UNESP - Universidade Estadual Paulista

"Júlio de Mesquita Filho"

Faculdade de Odontologia de Araraquara

Lívia Jacovassi Tavares

Eficácia da terapia fotodinâmica antimicrobiana associada ao metronidazol em biofilmes de *Fusobacterium nucleatum* e *Porphyromonas gingivalis*

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Resumo

O objetivo deste estudo foi avaliar a eficácia da terapia fotodinâmica antimicrobiana associada (aPDT) ao metronidazol (MTZ) em biofilmes periodontopatogênicos. Para tal finalidade, foram realizadas as seguintes etapas: (1) determinação do tempo de adesão (24 e 48 horas) e formação de biofilme mono e duo-espécie (3, 5 e 7 dias) de *Fusobacterium nucleatum* (NCTC 11326) e *Porphyromonas gingivalis* (ATCC 33277); (2) aplicação da aPDT mediada por PDZ associada ao MTZ em biofilmes mono-espécie de *F. nucleatum* e *P. gingivalis.* Foram avaliadas diferentes concentrações do PDZ (50, 75 e 100 mg/L) e dose de luz de 50 J/cm^2 (660nm). Após a aplicação da aPDT, os biofilmes foram incubados com diferentes concentrações do MTZ (MIC, 50x MIC e 100x MIC) por 24 horas. Os grupos controles positivos (L-F-) não receberam fotossensibilizador e não foram iluminados. A viabilidade dos microrganismos após os tratamentos foi avaliada por meio da contagem de UFC/ml. Os resultados demonstraram que o período de adesão de 24 horas, seguido de 5 dias de formação de biofilme foi satisfatório para a obtenção de biofilmes maduros mono-espécie. Para *F. nucleatum*, os resultados demonstraram que aPDT 75 mg/mL associado com MTZ 100x MIC e aPDT 100 mg/L associado com MTZ nas concentrações de 50x MIC e 100x MIC reduziu significativamente o número de UFC/mL, 2,99; 2,9 e 3,94 Log_{10} respectivamente. Para *P. gingivalis*, a redução mais significativa de UFC/mL foi obtida quando a associação de aPDT 100 mg/L e MTZ 100x MIC foi realizada, resultando em 5 Log_{10} de redução. Adicionalmente, houve redução significativa nos grupos que foram expostos apenas à luz ou à maior concentração de antibiótico, 1,71 e 3,07 Log_{10}, em comparação com o

grupo sem tratamento. O efeito do tratamento da aPDT associada à MTZ foi potenciado quando comparado aos tratamentos isolados.

Palavras chave: Fotoquimioterapia. Metronidazol. Fusobacterium nucleatum. Porphyromonas gingivalis.

Tavares LJ. Efficacy of antimicrobial photodynamic therapy associated with metronidazole on biofilms of Fusobacterium nucleatum and Porphyromonas gingivalis [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2017.

Abstract

The aim of this study was to evaluate the efficacy of metronidazole (MTZ) associated antimicrobial photodynamic therapy (aPDT) on periodontopathogenic biofilms. For this purpose, the following steps were performed: (1) determination of adhesion period (24 and 48 hours) and single and duo species biofilm formation (3, 5 and 7 days) of *Fusobacterium nucleatum* (NCTC 11326) and *Porphyromonas gingivalis* (ATCC 33277); (2) Photodithazine ® (PDZ)- mediated aPDT in association with MTZ in single-specie biofilms of *F. nucleatum* and *P. gingivalis*. Different concentrations of PDZ (50, 75 e 100 mg/L) and light dose of 50 J / cm2 (660nm) were evaluated. After application of aPDT, the biofilms were incubated with different concentrations of MTZ (MIC, 50x MIC and 100x MIC) for 24 hours. Positive control groups (L-F-) received no photosensitizer and were also not illuminated. The viability of the microorganisms after the treatments was evaluated by counting CFU/ml. The results demonstrated that the 24 hours adhesion period followed by 5 days of biofilm formation was satisfactory for obtaining a mature biofilm in single-specie. For *F. nucleatum*, the results demonstrated that 75 mg/L aPDT associated with MTZ 100x and 100 mg/mL aPDT associated with MTZ at 50x MIC and 100x MIC concentrations significantly reduced the number of CFU/mL, 2.99; 2.9 and 3.94 Log_{10} respectively. For *P. gingivalis*, the greatest reduction of CFU/mL was obtained when the association of aPDT 100 mg/L and MTZ 100x MIC was performed, resulting in 5 Log_{10} reduction. Additionally, there was a significant reduction in the groups that were exposed only to the light or the highest concentration of antibiotic, 1.71 and 3.07 Log_{10}, compared to the group without treatment. The

treatment effect of MTZ-associated aPDT was potentiated when compared to the isolated treatments.

