irradiado com Laser). Para a citotoxicidade no escuro, foram adicionados azul de metileno associado ou não, incubados no escuro por 5 minutos. Os grupos da terapia fotodinâmica foram incubados no escuro por 5 minutos e irradiados por Laser 30 segundos. Alíquotas de cada diluição foram cultivados em Placas de Petri com BHI ágar e incubados em estufa por 48h para posterior contagem de UFC/mL. Para ambos os trabalhos, os dados foram transformados em $\log_{10}$, analisados por ANOVA um e dois fatores e teste de Tukey $p < 0,05$. Os resultados mostraram reduções significativas na viabilidade no biofilme de *Streptococcus mutans* quando expostos ao azul de metileno associado ao nitrato de sódio,

2,13 log_{10} para [2,5µM] e 2,37 log_{10} para [5µM], comparado com o azul de metileno não associado. Pode-se concluir que em ambos os estudos a associação do fotossensibilizador quando associado ao Laser promoveram redução significativa tanto para cultura planctônica quanto para o biofilme de *Streptococcus mutans* quando comparado com o fotossensibilizador não associado, sendo mais eficiente que a clorexidina, podendo ser uma técnica viável para eliminar ou reduzir essas bactérias na cavidade oral.

PALAVRAS-CHAVES: Fotoquimioterapia. Azul de Metileno. *Streptococcus mutans*. Biofilmes. Cárie Dentária.

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ABSTRACT

The aim of this work, divided into two studies, was to evaluate: (1) the *in vitro* the effect of photodynamic therapy on the viability of *Streptococcus mutans* planktonic culture mediated by methylene blue associated or not by sodium nitrate in association with red Laser; and (2) the *in vitro* the effect of photodynamic therapy on the viability of *Streptococcus mutans* biofilm mediated by methylene blue associated or not by sodium nitrate in association with red Laser (660nm). In the first study the bacterial suspension containing *Streptococcus mutans* $1x10^8$ CFU/mL was prepared and 250µL pipetted according to the groups: L-P- (negative control), L-P+ (photosensitizer associated or no at 6 concentrations: 0.625, 1.25, 2.5, 5, 10 and 20µM), L+P- (Laser 30J/cm^2), chlorhexidine (positive control), sodium nitrate and group L+P+ (methylene blue associated or not with sodium nitrate and irradiated with Laser). For the dark cytotoxicity test, 250 µL of methylene blue associated or not, were added and incubated in the dark for 5 minutes. The groups of photodynamic therapy were incubated in the dark for 5 minutes and irradiated by red Laser during 30 seconds. Samples were incubated at 37° C during 48h for subsequent visual counting of CFU/mL. The results showed that there was no cytotoxicity in the dark for the different concentrations of methylene blue associated or not. The photodynamic therapy promoted complete reduction in the viability of *Streptococcus mutans* at concentrations between 0.625 and 5µM for associated methylene blue. For the second study was induced biofilms on the bovine dentin fragments (3x3x2mm) and was divided in groups: PS-L- (negative control), PS+L- (photosensitizer associated or no at 6 concentrations: 0.625, 1.25, 2.5, 5, 10 and 20µM), P-L+ (Laser 30J/cm^2), chlorhexidine (positive control), sodium nitrate and group P+L+ (methylene blue associated or not with sodium nitrate and irradiated with Laser). For the dark cytotoxicity test, methylene blue associated or not were added and incubated in the dark for 5 minutes. The groups of photodynamic therapy were incubated in the dark for 5 minutes and irradiated by red Laser during 30 seconds. Samples were incubated at 37° C during 48h for subsequent visual counting of CFU/mL. For both works, data were transformed into $\log_{10}$, analyzed by one and two-way ANOVA and Tukey's test at $p < 0.05$. The results showed significant decreases in the viability of biofilm *Streptococcus mutans* when exposed to associated methylene blue, 2.13 $\log_{10}$ to [2.5µM] and 2.37 $\log_{10}$ to [5µM], compared with methylene blue not associated. Can be concluded that in both works photosensitizer association, associated with red Laser

caused significant reduction in both the planktonic culture as for the biofilm of *Streptococcus mutans* biofilm when compared with the photosensitizer not associated, being more effective than chlorhexidine, may be a viable technique to eliminate or reduce these bacteria in the oral cavity.

KEYWORDS: Photochemotherapy. Methylene blue. Biofilms. *Streptococcus mutans*. Dental Caries.

LISTA DE ABREVIATURAS E SIGLAS

aPDT	Antimicrobial Photodynamic Therapy (Terapia Fotodinâmica Antimicrobiana)
ALA	Ácido 5-aminolevulínico
AM	Azul de Metileno
ANOVA	Análise de Variância
BHI	Brain Heart Infusion
CEUA	Comitê de Ética no Uso de Animais
CLX	Clorexidina
DNA	Ácido Desoxirribonucleico
Laser	Light Amplification by Stimulated Emission of Radiation (Luz Amplificadora por Estimulo Emissor de Radiação)
LED	Light Emitting Diode (Luz Emissora de Diodo)
N	Nitrato de Sódio
$NaNO_3$	Nitrato de Sódio
NI	Nitrado de Sódio Irradiado
PBS	Phosphate Buffered Saline (Salina Tamponada Fosfatada)
PDT	Photodynamic Therapy (Terapia Fotodinâmica)
PIA	Polissacarídeo Adesina Intercelular
SD	Standard Deviation (Desvio Padrão)
TBO	Toluidine Blue O (Azul de Orto-Toluidina)
TFDA	Terapia Fotodinâmica Antimicrobiana
UFC	Unidades Formadoras de Colônias
UV-Vis	Energia Eletromagnética na Região do Ultravioleta e Visível

SUMÁRIO

1 INTRODUÇÃO

Na cavidade oral dos seres humanos já foi detectada a presença de grande diversidade de microrganismos, como bactérias, vírus, protozoários e fungos (Marcotte, Lavoie[17], 1998), que são denominados como microbiota residente (Zanin et al[37], 2003). Esses microrganismos normalmente se encontram organizados na forma de biofilmes (Zanin[36], 2005).

O motivo para a colonização destes microrganismos na cavidade oral se deve pelo ambiente favorável que lhes é proporcionado, como a presença de secreções, nutrientes e restos epiteliais (Araújo et al.[4], 2012).

Algumas espécies de microrganismos, organizados na forma de biofilmes, são responsáveis por várias doenças na cavidade oral, como a cárie dental, doença periodontal, candidíase e mal hálito, além de infecções de natureza endodôntica (Soukos, Goodson[30], 2011). Biofilmes orais são complexos constituídos por várias espécies de microrganismos competindo por um espaço limitado e por nutrientes para sobreviverem e em condições saudáveis mantem um equilíbrio ecológico (Kreth et al.[13], 2008).

Na superfície dos elementos dentários ocorre a formação e deposição de uma camada acelular, denominada de película adquirida, que é formada por componentes bacterianos, salivares e do fluido gengival (Zanin[36], 2005). Em seguida ocorre a colonização pioneira por microrganismos (*Streptococcus mitis, Streptococcus oralis e Streptococcus sanguinis*), que crescem rapidamente formando colônias envoltas por matriz extracelular de polissacarídeos. Em aproximadamente 2 ou 3 semanas, devido à heterogeneidade das bactérias, este biofilme chega ao seu grau máximo de desenvolvimento, denominado comunidade clímax (Zanin et al.[37], 2003). A maturação do biofilme se torna oportuna pelo fato da superfície dentária ser dura, podendo assim se localizar supra ou subgengivalmente (Zanin[36], 2005).

Quando ocorre um desequilíbrio no ecossistema do biofilme dentário, como por exemplo aumento na frequência e ingestão de sacarose (carboidrato fermentável), ocorrerá aumento do desenvolvimento de algumas espécies de bactérias sacarolíticas acidúricas e acidogênicas, como os *Streptococcus* do grupo *mutans* (Alakker, Memarzadeh[1], 2014; Zanin et al.[37], 2003) e outras espécies de *Streptococcus* e *Lactobacillus*, que levam à produção de ácidos, principalmente o láctico, havendo assim a dissolução do fosfato de cálcio da camada superficial da estrutura dentária, liberando cálcio e fosfato para a cavidade oral (Marcotte, Lavoie[17], 1998). Logo, leva a queda de pH, que com a ação do tempo, ocorrem sucessivas desmineralizações do esmalte, fazendo com que a lesão de cárie avance para a dentina, e caso

esse progresso continue e não seja interrompido, pode haver a destruição completa e, consequentemente, a perda do elemento dentário (Loesche[14], 1993).

O tratamento da doença cárie no seu estágio inicial consiste na remoção mecânica e química do biofilme bacteriano, eliminando assim, de maneira preventiva, os microrganismos cariogênicos, principalmente *Streptococcus mutans*, no aumento da resistência dental, por meio da aplicação de selante por profissionais, e na aplicação tópica de flúor, bem como a modificação da dieta (Van Houte[32], 1994). E quando em estágio mais avançado como a presença de cavitações, o tratamento consiste na remoção da estrutura dentária infectada e realização de restaurações (Soukos, Goodson[30], 2011).

A remoção mecânica do biofilme dentário é o método mais aceito, já que a higienização bucal ineficiente, associada à presença do biofilme dental, são fatores relacionados à causa da doença cárie (Alves et al.[2], 2009). Entretanto, alguns indivíduos apresentam dificuldade na remoção mecânica do biofilme ou até mesmo, não possuem motivação para realizá-la, sendo, por isso, necessário associar ao método mecânico, o químico por meio do uso complementar de substâncias antimicrobianas ou antibióticos (Pereira et al.[23], 2006; Zanin[36], 2005). Infelizmente, o uso frequente destas substâncias pode levar à seleção de espécies resistentes (Zanin[36], 2005).

Atualmente, a solução antimicrobiana mais utilizada ainda é a clorexidina que tem sido utilizada para limpar as cavidades dentárias infectadas antes destas serem restauradas, para reduzir ou impedir a incidência de cáries recorrentes, como solução irrigadora durante um tratamento endodôntico, na forma de bochecho, assim como para promover a desinfecção na fase pré e pós operatória em cirurgias (Santin et al.[27], 2014). Atua na diminuição dos níveis de *Streptococcus mutans* (Santin et al.[27], 2014), altera o arranjo das bactérias do biofilme dental, e, assim, resulta na redução imediata do número de bactérias salivares devido seu amplo espectro, quando neste caso utilizada na forma de bochecho (Pereira et al.[23], 2006), devido à uma propriedade denominada substantividade, no qual a molécula permanece aderida aos tecidos, promovendo atividade antimicrobiana por até 12 horas (Santin et al.[27], 2014). Atua em bactérias gram positivas e negativas, tanto aeróbias quanto anaeróbias, fungos e leveduras (Franco et al.[9], 2007). Porém, a clorexidina apresenta alguns efeitos colaterais que perduram por mais de 14 dias, como: alteração no paladar, sensação de queimação, coloração dos dentes e restaurações, aumento da formação de cálculo e, mais raramente, descamação do epitélio oral (Arweiler et al.[5], 2002; Paschoal et al.[21], 2013; Santin et al.[27], 2014).

Justamente, em função da quantidade de microrganismos presentes na cavidade oral, pelos problemas apresentados pela clorexidina e também devido à resistência desenvolvida

pelas bactérias em função do uso frequente e impróprio de antibióticos, se faz necessário o uso de técnicas terapêuticas antimicrobianas alternativas para o controle do crescimento microbiano, afim de suprimir a doença de forma conservadora. É neste contexto que entra a terapia fotodinâmica antimicrobiana (TFDA) (Silva[29], 2012).