Key words: Photochemotherapy. Metronidazole. Fusobacterium nucleatum. Porphyromonas gingivalis.

SUMÁRIO

1 INTRODUÇÃO

De acordo com o "Instituto Nacional da Saúde" (NIH), infecções causadas por microrganismos organizados em biofilmes, são consideradas um problema crônico de saúde pública, uma vez que as doenças decorrentes desta comunidade microbiana estão associadas a 80% de todas as infecções em seres humanos (http://grants.nih.gov/bolsas/guia/pa-files/PA-07-288.html). Biofilmes são estruturas biológicas constituídas por microrganismos envoltos por uma matriz extracelular de polissacarídeos , que assegura a sobrevivência dos mesmos (Lamfon et al.[35], 2005; Ramage et al.[53], 2006), agindo como uma barreira protetora a agentes físicos e químicos externos, o que pode limitar a penetração de agentes antimicrobianos (Evans et al.[13], 1990). Particularmente na cavidade oral, o biofilme é responsável pelo desenvolvimento de diversas patologias, incluindo a doenças periodontal e peri-implantar.

Peri-implantite, assim como a periodontite, é caracterizada pela perda de inserção e destruição do osso alveolar adjacente. Embora estas patologias sejam mediadas e reguladas por processos inflamatórios advindos do próprio hospedeiro, as bactérias são responsáveis pelo seu desenvolvimento (Rosen et al.[56], 2013). De acordo com Socransky, Haffajee[62], 2002, as bactérias envolvidas nestas patologias agrupam-se em complexos, cujo o complexo conhecido como laranja, representado pelas espécies *Peptostreptococcus micros, Prevotella intermedia, Prevotella nigrescens, Fusobacterium periodonticum* e *Fusobacterium nucleatum* têm a capacidade de interagir e favorecer a implantação do complexo vermelho, representado pelas espécies *Porphyromonas gingivalis, Treponema denticola e Tannerella forsythia* (Socransky et al.[61], 1998; Socransky, Haffajee[62], 2002).

P. gingivalis e *F. nucleatum*, são bactérias anaeróbias Gram-negativas associadas com a periodontite crônica, periodontite agressiva localizada e doença peri-

implantar. *P. gingivalis* tem sido considerada uma das principais espécies que desenvolvem a doença periodontal, devido aos seus numerosos fatores de virulência (Hajishengallis, Lamont[26], 2012). *F. nucleatum* é considerado fundamental para a maturação dado biofilme dental, devido à grande capacidade de co-agregação com outros microrganismos, tais como *P. gingivalis* (Kolenbrander, Andersen[32], 1989; Bradshaw et al.[7], 1998). Estudos relatam aumento no grau de patogenicidade, determinado pelos fatores de virulência expressos durante a interação entre essas espécies (Sundqvist et al.[65], 1979; Baumgartner et al.[4], 1992; Feuille et al.[23], 1996; Ebersole et al.[17], 1997). Estas interações são definidas como um sinergismo patogênico que conduzem a uma relação de cooperação que contribui para a sobrevivência e a persistência de ambas em diversos nichos orais (Metzger et al.[41], 2009).