A terapia fotodinâmica surgiu como forma de tratamento antimicrobiano não invasivo para infecções causadas por bactérias, fungos e vírus (Jori et al.[12], 2006). Ao associar uma substância fotossensibilizadora com a luz em comprimento de onda ressonante, no tratamento contra microrganismos, a terapia passa a se chamar de terapia fotodinâmica antimicrobiana (TFDA), de maneira que as aplicações não desenvolvem resistência microbiana (Muller[18], 2006; Silva[29], 2012; Tennert et al.[31], 2015). Outras características que tornam a terapia fotodinâmica vantajosa, é o fato da morte celular ser rápida e mediada por radicais livres que acaba por não desenvolver resistência por parte do microrganismo, a morte celular se restringe a área irradiada (Dovigo et al.[8], 2011; Zanin[36], 2005), alta especificidade do alvo (Jori et al.[12], 2006) e não causa efeitos colaterais como a clorexidina (Araújo et al.[4], 2012).

Em 1993 iniciaram-se os estudos da aplicação da terapia fotodinâmica nas bactérias causadoras da doença cárie, onde se observou que tais bactérias eram sensíveis à luz Laser associada a fotossensibilizador, e no ano subsequente, alguns autores observaram reduções significativas destas bactérias cariogênicas utilizando a TFDA (Burns et al.[7], 1994; Loesche[14], 1993).

Na terapia fotodinâmica podem ser utilizados vários tipos de fontes de luz, como os Lasers, LEDs, luz halógena, entre outros, sendo que o comprimento de onda deve estar sempre em ressonância com o fotossensibilizador a ser utilizado (Giusti et al.[10], 2008; Paulino et al.[22], 2005).

O Laser, cuja sigla em inglês significa *Light Amplification by Stimulated Emission of Radiation*, tem ganho espaço na Odontologia com o intuito de reduzir o número de microrganismos que venham a causar patologias na cavidade oral, (Muller[18], 2006) principalmente as bactérias que estão envolvidas na doença cárie e doença periodontal, (Zanin et al.[37], 2003) por apresentar características como monocromaticidade (comprimento de onda bem definido), coerência (as ondas dos fótons que compõem o feixe estão em fase) e colimação (a radiação propaga-se como um feixe de ondas praticamente paralelas) (Muller[18], 2006).

Especificamente o Laser de baixa intensidade apresenta potência ao redor de 30 à 100 mW, comprimento de onda que varia entre 630 e 904 nm, efeito térmico insignificante, sua aplicação depende da quantidade de luz absorvida, (Muller[18], 2006) sua ação é restabelecer o

equilíbrio biológico das células, apresenta ação analgésica e anti-inflamatória sobre os tecidos (Zanin et al.[37], 2003) e associado à um fotossensibilizador leva à morte dos microrganismos (Yamada et al.[35], 2004).

A fonte de luz vermelha, que opera entre 630 e 700 nm, tem sido muito utilizada na TFDA, pois apresenta comprimento de onda longo, que facilita a difusão da luz por tecidos biológicos (Wilson et al.[34], 1992). Assim, a interação entre essas fontes de luz vermelha e um fotossensibilizador que absorva a luz neste comprimento de onda, como o azul de metileno (AM), promove morte bacteriana significativa (Rolim et al.[26], 2012).

A eficácia da fonte de luz depende do comprimento de onda, dose de energia, potência/fluência de energia e da quantidade de oxigênio resultante da combinação do fotossensibilizador e da fonte de luz (Santin et al.[27], 2014).

O fotossensibilizador se faz necessário para que este penetre e se fixe na parede das bactérias e possa atrair para si a luz da fonte utilizada (Zanin et al.[37], 2003), promovendo, dessa forma, a morte bacteriana (Yamada et al.[35], 2004), por meio da produção dos radicais que são fundamentais para os fotoprocessos (Zanin et al.[37], 2003). Assim, quando as bactérias apresentam-se coradas com o fotossensibilizador, são irradiadas e absorvem os fótons da fonte de luz e, consequentemente, os elétrons ficam excitados e passam para um nível de energia superior. Neste momento, o fotossensibilizador pode interagir com o oxigênio molecular e formar uma molécula altamente reativa, o oxigênio singleto, denominada de fotoprocesso tipo II, ou agir com outras moléculas e produzir hidroxilas e radicais livres, ocorrendo o fotoprocesso tipo I. Os produtos dos fotoprocessos I e II levam à morte bacteriana (Yamada et al.[35], 2004; Zanin[36], 2005; Zanin et al.[37], 2003). Contudo, nem toda substância capaz de atrair a luz para si, será denominada de fotossensibilizador, pois para ser eficaz na morte bacteriana, esta substância deve apresentar algumas características como baixa toxicidade local na ausência ou presença de luz, (Rolim et al.[26], 2012) absorver luz em comprimento de onda adequado (Muller[18], 2006), não trazer danos tóxicos ao hospedeiro (Zanin[36], 2005), apresentar elevada afinidade de ligação com microrganismos (Soukos, Goodson[30], 2011), conservar-se no estado excitado por tempo considerável para que haja a interação com moléculas adjacentes e produzir produtos com toxicidade suficiente para causar a morte bacteriana (Zanin et al.[37], 2003).

No que diz respeito à morte bacteriana, esta é dependente das estruturas constituintes da parede celular das bactérias gram positiva e gram negativa, na qual as bactérias gram positivas são sensibilizadas pela formação do oxigênio singleto ou radicais livres e as

bactérias gram negativas por um fotossensibilizador que consiga romper a membrana celular (Muller[18], 2006).

As bactérias gram positivas são mais susceptíveis à terapia fotodinâmica, pois apresentam parede celular com única camada espessa, que é ocupada em sua maior parte por substância peptídeoglicana que lhe confere rigidez e permite inúmeras interligações. Apresentam como componentes, ácidos teicóicos e lipoteicóicos que facilitam a ligação e regulação de entrada e saída de cátions das bactérias (Muller[18], 2006; Perussi[24], 2007).

Já as gram negativas, são mais resistentes à terapia fotodinâmica e apresentam em sua parede celular várias camadas, com poucas substâncias peptídeoglicanas e, consequentemente, menos interligações, e uma membrana externa, havendo entre estas o periplasma, que é composto por enzimas hidrolíticas e enzimas que inativam drogas. Não apresentam ácidos teicóicos e a principal proteína da membrana externa é a porina, que apresenta permeabilidade parcialmente seletiva (Muller[18], 2006; Perussi[24], 2007).

Durante a inativação das bactérias, com a TFDA podem ocorrer danos em sua membrana celular (Muller[18], 2006) e danos no DNA (Soukos, Goodson[30], 2011). Entretanto, na aplicação da TFDA, nos deparamos com um problema, pois existem diferenças na sensibilidade entre espécies de mesma classificação gram, como por exemplo na permeabilidade da membrana, tamanho da célula, enzimas antioxidantes que variam em cada espécie e diferentes mecanismos de reparo no DNA (Silva[29], 2012).

Diversos fotossensibilizadores já foram testados, tanto na área médica quanto na odontológica. No tratamento de tumores, derivados porfirínicos de primeira geração, clorinas e ftalocianinas de segunda geração e até agentes fototerapêuticos endógenos, como o ALA (Machado[16], 2000) são utilizados. Na Odontologia, vários estudos mostraram a eficiência do azul de toluidina O (TBO), azul de metileno (Zanin et al.[37], 2003) e curcumina (Araújo et al.[4], 2012; Yamada et al.[35], 2004). Por isso, os derivados de fenotiazina, como o azul de metileno e azul de toluidina, são os fotossensibilizadores mais utilizados na Odontologia (Muller[18], 2006; Longo et al.[15], 2010).

O azul de metileno, é utilizado como agente de fotossensibilização desde a década de 1920, para detectar lesões pré malignas da mucosa, como marcador, e apresenta-se muito eficiente em bactérias gram negativas porque consegue passar entre os canais de porina (proteína), e isso se deve à sua hidrofilicidade, ao seu baixo peso molecular e a sua carga positiva (Soukos, Goodson[30], 2011). Pertence ao grupo dos fenotiazínicos, apresenta máxima absorção na região do vermelho visível (Silva[29], 2012), é um corante orgânico, aromático, heterocíclico e é solúvel em água ou álcool (Poggere et al.[25], 2011).

Modificações em diferentes fotossensibilizadores vêm sendo propostas com o objetivo de aumentar sua eficiência e rendimento fotodinâmico. Dentro deste contexto, o composto químico nitrato de sódio vem sendo investigado por alguns autores (Horn, Morgenroth[11], 2006; Schlag et al.[28], 2007; Vatansever et al.[33], 2013).

O nitrato de sódio tem chamado a atenção dos pesquisadores pois pode ser utilizado como captador de elétrons em cadeias anaeróbicas e é relevante no processo anôxico em biofilmes, na qual a enzima nitrato-redutase respiratória se liga à membrana, se reduz a nitrito e sob condições anaeróbicas pode ainda se reduzir a amônio, provocando uma resposta ao estresse ambiental que ocorre na formação do biofilme, que concomitantemente leva à deficiência do biofilme mediada pela síntese do polissacarídeo adesina intercelular (PIA) (Horn, Morgenroth[11], 2006; Schlag et al.[28], 2007; Vatansever et al.[33], 2013).

Dessa forma este estudo teve como objetivo associar o fotossensibilizador azul de metileno com nitrato de sódio, para que possamos aumentar a sua eficiência na terapia fotodinâmica antimicrobiana quando aplicada em biofilmes orais (TFDA).

2 PROPOSIÇÃO

Abaixo temos o objetivo geral e os objetivos específicos referente aos artigos realizados.

2.1 OBJETIVO GERAL

O objetivo deste estudo foi investigar a citotoxicidade no escuro para as diferentes concentrações do azul de metileno associado e não associado por nitrato de sódio em células bacterianas planctônicas e em biofilme de *Streptococcus mutans*, bem como o efeito antimicrobiano da TFDA em células bacterianas planctônicas e em biofilme de *Streptococcus mutans*, utilizando-se azul de metileno associado ou não por nitrato de sódio.

2.2 OBJETIVOS ESPECÍFICOS

Os objetivos específicos dos artigos serão abordados em cada artigo.

Artigo 1- Photodynamic effects of methylene blue associated by sodium nitrate against *Streptococcus mutans* in a planktonic culture.

Artigo 2- Susceptibility of *Streptococcus mutans* biofilms to photodynamic therapy using methylene blue associated with sodium nitrate.

3 PUBLICAÇÕES

A seguir seguem os artigos referente à esta Dissertação, que estão em fase de redação para posterior submissão.

3.1 Artigo 1- Artigo elaborado nas normas da revista "Lasers in Medical Science"

Photodynamic Effects of Methylene Blue Associated by Sodium Nitrate Against *Streptococcus mutans* in a Planktonic Culture

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Abstract

The aim of this work was to evaluate, the in vitro, the effect of photodynamic therapy on the viability of *Streptococcus mutans* planktonic culture mediated by methylene blue associated or not by sodium nitrate in association with a red Laser (660nm). Bacterial suspension containing *Streptococcus mutans* $1x10^8$ CFU/mL was prepared and 250µL pipetted according to the groups: L-P- (negative control), L-P+ (photosensitizer associated or not at 6 concentrations: 0.625, 1.25, 2.5, 5, 10 and 20µM), L+P- (Laser 30J/cm^2), chlorhexidine (positive control), sodium nitrate and group L+P+ (methylene blue associated or not with sodium nitrate, irradiated with Laser). For the dark cytotoxicity test, 250µL of methylene blue associated or not, were added and incubated in the dark for 5 minutes. The groups of photodynamic therapy were incubated in the dark for 5 minutes and irradiated by red Laser during 30 seconds. Samples were incubated at 37°C during 48h for subsequent visual counting of CFU/mL. Data were transformed into $\log_{10}$, analyzed by one and two-way ANOVA and Tukey's test at $p < 0.05$. The results showed that there was no dark cytotoxicity for the different concentrations of methylene blue associated or not. The photodynamic therapy promoted complete reduction in the viability of *Streptococcus mutans* at concentrations between 0.625 and 5µM for associated methylene blue. Based on the results obtained, it was possible to conclude that, methylene blue associated by sodium nitrate is a viable technique for the elimination of *Streptococcus mutans* in planktonic culture.