Indivíduos portadores de doença periodontal apresentam grande quantidade de microrganismos patogênicos na cavidade oral. Em caso da perda de dentes comprometidos, estas bactérias permanecem sobre os dentes remanescentes e podem influenciar a microbiota peri-implantar (Metzger et al.[41], 2009). Estudos anteriores identificaram alta prevalência de bactérias anaeróbias Gram-negativas ao redor de implantes com sinais clínicos de peri-implantite (Mombelli et al.[44], 1987; Mombelli, Mericske-ster[43], 1990; Shibli et al.[60], 2008; Tabanella et al.[66], 2009). As bactérias encontradas nestas regiões foram similares as espécies envolvidas na periodontite, incluindo as bactérias do complexos vermelho (*P. gingivalis*, *Treponema dentícola* e *Tannerella forsythia*) e laranja (*Fusobacterium sp*. and *Prevotella intermedia*) (Socransky et al.[61], 1998). Espécies bacterianas como *P. gingivalis* and *P. intermedia* mostraram ter alta afinidade pelo titânio. Esta capacidade de se aderirem diretamente ao titânio poderia causar infecções na região peri-implantar (Kuula et al.[34], 2004). A inflamação em consequência da presença do biofilme bacteriano na região subgingival,

é considerada um dos principais responsáveis pela perda dos implantes após o processo de osseointegração (Hayek et al.[27], 2005; Elter et al.[20], 2008).

O tratamento da peri-implantite deve ser focada na descontaminação da superfície do implante e na regeneração dos tecidos perdidos (Schou et al.[59], 2004; Bautista, Huynh-Ba[5], 2013). No entanto, o desenho e os tratamentos de superfície do titânio podem facilitar a adesão de bactérias e desenvolvimento de biofilme bacteriano (Schou et al.[59], 2004; Bautista, Huynh-Ba[5], 2013). O tratamentos convencional, tal como o debridamento mecânico, é insuficiente para a remoção completa do biofilme (Karring et al.[29], 2005). Então, o uso de antibiótico pode ser indicado para potencializar a redução bacteriana desde exista o reconhecimento da etiologia infecciosa da doença. O metronidazol foi considerado o antibiótico de escolha, dada a sua capacidade de causar dano ao DNA bacteriano, especialmente em anaeróbios como *P. gingivalis* (Müller[47], 1983). Embora os antibióticos tenham seus benefícios, a utilização de grandes quantidades por longos períodos é indesejável na prática clínica devido aos efeitos adversos que causam na microbiota e ao aumento do potencial para induzir resistência.

Recentemente, a terapia fotodinâmica antimicrobiana (aPDT) foi introduzida como uma nova abordagem na descontaminação de superfície de implantes (Marotti et al.[40], 2008; Lima et al.[36], 2009). No processo fotodinâmico, a célula-alvo deve ser tratada com o fotossensibilizador (FS) de absorção máxima de luz específica, em um processo conhecido como fotossensibilização. Em seguida, a interação da luz com comprimento de onda adequado, com o FS e na presença de oxigênio, resulta em espécies reativas de oxigênio capazes de induzir a morte celular. Esse mecanismo envolve a absorção de fótons da fonte de luz pelo FS, o que leva os elétrons a um estado excitado. Na presença de oxigênio, o FS excitado pela luz pode reagir com moléculas vizinhas, por meio da transferência de elétrons ou hidrogênio (reação do tipo I) ou pela

transferência de energia ao oxigênio (reação do tipo II), levando à produção de espécies reativas (Bonnett, Martınez[6], 2001) e, consequentemente, à morte celular. Estudos têm demonstrado que aPDT é mais eficaz na inativação de bactérias Gram-positivas que Gram-negativas, possivelmente pela estrutura química da parede celular das mesmas (Malik et al.[38], 1992). O efeito bactericida da aPDT associada ao azul de metileno (MB) foi avaliado em culturas planctônicas de *Aggregatibacter actinomycetemcomitans, F. nucleatum, P. gingivalis, Prevotela Intermedia e Streptococcus. sanguis.* Os dados mostraram que as espécies Gram-negativas foram mais resistentes e *S. sanguis* foi a espécie mais suscetível (Chan, Lai[9], 2003), sugerindo uma relação direta da composição química da parede celular com o fotossensibilizador utilizado. Estudos anteriores relataram que as suspensões de *P. gingivalis* e *F. nucleatum* são suscetíveis a aPDT (Chan, Lai[9], 2003; Habiboallah et al.[25], 2014). Por outro lado, não foi observada a inativação completa desses microrganismos, quando organizados em biofilmes (Street et al.[64], 2010). Estudos anteriores sugeriram que a aplicação prévia de aPDT associada a um antibiótico poderia potencializar a redução bacteriana (Di Poto et al.[14], 2009; Barra et al.[3], 2015; Ronqui et al.[55], 2016).