Keywords: Photochemotherapy, Methylene Blue, *Streptococcus mutans*, Dental Caries.

Introduction

In healthy individuals, there is a large amount of microrganisms that are in the form of biofilm on the tooth surface or on any other hard structure. Their structures are complex and they are constituted of an array of microrganisms that compete for a limited space and

nutrients to survive [1,2]. Biofilms are responsible for many diseases, among them, the dental caries [3].

When an imbalance occurs in the biofilm, due to the increase in the frequency and intake of sucrose, some species of bacteria saccharolytic aciduric and acidogenic, such as *Streptococcus mutans* [4,5], has increased its development as well as other species of *Streptococcus* and *Lactobacillus.* Thus, there is the production of acids, there is a decrease in pH and subsequent demineralization of the enamel and in some cases the caries progress to the dentin [6].

One option for the treatment of dental caries, is the association between mechanical methods and chemical agents. The chemical agent most used is Chlorhexidine which has a broad spectrum of action, however, it presents numerous side effects such as discoloration of resins and teeth, taste alteration, burning sensation, among others [7].

Therefore, alternative antimicrobial products that cause bacterial reduction, without causing adverse side effects are needed. Aiming to overcome this problem, photodynamic therapy (PDT) has touted as a promising alternative for the treatment, enabling an efficient disinfection of the oral cavity [8]. PDT uses a photosensitizer in resonance with a light source, because of the resulting products of the reaction, called reactive oxygen species (ROS), bacterial death occurs. The photosensitizer and light alone do not promote antimicrobial activity [9,10].

Many photosensitizers in PDT have been used, as is the case of methylene blue. The methylene blue belongs to the phenothiazinium family, over a century it has been used for surgical identification, very effective against bacteria, fungi and viruses, it features high production of singlet oxygen, it absorbs light at the wavelength of the red region (650-670 nm), it presents a reduced cost when compared to other photosensitizers and it does not present dark cytotoxic at low concentrations [11].

Although methylene blue showed to be effective when used against the most important bacteria associated with dental caries, *Streptococcus mutans*, a study to associate the photosensitizer to make it even more effective is necessary. In this way, sodium nitrate has raised interest due to its properties of electrons acceptors in anaerobic chains and to be relevant in the anoxic process biofilms, triggering a response to environmental stress that occurs in the formation of biofilm, concomitantly leads to deficiency of biofilm mediated synthesis of polysaccharide intercellular adhesin (PIA) [12-14].

Thus, the aim of this study was to evaluate, in vitro, the effect of photodynamic therapy on the viability of *Streptococcus mutans* planktonic bacterial culture mediated by methylene blue associated or not by sodium nitrate in combination with a red Laser.

Material and Methods

Photosensitizer and Chemical Compound used for Photosensitizer Association

Methylene blue [3,7 bis (dimethylamino) phenazathionium chloride, Tetramethylthionine chloride**]** (Sigma Aldrich, St. Louis, Missouri, EUA) was used as photosensitizer (P). The photosensitizer was prepared in sterile distilled water in different concentrations (0.625, 1.25, 2.5, 5, 10 and 20µM), associated or not with sodium nitrate. The sodium nitrate (Sigma Aldrich, St. Louis, Missouri, EUA) was prepared in sterile distilled water to associate the photosensitizer, in concentration of 20mM. The photosensitizer was mixed with sodium nitrate, in the same proportions, the wells of the microculture plate, according to the different concentrations. The photosensitizer associated or not was excited with Laser in the red region of the spectrum, where photosensitizer associated or not absorbs the light delivered, with maximum wavelength of 660nm. The characteristics of ultraviolet – visible absorption spectrum of the compounds, before (a) and after (b) irradiation, are illustrated in Figure 1.

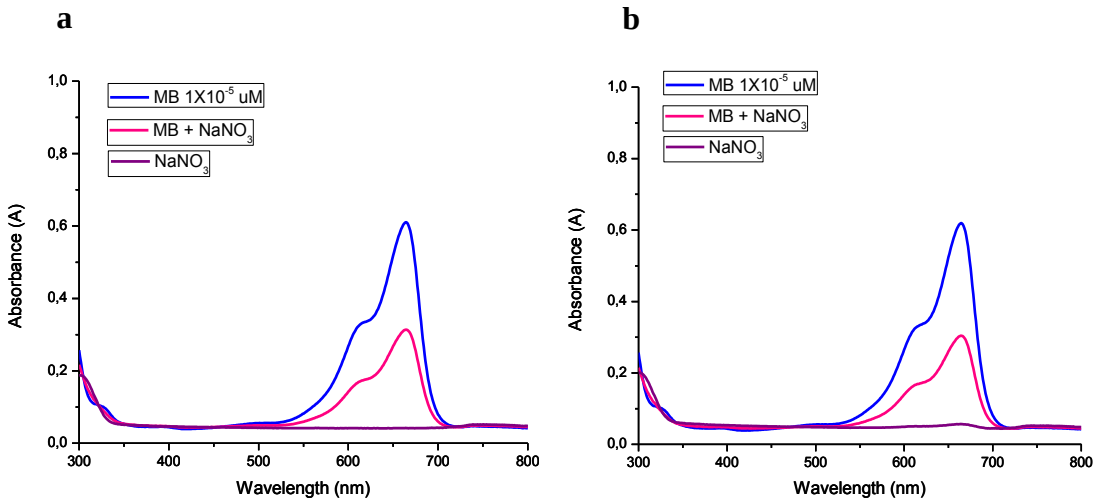

Figure 1. Absorption spectrum of methylene blue (MB), methylene blue associated with sodium nitrate (MB + $NaNO_3$) and sodium nitrate ($NaNO_3$), before (a) and after (b) irradiation.

Light Source

A low level Laser (gallium arsenide) [TWIN FLEX II muntifunctional system] (MM Optics Ltda., São Carlos, Brazil) in the red wavelength was used to excite the methylene blue associated or not with sodium nitrate. The equipament's power was checked with the power meter. The parameters used in this study were: wavelength of 660nm, power of 40mW, energy fluency of 30J/cm^2 and irradiation time of 30s and with respect to working distance, the tip of the Laser touched the well edge. The calculation regarding the irradiation time was determined by the equipment itself.

Selection of Bacteria and Preparation of Bacterial Suspension Standardized

Streptococcus mutans (strain ATCC 25175) was used, provided by the Foundation Fio Cruz (Department of Microbiology, Reference Materials Laboratory, Sector Reference

Bacteria located in the Oswaldo Cruz Foundation, National Institute of Health Quality Control -. INCQS, Av Brazil , 4365 - Manguinhos, Cep: 21045-900, Rio de Janeiro, RJ, Brazil). A *Streptococcus mutans* suspension standard containing $1X10^8$ CFU/mL was prepared as follows: initially, 3 to 5 colonies were collected from the Petri dish containing BHI agar (BHI Agar-Brain Heart Infusion, Difco Laboratories, Becton Dickinson and Company, USA) + 1% sucrose, placed in a 15mL falcon tube containing 5mL Bacto™ BHI broth + 1% sucrose and incubated at 37° C (± 1° C) for 18 hours. After growth, the bacterial culture was centrifuged (Excelsa® II Centrifuge, FANEM, Mod. 206 BL, serial number: HV 9462) at 3000 rpm for 15 minutes, providing the obtaining of the pellet. The supernatant was discarded and the pellet resuspended in PBS (phosphate buffered saline) until reaching the absorbance of 0.08 read at 600nm, with amount of cells in the order of 10^8 CFU/mL, with a spectrophotometer (Eppendorf AG, Hamburg, Germany).

Dark Cytotoxicity Evaluation in Methylene Blue Associated and Non-Associated by Sodium Nitrate

After preparation of standardized bacterial suspension, 250µL of this was pipetted into microculture plates of 48 wells. Then, different concentrations of the photosensitizer associated or not by sodium nitrate (L-P+) was prepared and added to 250µL of the photosensitizer of the standardized suspension. For the group without light and without photosensitizer (L-P-), 250µL of distilled water was pipetted, for the positive control group, 250µL of chlorhexidine Colgate PerioGard (0.12%) and 250µL to the group only of sodium nitrate. Each well had all its contents homogenized 3 times. The photosensitizer remained in touch with the planktonic cells for 5 minutes, which is called pre-irradiation time. After this period, each well was homogenized again for 3 times and then the quantification of viable planktonic bacterial cells was made. Each experiment was realized in three different occasions. Aliquots of each group (10µL) (drop technique) and serial dilution of 10^{-1} to 10^{-5}

times (100µL) the original concentrations were transferred in triplicate to Petri dishes containing BHI agar. After the incubation period (37° C ± 1° C for 48 hours), the total number of colony forming units (CFU/ml) was determined. The number of CFUs per millimeter (CFU mL^{-1}) was obtained and transformed into logarithm (log_{10}).

Photodynamic Therapy

Aliquots of 250µL of *Streptococcus mutans* standard suspension were placed in 48-well culture cell plate. Then, the distribution of the solution with the photosensitizer associated or not by sodium nitrate (250µL) in the wells in accordance with the groups was made. For the control groups of photodynamic therapy, L-P- (without light and photosensitizer) was added to 250µL of distilled water, L-P+ (no light and with photosensitizer) 250µL of photosensitizer at different concentrations associated or not by sodium nitrate, positive control (250µL chlorhexidine at 0.12%) and sodium nitrate (250µL). The entire contents of wells were homogenized 3 times and the photosensitizer remained in touch with the planktonic cells for 5 minutes and 30 seconds, to check any toxicity. Regarding photodynamic therapy groups (L+P+) was added 250µL of various concentrations of the photosensitizer associated or not by sodium nitrate and the plates were incubated in the dark for 5min (pre-irradiation time). After this period, samples from groups L+P+ and L+P- were exposed to Laser light and were illuminated for 30s (660nm and 30J/cm^2). The control groups (L-P- and chlorhexidine), L-P+ and sodium nitrate, remained in the dark for the period (5min and 5s). After irradiation, the wells were homogenized again for three times and a quantification of viable planktonic bacterial cells by counting colony forming units (CFU/mL) was performed. Each experiment was realized in three different occasions. Aliquots of each group (10µL) (drop technique) and serial dilution of 10^{-1} to 10^{-5} times (100µL) the original concentrations were transferred in triplicate to Petri dishes containing BHI agar. After the incubation period (37° C ± 1° C for

48 hours), the total number of colony forming units (CFU/mL) was determined. The number of CFUs per millimeter (CFU mL^{-1}) was obtained and transformed into logarithm (log_{10}).

Statistical Analysis

The results presented normality and homocedasticity, and were evaluated by the Analysis of Variance (ANOVA) of one and two factors and Tukey's test. Was evaluated the logarithm CFU/mL, respectively, the dark cytotoxicity of methylene blue associated or not at various concentrations and aPDT effect on planktonic bacterial cells, using methylene blue in varying concentrations irradiated or not by Laser light source. Differences were considered statistically significant at $p < 0.05$.

Results

Dark Cytotoxicity

Table 1 shows the descriptive statistics of decimal logarithm of CFU/mL, obtained from three different occasions for each experimental condition, to evaluate the dark cytotoxicity of methylene blue associated or not with sodium nitrate, at various concentrations. The microrganisms survival rates are also given compared to the control, calculated on the average log (CFU/mL) and should be interpreted in the logarithmic scale.

Table 1. Mean values (standard deviation) of logarithms of CFU/mL and percentage of survival of planktonic bacteria compared to control for dark cytotoxicity for methylene blue (MB), associated or not by sodium nitrate, at various concentrations.

Concentration	MB				MB associated			
(μM)	Mean	(SD)	% of survival		Mean	(SD)	% of survival	
0	7.29	(0.10)	100.0	b	7.27	(0.01)	100.0	c
0.625	7.17	(0.11)	98.3	b	7.14	(0.02)	98.2	c
1.25	7.29	(0.11)	99.9	b	7.16	(0.02)	98.5	c
2.5	7.28	(0.13)	99.8	b	7.19	(0.02)	99.0	c
5	7.21	(0.12)	98.9	b	7.16	(0.01)	98.5	c
10	7.24	(0.17)	99.3	b	7.15	(0.03)	98.4	c
20	7.28	(0.12)	99.8	b	7.16	(0.02)	98.5	c
Chlorhexidine	0.00	(0.00)	0.0	a	0.00	(0.00)	0.0	a
Nitrate					6.54	(0.04)	90.0	b

Mean values accompanied by the same letters, in column, are not significantly different to Tukey test ($p > 0.05$)

The analysis of variance on the methylene blue associated and not associated, did not accuse significant difference between groups ($p > 0.05$) and the Tukey test showed equivalence between the mean log (CFU/mL) demonstrating that the photosensitizer not irradiated by the light source, in its different concentrations, was not cytotoxic. The chlorhexidine led to the complete reduction of the bacteria. While sodium nitrate showed no significant reduction.

Figure 2 shows the mean values of decimal logarithm of CFU/mL on the dark cytotoxicity of methylene blue, with intervals of 95% for the population average. These intervals estimate the accuracy of the medium, having been built based on the average standard deviation used in the analysis of variance. This standard deviation compensates to some extent the variation between the standard deviations of the experimental groups shown in Table 1.

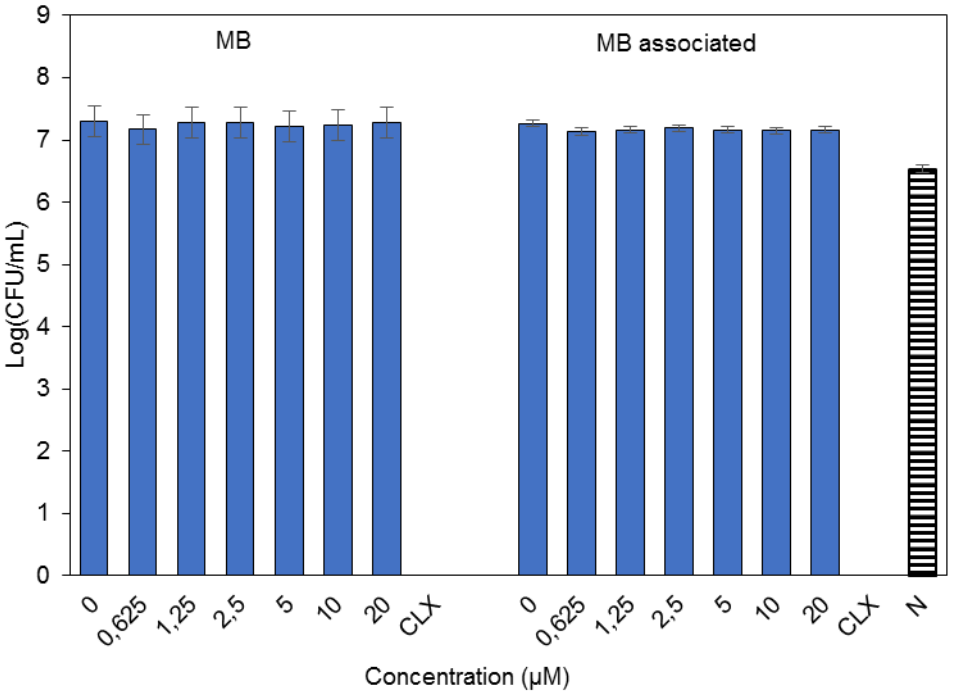

Figure 2. Mean values (columns) of logarithms of CFU/mL and confidence intervals of 95% for the population means (error bars) for dark cytotoxicity for methylene blue (MB), associated or not with sodium nitrate, in varying concentrations, including chlorhexidine (CLX) and sodium nitrate (N) applied to planktonic bacterial cells.

Photodynamic Therapy

Table 2 presents the descriptive statistics of decimal logarithm of CFU/mL of each experimental group, to evaluate the aPDT efficiency in elimination of planktonic bacterial cells, using a methylene blue associated or not by sodium nitrate, in varying concentrations, with and without Laser irradiation (30J/cm^2), besides groups with application of chlorhexidine and sodium nitrate. Planktonic cells survival rates compared to the control, calculated on the average log (CFU/mL), are also in this table and are interpreted on a logarithmic scale. It is seen that methylene blue associated with sodium nitrate led to complete elimination of bacteria in concentrations between 0.625 and 5µM, as well as chlorhexidine.

Table 2. Mean values (standard deviation) of logarithms of CFU/mL and survival percentage

of microrganisms compared to control, as the efficiency of aPDT in planktonic bacterial cells, using methylene blue (MB), associated or not with sodium nitrate, in varying concentrations, with and without Laser irradiation, including the application of chlorhexidine (CLX), sodium nitrate (N) and irradiated sodium nitrate (NI).

Light	Concentration	MB				MB associated			
(J/cm²)	(μM)	Mean	(SD)	% of survival		Mean	(SD)	% of survival	
0	0	7.40	(0.01)	100.0	h	7.21	(0.01)	100.0	d
	0.625	6.87	(0.02)	92.8	fgh	7.16	(0.01)	99.3	d
	1.25	7.25	(0.02)	97.9	gh	7.15	(0.01)	99.3	d
	2.5	7.21	(0.04)	97.3	gh	7.16	(0.01)	99.3	d
	5	7.27	(0.06)	98.1	gh	7.17	(0.02)	99.5	d
	10	6.84	(0.26)	92.4	fgh	7.17	(0.01)	99.5	d
	20	6.98	(0.06)	94.3	fgh	7.16	(0.01)	99.3	d
30	0	6.69	(0.25)	90.3	efg	7.13	(0.08)	98.9	d
	0.625	6.10	(0.00)	82.3	de	0.00	(0.00)	0.0	a
	1.25	4.38	(0.04)	59.2	c	0.00	(0.00)	0.0	a
	2.5	3.40	(0.23)	45.9	b	0.00	(0.00)	0.0	a
	5	4.49	(0.00)	60.7	c	0.00	(0.00)	0.0	a
	10	5.89	(0.75)	79.6	d	6.34	(0.12)	88.0	c
	20	6.35	(0.16)	85.7	def	6.27	(0.09)	87.1	c
Chlorhexidine		0.00	(0.00)	0.0	a	0.00	(0.00)	0.0	a
N						6.43	(0.05)	89.3	c
NI						6.00	(0.13)	83.2	b

Mean values accompanied by the same letters, in column, are not significantly different to Tukey test ($p > 0.05$)

The analysis of variance on the methylene blue not associated showed significant interaction between light and photosensitizer concentrations ($p < 0.001$). The Tukey test was used to compare means two by two, without the inclusion of chlorhexidine who presented total reduction of planktonic cells. A significant reduction was observed at concentrations of 1.25, 2.5 and 5μM, which promoted reductions of 3.02 log_{10}, 4 logs and 2.91 log_{10}, respectively, in the control group (7.4 log_{10}). For the L+P- group the reduction was less than 1 log_{10}, demonstrating that only when the light source combined with photosensitizer were able to promote greater antibacterial activity. And chlorhexidine led to complete reduction of bacteria.

In relation to methylene blue associated, analysis of variance applied in an incomplete factorial, did not include the detected nonsurvival groups, also indicated significant interaction between light and methylene blue concentrations ($p < 0.001$). The Tukey test was applied for multiple comparisons of mean log (CFU/mL) not null, the result is shown in Table 2. A complete reduction in viability of *Streptococcus mutans* in concentrations between 0.625 and 5µM, relative in the control group (7.21 $\log_{10}$), as well as chlorhexidine was achieved. With sodium nitrate and sodium nitrate irradiated reductions were lower, close to 10% and 20% respectively.

Figure 3 shows the mean values of decimal logarithm of CFU/mL to assess the efficiency of aPDT in planktonic bacterial cells, with intervals of 95% for the population average. In addition to estimating the accuracy on the average, these intervals help you interpret the results of statistical tests, in that, the smaller the overlap, greater evidence of differences between the means.

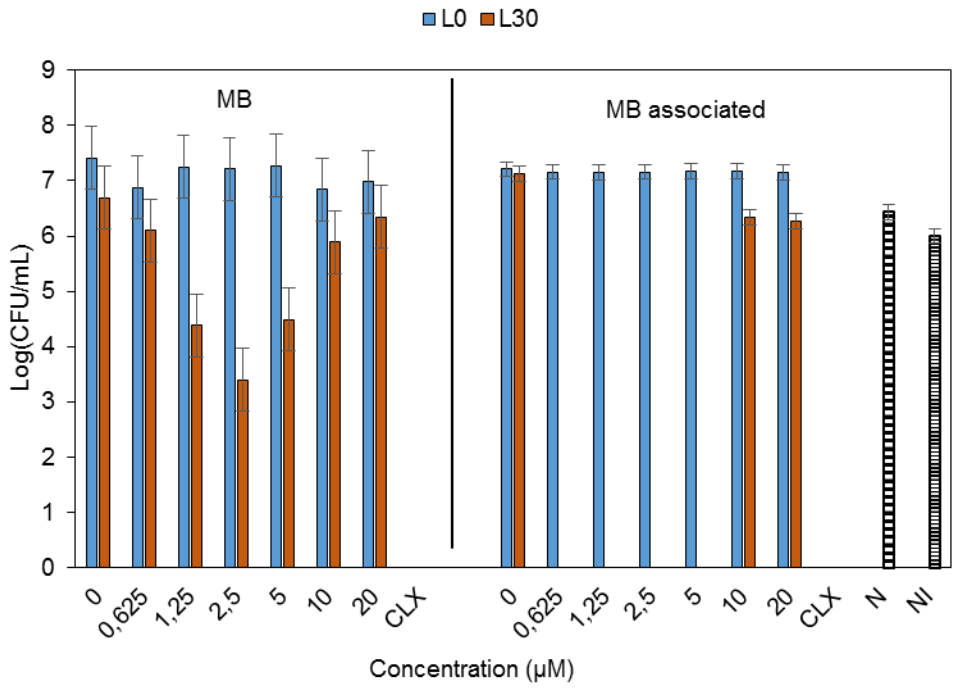

Figure 3. Mean values (columns) of logarithms of CFU/mL and confidence intervals of 95%

for the population means (error bars) as the aPDT in planktonic bacterial cells, using methylene blue (MB), associated or not with sodium nitrate, in varying concentrations, without light (L0) and Laser irradiation (L30), including the application of chlorhexidine (CLX), sodium nitrate (N) and irradiated sodium nitrate (NI).

Discussion

The results showed that the association of the photosensitizer methylene blue with sodium nitrate in PDT when irradiated with an appropriate light source, presented a great potential compared to the methylene blue not associated. Additionally, *Streptococcus mutans* is the main etiological agent of dental caries and showed a high sensitivity to the studied parameters. The synergism between the methylene blue associated by sodium nitrate and the light was confirmed with the total log reduction of planktonic bacteria, in concentrations between 0.625 and 5µM, as well as chlorhexidine ($p < 0.001$). In only light groups and only photosensitizer, the reduction observed was less than 1 $\log_{10}$. While the methylene blue not associated in the presence of light led to a reduction of 3.02 $\log_{10}$ (1.2µM), 4 $\log_{10}$ (2.5µM) and 2.91 $\log_{10}$ (5µM), and the chlorhexidine led to the complete reduction of bacteria in the planktonic phase ($p < 0.001$).

The absorption spectrum of methylene blue associated or not fits with the region of emission of light from the light source used (Laser). This combination of methylene blue with Laser, has been used effectively in PDT by Fontana et al. [15].