Atualmente, uma nova classe de FS vem sendo empregada em aPDT, os fotossensibilizadores de segunda geração. Dentre estes compostos estão as clorinas, porfirinas hidrofílicas reduzidas que apresentam forte banda de absorção na região vermelha do espectro fotomagnético. O Photodithazine ® (PDZ) é uma clorina e6 que mostrou ter efeito significativo em células tumorais (Corrêa[11], 2006). Em estudos preliminares (Dovigo et al.[16], 2013; Quishida et al.[52], 2015; Carmello et al.[8], 2016) foi avaliado a eficácia da PDZ na fotoinativação de cepas de *Candida albicans, Candida glabrata* e *Candida tropicalis* isoladas de pacientes com estomatite protética. As suspensões foram tratadas com 25, 50 e 75mg/L de PDZ e expostas a luz LED a 37,5;

25,5 e 18,0J/cm^2 e os biofilmes foram tratados com maiores concentrações de PDZ (100 e 125mg/L). Os resultados demonstraram que a aPDT promoveu redução significativa na viabilidade da *C. tropicalis* e da *C. glabrata* enquanto cinco cepas de *C. albicans* foram completamente inativadas após a aPDT. A maior redução do biofilme foi observada com a utilização de 125 mg/L de PDZ. Para *C. albicans,* houve uma redução de 62,1% enquanto para a *C. tropicalis* e *C. glabrata* foi observada uma redução de 76 e 76,9%, respectivamente (Dovigo et al.[16], 2013). Quando um biofilme misto formado por *C albicans*, C *glabrata*, e *S mutans* foi submetido a aPDT com o PDZ foi observada redução significativa na viabilidade das colônias das três espécies avaliadas, e redução significativa na atividade metabólica dos biofilmes submetidos a aPDT (Quishida et al.[52], 2015). No tratamento da candidíase oral em um modelo murino, aPDT mediada por PDZ foi tão eficaz quanto a nistatina na inativação de *C. albicans* e apresentou regressão completa das lesões orais após seis aplicações (Carmello et al.[8], 2016)

Apesar dos resultados promissores, seria interessante o desenvolvimento de estratégias que aumentassem a suscetibilidade dos microrganismos aos métodos antibacterianos já conhecidos (Di Poto et al.[14], 2009; Ronqui et al.[55], 2016). A avaliação do pré-tratamento do biofilme maduro de *Streptococcus aureus* com aPDT seguida pela aplicação de vancomicina reduziu significantemente a concentração bacteriana, sugerindo que aPDT poderia provocar a desintegração da matriz extracelular e, consequentemente, aumentaria a suscetibilidade ao antibiótico (Di Poto et al.[14], 2009). Em biofilmes de *S. aureus* e *Escherichia coli* resultados significativos também foram observados na combinação entre aPDT e o ciprofloxaxino, com redução de 5.4 Log_{10} para *S. aureus* e aproximadamente 7 Log_{10} para *E. coli* (Ronqui et al.[55], 2016).

Até o presente momento, os autores não localizaram informações de aPDT mediada pela PDZ sobre bactérias periodontopatogênicas, tão pouco sobre a influência

no tratamento com antibióticos. Dessa forma, o objetivo do presente estudo será avaliar se a aplicação da aPDT poderia atuar no biofilme bacteriano formado pela *P. gingivalis* e *F. nucleatum* e favorecer a suscetibilidade destas bactérias ao antibiótico MTZ.

2 PROPOSIÇÃO

O presente trabalho tem como objetivo geral avaliar in vitro a eficácia da aPDT associada ao MTZ em biofilmes mono-espécie. Para isso, este projeto foi subdividido em 3 publicações, com os seguintes objetivos:

Objetivos Específicos

Publicação 1- Revisão de literatura para melhor entendimento dos mecanismos que envolvem aPDT na peri-implantite

Publicação 2 - Avaliar a adesão e o período satisfatório para a formação de biofilme maduro mono e duo-espécie de *P. gingivalis* e *F. nucleatum*

Publicação 3– Avaliar a eficácia da aPDT associada ao MTZ em biofilme mono-espécie de *P. gingivalis* e *F. nucleatum*

3 PUBLICAÇÃO 1*

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Review

The impact of antimicrobial photodynamic therapy on peri-implant disease: What mechanisms are involved in this novel treatment?