The methylene blue concentrations associated or not with sodium nitrate tested in this study (0.625, 1.25, 2.5, 5, 10 and 20µM) were very low, which does not cause toxicity to oral tissues [16] and dark cytotoxicity occurs at concentrations greater than 20µM [11], as confirmed by the results of this study wherein the photosensitizer in its associated form or not,

and not irradiated by the light source, at different concentrations, was not cytotoxic to planktonic bacteria. The concentrations between 1.25 and 5µM of methylene blue not associated irradiated promoted reduction of 40% and 2.5µM concentrations promoted reduction of 55% in the planktonic phase. The lowest concentration (0.625µM) or highest (10 and 20µM) showed reductions between 10 and 20%.

Many studies on PDT have used methylene blue as photosensitizer to reduce the number of microrganisms in the oral cavity in planktonic phase and in biofilms [15,17-19]. Such as the study by Fontana et al. [15], which evaluated the effect of PDT using methylene blue in microrganisms of human dental plaque in the planktonic phase and biofilm, and observed that 25µg/mL methylene blue in combination with red light (665nm and 30J/cm^2), led to the death of 63% of the bacteria causing of chronic periodontitis in planktonic phase and 32% in biofilm. The use of methylene blue has become common due to some inherent characteristics of this photosensitizer, as in low concentrations it does not show toxicity to the host, low cost, effective against bacteria and fungi and it still presents itself to be clinically effective in some cancers [9,11,17].

In the planktonic phase, microrganisms present more susceptibility to the photosensitizer, because the surface of the microrganism wall is free [15]. However, antimicrobial strategies to increase the potential of PDT against the main cause of dental caries has been proposed, and this study proposed to associate the photosensitizer with sodium nitrate, and the results showed that concentrations of methylene blue varied between 0.625 and 5µM in association with the light source, resulted in the total elimination of planktonic bacteria and that the two highest concentrations resulted in reductions slightly higher than 10%. With sodium nitrate and sodium nitrate irradiated reductions were lower, close to 10% and 20% respectively.

Only recently bactericidal potential has been shown for sodium nitrate and also sodium nitrite, because the characteristic of being electron acceptor under anaerobic conditions as quoted beforehand [12-14]. However, no studies in literature were found on methylene blue associated by sodium nitrate, which makes this study unique. What is reported in literature are a few studies on the use of sodium nitrate, as the study of Horn et al. [12], which investigated the sodium nitrate diffusion coefficients in heterotrophic biofilm and concluded that the diffusion coefficient decreases within the biofilm with increasing biofilm density. In another study [13] the authors provide evidence that nitrite, either as endogenous respiration of nitrate reduction products or after external addition, causes suppression of gene expression during growth of the biofilm and further causes a response to oxidative and nitrosative stress.

Only light or only the photosensitizer associated or not by sodium nitrate showed no significant antibacterial effect in this study performed. These results highlight the need to use the photosensitizer in combination with light to ensure the effectiveness of aPDT.

The high concentrations used in study for methylene blue associated or not with sodium nitrate (10 and 20μM) when associated with Laser, showed no significant antibacterial effect. Probably this occurred because the light present difficulty spreading, because the photosensitizer is darker in high concentrations [11].

Additionally, chlorhexidine solution also showed significant antimicrobial effect, complete elimination of bacteria in the planktonic phase, which could be observed in Figures 2 and 3. Although chlorhexidine has made a great antimicrobial effect, the adverse effects that it may cause cannot be pointed out, as previously mentioned [20].

However, although these results are highly promising, other studies should be developed in order to make the use of photodynamic therapy applicable in clinical dentistry

and parameters are set for what is also effective in eliminating bacteria in biofilms and the carious lesions where bacteria are more resistant than bacteria grown in planktonic form.

Conclusion

Antimicrobial photodynamic therapy is an interesting alternative to antibiotics and antimicrobial solution to reduce the microrganisms present in the oral cavity. Therefore, this study may suggest that the use of methylene blue associated by sodium nitrate at concentrations between 0.625 and 5µM associated with the red light source (Laser) promotes significant reduction of *Streptococcus mutans* in planktonic phase, and may be a viable technique to eliminate and control these bacteria that are present in the oral cavity.

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Conflict of interest The authors declare that they have no conflict of interest.

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3.2 Artigo 2- Artigo elaborado nas normas da revista "Photochemistry and Photobiology"

Susceptibility of *Streptococcus mutans* Biofilms to Photodynamic Therapy using Methylene Blue Associated with Sodium Nitrate.

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ABSTRACT

The aim of this work was to evaluate, the in vitro, the effect of photodynamic therapy on the viability of *Streptococcus mutans* biofilm mediated by methylene blue associated or not by sodium nitrate in association with red Laser (660nm). The biofilms was induced on bovine

dentin fragments and was divided into groups: PS-L- (negative control), PS+L- (photosensitizer associated or not at 6 concentrations: 0.625, 1.25, 2.5, 5, 10 and 20μM), P-L+ (Laser 30J/cm^2), chlorhexidine (positive control), sodium nitrate and group P+L+ (methylene blue associated or not with sodium nitrate and irradiated with Laser). For the dark cytotoxicity test, methylene blue associated or not were incubated in the dark for 5 minutes. The groups of photodynamic therapy were incubated in the dark for 5 minutes and irradiated by red Laser during 30 seconds. Samples were incubated at 37° C during 48h for subsequent visual counting of CFU/mL. Data was transformed into $\log_{10}$, analyzed by one and two-way ANOVA and Tukey's test at $p < 0.05$. The results showed significant decreases in the viability of biofilm when exposed to methylene blue associated. In conclusion, methylene blue associated by sodium nitrate is a viable technique for reduction of *Streptococcus mutans* biofilm.

KEYWORD: Photochemotherapy, Methylene Blue, *Streptococcus mutans*, Dental Caries, Biofilms.

INTRODUCTION

The presence of a wide variety of microrganisms, such as bacteria, viruses, protozoa and fungi (1), which are arranged in the form of biofilms (2) and are responsible for various diseases such as dental caries (3) has been detected in the oral cavity of humans. When an disorganization occurs in the ecosystem of the biofilm, leading to the production of acid and dissolution of calcium phosphate surface on the layer of the tooth structure (1) leading to progression and destruction of the organic fraction (4).

The treatment of caries at an early stage consists of mechanical and chemical removal of the bacterial biofilm, thus eliminating, in a preventive manner, cariogenic microrganisms,

particularly *Streptococcus mutans* (5). For chemical removal, the most commonly used antimicrobial solution is chlorhexidine which has been used to clean infected dental cavities before they are restored to reduce or prevent the incidence of recurrent cavities (6), works in reducing *Streptococcus mutans* levels (6) changes the arrangement of the bacterial biofilm, and thus results in an immediate reduction in salivary bacteria due to its broad spectrum when used, in this case, in the form of mouthwash (7). However, chlorhexidine has some side effects such as: altered taste, burning sensation, staining of teeth and restorations, increased formation of calculation and, more rarely, desquamation of oral epithelium (6, 8-9).

Precisely, according to the quantity of microrganisms present in the oral cavity and the problems presented by chlorhexidine, it is necessary the use of alternative antimicrobial therapeutic techniques for the control of microbial growth, in order to suppress the disease in a biological and conservative manner. Antimicrobial photodynamic therapy (aPDT) emerges in this context (10).

Photodynamic therapy has emerged as a means of noninvasive antimicrobial treatment for infections caused by bacteria, fungi and viruses (11). By associating a photosensitizing substance with light, in the treatment against microrganisms, therapy will be called antimicrobial photodynamic therapy (aPDT), so that the applications do not develop microbial resistance (10, 12-13).

The photosensitizer is necessary to penetrate and fasten in the walls of bacteria and can draw its light from the source (14), promoting thus bacterial kill (15), through the production of radicals that are fundamental for photoprocesses (14). The photosensitizer should exhibit low local toxicity in the absence or presence of light (16), absorbing light in appropriate wavelength (12), not bringing toxic damage to the host (2) and it has a very high binding affinity with microrganisms (3).

Various photosensitizers have been tested in dentistry, many studies show the efficiency of toluidine blue O (TBO), methylene blue (14) and curcumin (15, 17). Therefore, phenothiazine derivatives such as methylene blue, and toluidine blue, are photosensitizers that are mostly used in dentistry (12, 18).

Methylene blue is used as a photosensitizing agent since the 1920s, to detect premalignant lesions of the mucosa, as a marker, and it is very efficient in gram-negative bacteria because it can pass between the porin channels (protein) and this is due to its hydrophilicity, its low molecular weight and its positive charge (3). It belongs to the group of phenothiazines, it is an organic colorant, aromatic, heterocyclic, it is soluble in water or alcohol (19) and has maximum absorption in the visible red region (10). The red light source, which operates between 630 and 700 nm has been widely used in aPDT, it has long wavelength, which facilitates the diffusion of light by biological tissues (20). Thus, the interaction between these sources of red light and a photosensitizer that absorbs light in this wavelength such as Methylene Blue (MB) promotes significant bacterial kill (16).

Changes in different photosensitizers have been proposed in order to increase their efficiency and photodynamic performance against biofilm responsible for dental caries. In this context, sodium nitrate, has been investigated by several authors (21-23). It can be used as a electron acceptors under anaerobic conditions and is relevant in anoxic biofilm process, in which the enzyme respiratory nitrate reductase binds to the membrane, it is reduced to nitrite and under anaerobic conditions may also reduce the ammonium, causing a response to environmental stress occurring in biofilm formation, which concurrently leads to a deficiency of biofilms mediated by synthesis of the polysaccharide intercellular adhesin (PIA) (21-23) (Figure 1). The objective of this study was to associate the methylene blue photosensitizer with sodium nitrate, to increase its efficiency in antimicrobial photodynamic therapy when applied in oral biofilms.

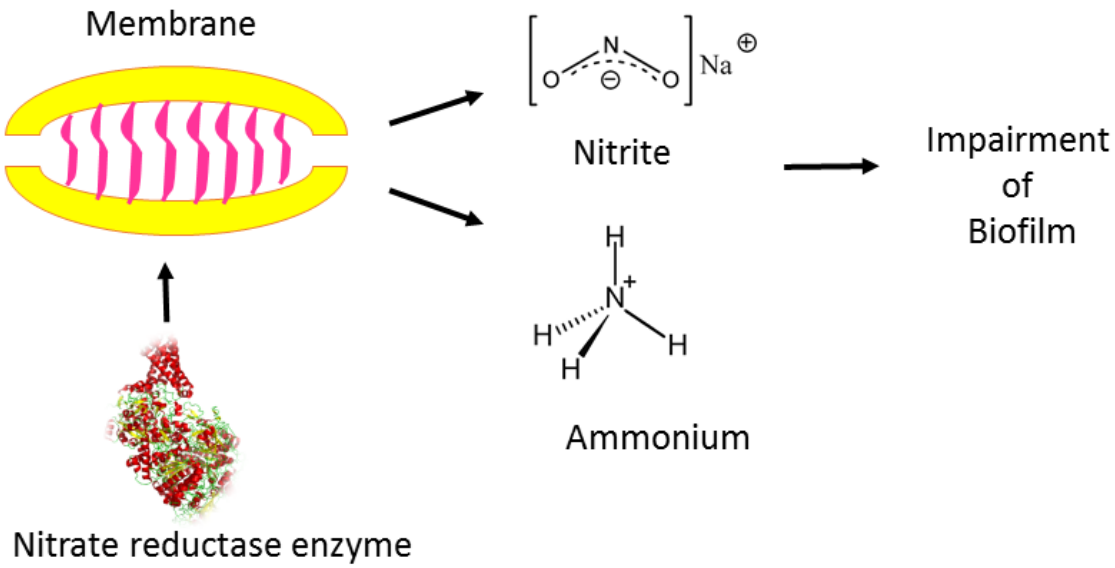

Figure 1. Scheme sodium nitrate mechanism. (Adapted from Vatansever et al 2013 (23)).

MATERIALS AND METHODS

Ethics Committee on Animal Use: This study was approved by the Ethics Committee on Animal Use for the Faculty of Dentistry of Araraquara (UNESP, Brazil), Proc. CEUA No. 22/2014, to use bovine dentin fragments. The bovine teeth were provided by Mondelli.

Photosensitizer and Chemical Compound used for Photosensitizer Association: The methylene blue [3,7 BIS (Dimethylamino) Phenazathionium chloride, tetramethylthionine chloride] (Sigma Aldrich, ST. Louis, Missouri, EUA) was prepared in sterile distilled water in different concentrations (0.625; 1.25; 2.5; 5; 10 and 20µM), associated or not with sodium nitrate. Sodium Nitrate (Sigma Aldrich, ST. Louis, Missouri, EUA) was prepared in sterile distilled water to associate the photosensitizer. The photosensitizer was mixed with sodium nitrate, in the same proportions, the wells of the microculture plate, according to the different concentrations. The methylene blue associated or not with sodium nitrate was excited with

Laser in the red region of the spectrum, where photosensitizer associated or not absorbs the light delivered. The characteristics of ultraviolet – visible absorption spectrum of the methylene blue associated and not associated, and sodium nitrate, before (a) and after (b) irradiation, are illustrated in Figure 2.

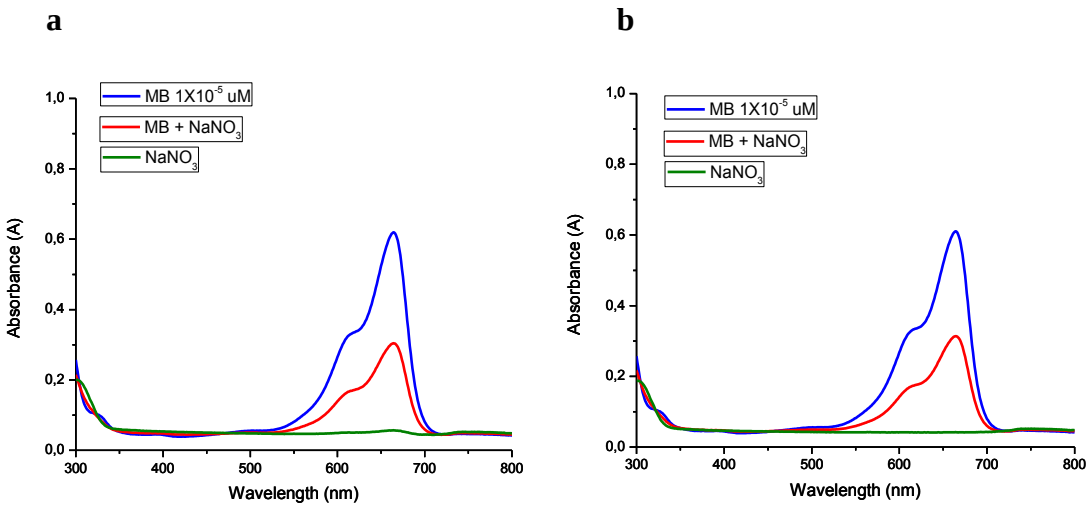

Figure 2. Absorption spectrum of methylene blue (MB), methylene blue associated with sodium nitrate (MB + $NaNO_3$) and sodium nitrate ($NaNO_3$), (a) before and (b) after irradiation.

Light Source: A low level Laser (Gallium arsenide) [TWIN FLEX II multifunctional system] (MM Optics Ltda., São Carlos, Brazil) in the red wavelength was used to excite the methylene blue associated or not with sodium nitrate. The equipment's power was checked with the power meter. The Laser used under the following conditions: wavelength 660nm, power 40mW, energy fluency 30J/cm^2 and irradiation time 30s and with respect to working distance, the tip of the Laser touched the well edge. The calculation regarding the irradiation time was determined by the equipment itself.

Obtaining Dentin Fragments: Central bovine incisors were used for the preparation of specimens. The teeth were provided by the Mondelli company. Firstly the bovine teeth were examined in magnifying glass stereoscopic Zeiss* (10x magnification), to detect possible cracking or structural changes that could cause experimental failure. They were separated from the roots with the aid of a metallographic cutting machine (Isomet 1000 -. Buehler Ltd., Lake Bluff, II, USA) with a diamond blade, under constant cooling water, to obtain dentin fragments in the dimensions of 3x3x2mm. They were sterilized according to the different groups in autoclave (121° C, 1 atm) for 15 minutes in suitable containers with distilled water prior to induction of biofilm.

Preparation of Standardized Bacterial Suspension: *Streptococcus mutans* ATCC 25175 strain was used, provided by the Foundation Fio Cruz (Department of Microbiology, Reference Materials Laboratory, Sector Reference Bacteria located in the Oswaldo Cruz Foundation, National Institute of Health Quality Control -. INCQS, Av Brazil , 4365 - Manguinhos, Cep: 21045-900, Rio de Janeiro, RJ, Brazil). Initially, 3 to 5 colonies were collected from the Petri dish containing BHI agar (BHI Agar-Brain Heart Infusion, Difco Laboratories, Becton Dickinson and Company, USA) + 1% sucrose, placed in a 15 mL falcon tube containing 5mL Bacto^{TM} BHI broth + 1% sucrose and incubated at 37° C (± 1° C) for 18 hours. After growth, the bacterial culture was centrifuged (Excelsa® II Centrifuge, FANEM, Mod. 206 BL, serial number: HV 9462) at 3000 rpm for 15 minutes, providing the obtaining of the pellet. The supernatant was discarded and the pellet resuspended in PBS (phosphate buffered saline) until reaching the absorbance of 0.08 read at 600nm, with amount of cells in the order of 10^8 CFU/mL, with a spectrophotometer (Eppendorf AG, Hamburg, Germany). This suspension was used for biofilm formation and incubation for 7 days.

Bacterial Biofilm Formation in vitro on Bovine Dentin Fragments: The dentine fragments were placed in wells of polystyrene plates of 24 wells (Well plate). One thousand microliters (1000µL) standardized bacterial suspension were added to 10mL of BHI broth + 1% sucrose. After vortex homogenization was placed in each well of 1000µL well plate. Plates were incubated at 37° C (± 1° C) for 7 days. The culture medium was changed every 48 hours.

Dark Cytotoxicity Evaluation in Methylene Blue Associated and Non-Associated by Sodium Nitrate for Biofilm: On the plate where the biofilm was induced, it had its culture medium removed, the fragments were washed 3 times with PBS and the fragments were transferred to a new 48-wells microculture plate. After the different tested concentrations of methylene blue associated or not with sodium nitrate (0.625, 1.25, 2.5, 5, 10 and 20µM) were prepared in distilled water and the *Streptococcus mutans* biofilm (aliquots 500µL for each well) was incubated for 5 minutes (pre irradiation time) to detect any inherent toxicity of the different compounds and if the drug showed antimicrobial activity in the absence of light. Control wells received 500µL of distilled water, chlorhexidine (0.12%) and sodium nitrate. After pre-irradiation time, the fragments were transferred for a tube containing PBS for breakdown in ultrasonic bath for 5 minutes. Each experiment was realized in three different occasions. Aliquots of each group (10µL) (drop technique) and serial dilution of 10^{-1} to 10^{-5} times (100µL) the original concentrations were transferred in triplicate to Petri dishes containing BHI agar. After the incubation period (37° C for 48 hours), the total number of colony forming units (CFU/mL) was determined. The number of CFUs per millimeter (CFU mL^{-1}) was obtained and transformed into logarithm (log_{10}).

Photodynamic Therapy: On the plate where the biofilm was induced, it had its culture medium removed, the fragments were washed 3 times with PBS and the fragments were transferred to a new 48-wells microculture plate. Aliquots of 500µL of each tested

concentrations was added to the wells and was dark incubated for 5 minutes (pre-irradiation time) and after that the wells were illuminated by a red Laser for 30s (treated with methylene blue associated or not with sodium nitrate and Laser: Group PS+L+). It was determined whether photosensitizer associated or not alone induced any toxic effects and sodium nitrate too, but not exposed to Laser (treated only with photosensitizer associated or not in different concentrations: Group PS+L-). The biofilm was exposed to irradiation to determine the isolated effect of Laser (treated only with Laser: Group PS-L+). The control wells consisted of *S. mutans* biofilm without treatment (no treatement: PS-L-) and positive control (Chlorhexidine Colgate PerioGard 0.12%). The effect of sodium nitrate only irradiated was also determined. After irradiation time, the fragments were transferred for a tube containing PBS for breakdown in ultrasonic bath for 5 minutes. Each experiment was realized in three different occasions. Aliquots of each group (10µL) (drop technique) and serial dilution of 10^{-1} to 10^{-5} times (100µL) the original concentrations were transferred in triplicate to Petri dishes containing BHI agar. After the incubation period (37° C for 48 hours), the total number of colony forming units (CFU/mL) was determined. The number of CFUs per millimeter (CFU mL^{-1}) was obtained and transformed into logarithm (log_{10}).

Statistical Analysis: The results presented normality and homocedasticity, and were evaluated by Analysis of Variance (ANOVA) of one and two factors and Tukey's test. Was evaluated the logarithm CFU/mL, respectively, the dark cytotoxicity of methylene blue associated or not at various concentrations and aPDT effect on biofilm, using methylene blue in varying concentrations irradiated or not by Laser light source. Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Dark cytotoxicity

Table 1 shows the descriptive statistics that are shown in decimal logarithm of CFU/mL, obtained from three different occasions of each experimental condition, to assess the dark cytotoxicity of methylene blue associated or not by sodium nitrate, at various concentrations. The survival percentages are also given to microrganisms compared to control, calculated on the average log (CFU/mL) and should be interpreted in the logarithmic scale.

Table 1. Mean values (standard deviation) of logarithms of CFU/mL and percentage of survival of biofilm compared to control for dark cytotoxicity for methylene blue (MB), associated or not by sodium nitrate, at various concentrations.

Concentration	MB				MB associated			
(μM)	Mean	(SD)	% of survival		Mean	(SD)	% of survival	
0	6.58	(0.29)	100.0	b	6.92	(0.08)	100.0	c
0.625	6.47	(0.38)	98.4	b	6.84	(0.14)	98.8	c
1.25	6.68	(0.33)	101.6	b	6.83	(0.04)	98.6	c
2.5	6.91	(0.40)	105.1	b	6.69	(0.09)	96.7	c
5	6.43	(0.24)	97.7	b	6.47	(0.35)	93.5	c
10	6.62	(0.19)	100.6	b	6.57	(0.34)	94.9	c
20	6.48	(0.86)	98.5	b	6.45	(0.43)	93.2	c
Chlorhexidina	5.50	(0.09)	83.6	a	5.30	(0.05)	76.5	a
N					6.27	(0.00)	90.5	b

Mean values accompanied by the same letters, in column, are not significantly different to Tukey test ($p > 0.05$)

Regarding the methylene blue not associated, equivalence between the mean of microrganism counts in various concentrations of methylene blue ($p > 0.05$) was identified. This way, methylene blue not associated did not cause significant reduction of microrganisms, reduction with chlorhexidine was almost 20% in logarithmic scale. While the methylene blue associated also presented equivalent to the control means ($p > 0.05$), no significant reductions, with the exception of chlorhexidine, which showed reduction close to 20%.

According to the results presented, both methylene blue not associated, as for the associated by sodium nitrate, there was no dark cytotoxicity, because the average values observed in the different groups in logarithmic scale (log 10) were next to the control group,

demonstrating that the photosensitizer associated or not with sodium nitrate and not irradiated by the light source, in its different concentrations, it was non-cytotoxic (Table 1). This result may be related to the fact this study only used very low concentrations (0.625, 1.2, 2.5, 5, 10 and 20µM) and according to the literature, only concentrations greater than 20µM exhibit dark cytotoxicity (24, 25).

Figure 3 shows the mean values of decimal logarithm of CFU/mL related to dark cytotoxicity of methylene blue, associated and not associated, with intervals of 95% for the population average. These intervals estimate accuracy on average, they have been built based on the average standard deviation used in the analysis of variance. This standard deviation compensates to some extent the variation between the standard deviations of the experimental groups shown in Table 1.

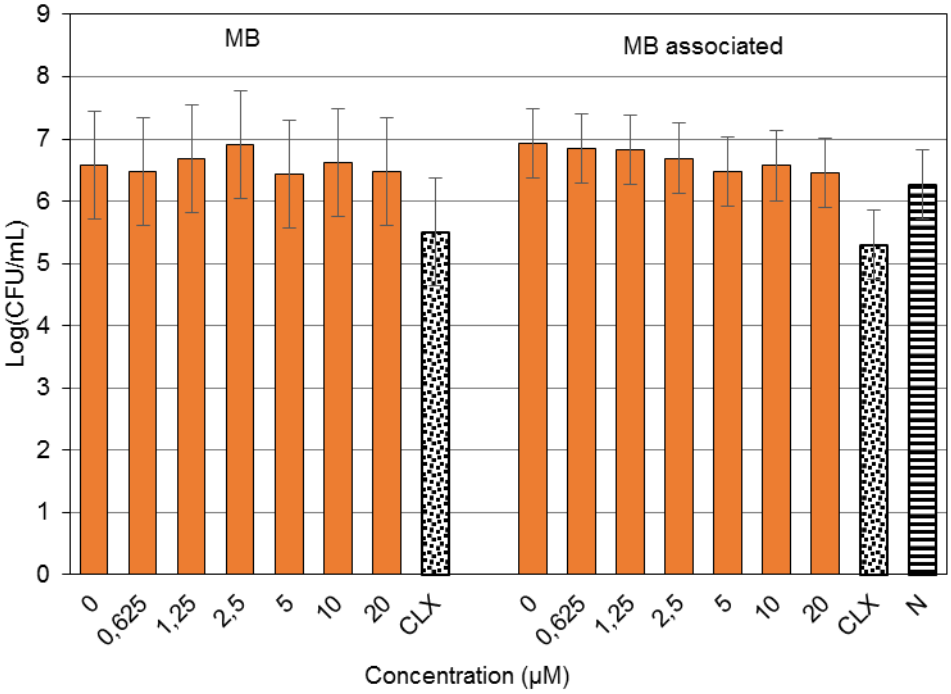

Figure 3. Mean values (columns) of logarithms of CFU/mL and confidence intervals of 95% for the population means (error bars) for dark cytotoxicity for methylene blue (MB), associated or not with sodium nitrate, in varying concentrations, including chlorhexidine (CLX) and sodium nitrate (N), applied in *Streptococcus mutans* biofilm.

Photodynamic therapy

Table 2 shows descriptive statistics logarithms CFU/mL obtained from three independent replicates for each experimental group, to assess the efficiency of aPDT on *Streptococcus mutans* biofilm, using methylene blue associated or not with sodium nitrate, in varying concentrations, with and without Laser irradiation (30J/cm^2) groups in addition to the application of chlorhexidine and sodium nitrate. The microrganisms survival rates compared to the control, were calculated on the average log (CFU/mL), are given in the table and must be interpreted on a logarithmic scale.

Table 2. Mean values (standard deviation) of logarithms of CFU/mL and survival percentage of microrganisms compared to control, as the efficiency of aPDT in *Streptococcus mutans* biofilm, using methylene blue (MB), associated or not with sodium nitrate, in varying concentrations, with and without Laser irradiation, including the application of chlorhexidine (CLX), sodium nitrate (N) and irradiated sodium nitrate (NI).

Light	Concentration	MB				MB associated			
(J/cm^2)	(μM)	Mean	(SD)	% of survival		Mean	(SD)	% of survival	
0	0	6.99	(0.28)	100.0	e	6.94	(0.02)	100.0	f
	0.625	6.43	(0.03)	91.7	de	6.93	(0.02)	99.8	f
	1.25	6.49	(0.05)	92.5	de	6.91	(0.01)	99.7	f
	2.5	6.67	(0.13)	95.1	de	6.89	(0.01)	99.3	f
	5	6.43	(0.18)	91.7	de	6.90	(0.05)	99.4	f
	10	6.38	(0.62)	91.1	de	6.92	(0.02)	99.7	f
	20	6.64	(0.44)	94.7	de	6.90	(0.01)	99.5	f
30	0	6.40	(0.22)	91.2	de	6.93	(0.04)	99.9	f
	0.625	6.17	(0.28)	88.0	cd	5.87	(0.01)	84.6	cd
	1.25	6.19	(0.35)	88.3	cd	5.91	(0.02)	85.2	cd
	2.5	5.21	(0.15)	74.3	a	4.81	(0.03)	69.4	a
	5	5.31	(0.08)	75.7	ab	4.57	(0.03)	65.9	a
	10	6.06	(0.31)	86.4	bcd	5.96	(0.02)	85.9	de
	20	6.34	(0.08)	90.4	de	6.01	(0.28)	86.6	de
Chlorhexidina		5.49	(0.08)	78.3	abc	5.30	(0.05)	76.4	b
N						6.21	(0.01)	89.5	e
NI						5.66	(0.23)	81.5	c

Mean values accompanied by the same letters, in column, are not significantly different to Tukey test ($p > 0.05$)

Relative to the methylene blue not associated, the analysis of variance showed that there was a significant effect of interaction between light and photosensibilizer concentrations ($p < 0.05$). Then, the Tukey test was used for comparison of the experimental groups two by two, with the results shown in Table 2. It is observed that, without the application of light, the average of any concentration of methylene blue was equivalent to the control. But, with irradiation, there was a significant decrease in methylene blue more pronounced in concentrations equal to 2.5µM and 5µM, a reduction of 1.78 log_{10} and 1.68 log_{10} in the control (6.99 log_{10}), respectively, equating to the reduction presented by chlorhexidine (1.5 log_{10}). The group without methylene blue and only light, and groups with higher concentrations of methylene blue (10 and 20µM) had lower logarithmic reductions 1 log, therefore, they did not show significantly important differences.

Regarding associated methylene blue, the variance analysis also indicated significant interaction between light and methylene blue concentrations ($p < 0.05$). As seen from the results of Table 2, the most evident decrease in mean logarithms microrganism counted the concentrations of 2.5µM and 5µM with the application of light, at about 2.13 log_{10} and 2.37 log_{10}, respectively, compared to the control (6.94 log_{10}). These values are higher than chlorhexidine (1.64 log_{10}). With respect to the irradiated sodium nitrate reduction that was smaller (1.32 log_{10}).

Figure 4 shows the mean values of decimal logarithm CFU/mL of aPDT on *Streptococcus mutans* biofilm, with intervals of 95% for the population means. In addition to estimating the accuracy of the medium, these intervals help you interpret the results of statistical tests, in that the smaller the overlap, the greater the evidence of difference between the averages.

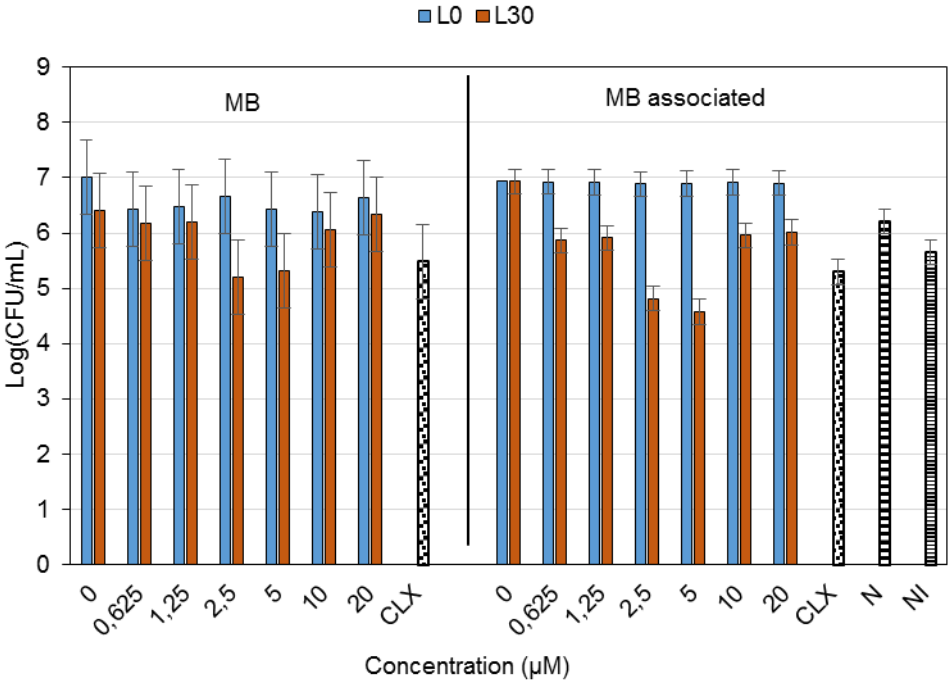

Figure 4. Mean values (columns) of logarithms of CFU/mL and confidence intervals of 95% for the population means (error bars) as the aPDT in *Streptococcus mutans* biofilm, using methylene blue (MB), associated or not with sodium nitrate, in varying concentrations, without (L0) and Laser irradiation (L30), including the application of chlorhexidine (CLX), sodium nitrate (N) and irradiated sodium nitrate (NI).

Several studies have shown that bacteria of the oral cavity are susceptible to photodynamic therapy, even in the form of biofilms (2, 16, 18, 26-29), and the results of this study showed that photodynamic therapy was effective in a significant reduction in the viability of *Streptococcus mutans* biofilm, especially when it associated the photosensitizer methylene blue.

Several studies of photodynamic therapy have also used methylene blue as photosensitizer because of its many advantages, such as its hydrophilicity, low molecular

weight, low cost and maximum absorption in the visible red region (3, 24). How Longo et al. 2010 (18) evaluating the influence of changes in the concentration of the photosensitizer methylene blue (25 and 50µg/mL) and red Laser irradiation energy fluence (6.8J/cm^2, 20.55J/cm^2 and 61.65J/cm^2; 660nm) in bacterial culture viability of human carious dentin, after application of photodynamic therapy and noted that the fluence of 20.55 and 61.65J/cm^2 was effective in reducing microbial load significantly. And Ricato et al. 2014 (29) evaluating, in vitro, the antimicrobial effect of photodynamic therapy using Laser or LED (94J/cm^2) associated with methylene blue (10mM) of *Streptococcus mutans* and *Lactobacillus casei* in bovine dentin and resulted in a great reduction in the number of microrganisms.

Currently, to improve the potential of photodynamic therapy against the microrganisms that cause caries, some photosensitizers association have been proposed, and it is to this end that this work proposes to associate the photosensitizer methylene blue with the inorganic salt sodium nitrate, since methylene blue is as set out in the literature.

Sodium nitrate, with the sodium nitrite, has drawn the attention of researchers because of some features mentioned above, but mainly because they are electrons acceptors under anaerobic conditions and relevance in the anoxic process in biofilms, leading to deficiency of biofilm (21-23, 30-33). Nothing can be found in the literature of methylene blue associated with sodium nitrate, what is found in the literature on sodium nitrate, are initial studies such as Neubauer et al. 1996 (34) whose studied tried to understand the reduction of sodium nitrate in *Staphylococcus carnosus* and concluded that the nitrate is reduced to nitrite and that the accumulated nitrite reduced the ammonia, and that the nitrate reduction seemed to be linked to a membrane bound enzyme, and thus acts as a electrons acceptors under anaerobic chains. Horn et al. 2006 (21), who investigated the sodium nitrate diffusion coefficients in heterotrophic biofilms by means of two tests that evaluated the quantitative density of

biofilms on the diffusion and concluded that the diffusion coefficient of sodium nitrate in biofilms decreases with increasing biofilm density.

The red light source (630-700nm) has also been widely used in photodynamic therapy, due to its long wavelength, facilitating the penetration of biological tissues (16) and when associated with photosensitizers, which absorb at this wavelength, such as methylene blue results in death of microrganisms (35).

Only light, only the photosensitizer or higher concentrations (10 and 20μM) of photosensitizer showed no significant antibacterial effect on the performed study, the reductions were less than 1 $\log_{10}$. These results highlight the need of the photosensitizer in conjunction with light to ensure the effectiveness of aPDT (9, 36).

About the high concentrations used in study for methylene blue associated or not with sodium nitrate (10 and 20μM) when associated with Laser in PDT, showed no significant antibacterial effect. According Yu J et al. 2015 (24) is probably this occurred because the light present difficulty spreading, because the photosensitizer is darker in high concentrations, as 10 and 20μM.

Although chlorhexidine has demonstrated significant antimicrobial effect, its adverse effects cannot be forgotten, as previously mentioned.

Thus, due to adverse effects presented by chlorhexidine and antibiotic resistance onset, antimicrobial photodynamic therapy has become a viable technique for diseases related to the biofilm, as in the case of dental caries.

CONCLUSION

This study indicated that the association of the photosensitizer methylene blue with sodium nitrate in photodynamic therapy was able to reduce the number of viable cells of *Streptococcus mutans* biofilm compared to methylene blue not associated according to the studied parameters.

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4 CONSIDERAÇÕES FINAIS

A terapia fotodinâmica tem sido amplamente utilizada na odontologia para o tratamento de várias doenças, entre ela a doença cárie [2] e tem demonstrado um eficaz efeito antibacteriano [29].

O tratamento da doença cárie consiste na remoção mecânica e química, quando em estágio inicial [32] e quando em estágio mais avançado com a presença de cavitações, o tratamento consiste na remoção da estrutura dentária infectada [30], de tal maneira que os microrganismos sejam removidos devido a impossibilidade de remineralização, e em áreas que são passíveis de remineralização, estas devem ser mantidas [20,38]. No entanto, a remoção precisa destas áreas que não são mais passíveis de remineralização, se torna muito difícil, havendo o risco de exposição pulpar [6,7]. Com a terapia fotodinâmica antimicrobiana, devido a morte bacteriana se dar por meio da formação de espécies reativas de oxigênio e se restringir a área irradiada, o risco de exposição pulpar pode ser evitado e ainda, não existe possibilidade de resistência bacteriana [8,36].

Visando reduzir os microrganismos presentes na cavidade oral, a clorexidina tem sido amplamente utilizada como solução antimicrobiana para limpar as cavidades dentárias infectadas antes de serem restauradas, além de outras funcionalidades [27], pois atua em todos os tipos de bactérias, além de fungos e leveduras [9]. Porém, apresenta inúmeros efeitos colaterais [5,21,27].

Desta forma, a terapia fotodinâmica tem apontado com o intuito de promover a descontaminação geral da cavidade oral [19]. No entanto, visando melhorar o potencial da terapia fotodinâmica contra os microrganismos causadores da doença cárie, estratégias antimicrobianas vêm sendo propostas, como a modificação de fotossensibilizadores.

Assim, este trabalho se propôs à associar o fotossensibilizador azul de metileno com nitrato de sódio. Em cultura planctônica de *Streptococcus mutans* o fotossensibilizador associado ao nitrato de sódio quando irradiado pela fonte de luz Laser, resultou em completa eliminação dos microrganismos nas concentrações entre 0,625 e 5 µM, quando comparado ao fotossensibilizador não associado que apresentou reduções de até 4 logs nas concentrações de 2,5 e 5 µM. Enquanto que no biofilme a associação do fotossensibilizador resultou em reduções entre 2,13 $\log_{10}$ e 2,37 $\log_{10}$ para as concentrações de 2,5 e 5 µM, e para o fotossensibilizador não associado resultou em reduções de 1,78 e 1,68 $\log_{10}$ para as mesmas concentrações.

Analisando ambos os resultados, pode-se concluir que a associação de fotossensibilizadores pode ser uma maneira viável para redução ou eliminação de microrganismos presentes na cavidade oral, principalmente os causadores da doença cárie. No entanto, muitos estudos ainda devem ser realizados visando padronizar os parâmetros para que a terapia fotodinâmica seja aplicada clinicamente e ainda, seja eficiente em biofilmes, visto que a maioria das doenças que acometem a cavidade oral, como a doença cárie, doença periodontal, candidíase, entre outras [30], são resultantes da organização dos microrganismos na forma de biofilme, o que dificulta o sucesso dos tratamentos, devido os biofilmes serem estruturas muito complexas [13].

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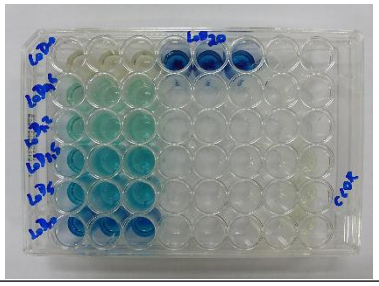
APÊNDICE A - METODOLOGIA ARTIGO 1

1) Espectroscopia na região do UV-visível para determinar as curvas de absorção do fotossensibilizador associado e não associado, antes e após irradiação.

2) Fotossensibilizador: Azul de metileno, nas concentrações de 0,625; 1,25; 2,5; 5; 10 e 20µM.
Composto químico associado ao fotossensibilizador: nitrato de sódio. Os dois compostos foram misturados posteriormente.

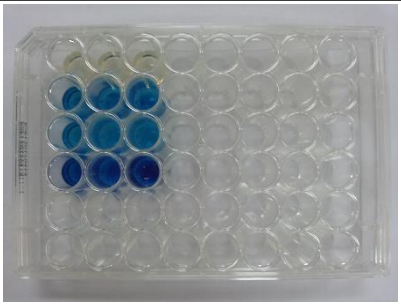
3) Fonte de Luz: Laser vermelho
Comprimento de onda: 660nm
Potência: 40mW
Dose de Energia: 30J/cm^2
Tempo de irradiação: 30s

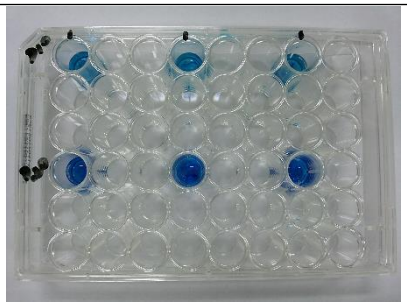
4) Suspensão bacteriana padronizada foi preparada com absorbância de 0,08 lida em 600nm (10^8 UFC/mL).



5) Citotoxicidade no Escuro: 250µL da suspensão + 250µL do fotossensibilizador associado ou não por nitrato de sódio; incubou-se no escuro por 5min e avaliação quantitativa de células viáveis, foi realizada.

6) Terapia Fotodinâmica: 250µL da suspensão + 250µL do fotossensibilizador associado ou não por nitrato de sódio; Controle: incubou-se no escuro por 5 min e 30s, e foi realizada a avaliação quantitativa de células viáveis.



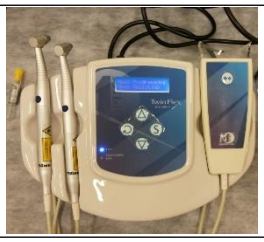


7) Terapia Fotodinâmica**:** 250µL da suspensão + 250µL do fotossensibilizador associado ou não por nitrato de sódio; TFDA: incubou-se no escuro por 5min e irradiou por 30s, e em seguida foi feita a avaliação quantitativa de células viáveis.

APÊNDICE B - METODOLOGIA ARTIGO 2

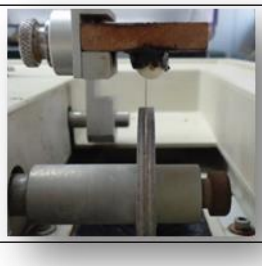
1) Espectroscopia na região do UV-visível: determinar as curvas de absorção do fotossensibilizador associado e não associado antes e após irradiação.

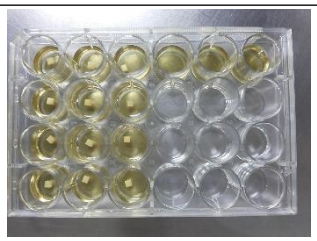
2) Fotossensibilizador: Azul de metileno, concentrações: 0,625; 1,25; 2,5; 5; 10 e 20µM. Composto químico associado ao fotossensibilizador: nitrato de sódio. Os dois compostos foram misturados posteriormente.



3) Fonte de Luz: Laser. Comprimento de onda: 660nm, potência: 40mW, dose de energia: 30J/cm^2 e tempo de irradiação: 30s.

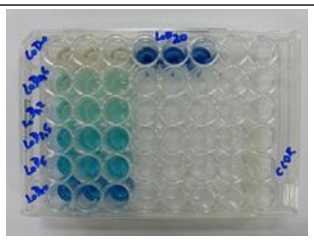
4) Fragmentos de dentina 3x3x2mm, obtidos por meio da máquina de cortes Isomet e disco diamantado. Os fragmentos foram autoclavados de acordo com os diferentes grupos para realização dos testes.

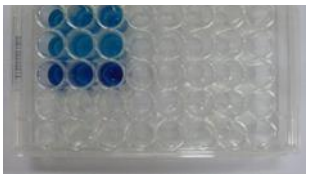




5) Suspensão bacteriana foi preparada com absorbância de 0,08 lida em 600nm (10^8 UFC/mL) e indução do biofilme por 7 dias.

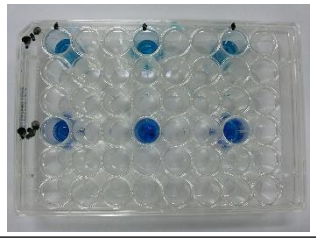
6) Citotoxicidade no Escuro: 250µL da suspensão + 250µL do fotossensibilizador associado ou não por nitrato de sódio; incubou-se no escuro por 5min e foi realizada a avaliação quantitativa de células viáveis.





7) Terapia Fotodinâmica: 250µL da suspensão + 250µL do fotossensibilizador associado ou não por nitrato de sódio; Controle: incubou-se no escuro por 5min e 30s, e foi realizada a avaliação quantitativa de células viáveis em seguida.

8) Terapia Fotodinâmica: 250µL da suspensão + 250µL do fotossensibilizador associado ou não por nitrato de sódio; TFDA: incubou-se no escuro por 5min e irradiou por 30s, e em seguida foi realizada a avaliação quantitativa de células viáveis.



ANEXO A – Aprovação Comitê de Ética

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Câmpus de Araraquara
FACULDADE DE ODONTOLOGIA

Proc. CEUA nº 22/2014

Araraquara, 16 de setembro de 2015.

Senhores Pesquisadores:

A Comissão de Ética no Uso de Animal – CEUA desta Faculdade, procedeu a análise do Relatório Parcial do projeto de pesquisa de sua responsabilidade intitulado ***"MODIFICAÇÃO EM FOTOSSENSIBILIZADOR E SUA APLICAÇÃO NA TERAPIA FOTODINÂMICA ANTIMICROBIANA EM SUBSTRATO DENTINÁRIO"*** (Proc. CEUA nº 22/2014), e considerou-o APROVADO, bem como sua solicitação de alteração da metodologia da pesquisa.

Lembramos que o Relatório Final deste projeto deverá ser entregue em **JUNHO/2016**.

Atenciosamente.

Prof. Dr. PAULO SÉRGIO CERRI
Coordenador da CEUA

À
Profa. Dra. ALESSANDRA NARA DE SOUZA RASTELLI
DD. Pesquisadora Responsável
Departamento de Odontologia Restauradora

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Araraquara, 31 de Março de 2016.

Emanuelle Teixeira Carrera