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ABSTRACT

According to the American Academy of Implant Dentistry, 3 million Americans have dental implants, and this number is growing by 500,000 each year. Proportionally, the number of biological complications is also increasing. Among them, peri-implant disease is considered the most common cause of implant loss after osseointegration. In this context, microorganisms residing on the surfaces of implants and their prosthetic components are considered to be the primary etiologic factor for peri-implantitis. Some research groups have proposed combining surgical and non-surgical therapies with systemic antibiotics. The major problem associated with the use of antibiotics to treat peri-implantitis is that microorganisms replicate very quickly. Moreover, inappropriate prescription of antibiotics is not only associated with potential resistance but also and most importantly with the development of superinfections that are difficult to eradicate. Although antimicrobial photodynamic therapy (aPDT) was discovered several years ago, aPDT has only recently emerged as a possible alternative therapy against different oral pathogens causing peri-implantitis. The mechanism of action of aPDT is based on a combination of a photosensitizer drug and light of a specific wavelength in the presence of oxygen. The reaction between light and oxygen produces toxic forms of oxygen species that can kill microbial cells. This mechanism is crucial to the efficacy of aPDT. To help us understand conflicting data, it is necessary to know all the particularities of the etiology of peri-implantitis and the aPDT compounds. We believe that this review will draw attention to new insights regarding the impact of aPDT on peri-implant disease.

Contents

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* ANEXO B

The impact of antimicrobial photodynamic therapy on peri-implant disease: What mechanisms are involved in this novel treatment?

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Abstract

According to the American Academy of Implant Dentistry, 3 million Americans have dental implants, and this number is growing by 500,000 each year. Proportionally, the number of biological complications is also increasing. Among them, peri-implant disease is considered the most common cause of implant loss after osseointegration. In this context, microorganisms residing on the surfaces of implants and their prosthetic components are considered to be the primary etiologic factor for peri-implantitis. Some research groups have proposed combining surgical and non-surgical therapies with systemic antibiotics. The major problem associated with the use of antibiotics to treat peri-implantitis is that microorganisms replicate very quickly. Moreover, inappropriate prescription of antibiotics is not only associated with potential resistance but also and most importantly with the development of superinfections that are difficult to eradicate. Although antimicrobial photodynamic therapy (aPDT) was discovered several years ago, aPDT has only recently emerged as a possible alternative therapy against different oral pathogens causing peri-implantitis. The mechanism of action of aPDT is based on a combination of a photosensitizer drug and light of a specific wavelength in the presence of oxygen. The reaction between light and oxygen produces toxic forms of oxygen species that can kill microbial cells. This mechanism is crucial to the efficacy of aPDT. To help us understand conflicting data, it is necessary to know all the particularities of the etiology of peri-implantitis and the aPDT compounds. We believe that this review will draw attention to new insights regarding the impact of aPDT on peri-implant disease.

Keywords: Photodynamic therapy; peri-implantitis; photosensitizer; microorganisms

1. Introduction

According to the National Institutes of Health (NIH), infections caused by microorganismal biofilms are considered to be a public health problem, as biofilm-associated diseases might be responsible for 80% of all infections in humans (http://grants.nih.gov/grants/guide/pa-files/PA-07-288.html). A biofilm is a complex interaction between a surface and microbial cells that are protected by an extracellular matrix of polymeric substances [1, 2], which confers resistance to antibiotic treatment [3]. In addition, these microbial networks are responsible for the most common oral diseases: dental caries, periodontitis, and peri-implantitis [4-6].

With the growing number of dental implant procedures, the prospective number of sites with implant-associated diseases has also increased [7]. Specifically, given the common incidence of peri-implantitis [8-10] and considering that the etiopathogenesis of peri-implantitis is not well delineated, the most effective treatment for peri-implantitis has not been conclusively established. Similarly to periodontal disease, peri-implantitis is a destructive inflammatory process that leads to pocket formation and loss of supporting bone; in peri-implantitis in particular, the disease site surrounds an osseointegrated implant. Peri-implantitis has been estimated to occur in 10.7–47.2% of dental implant patients within 10 years of post-treatment observation, and these data are considered alarming [11]. According to NHANES 2009-2010, the prevalence of periodontitis in the United States among adults aged 30 years and older was 47.2%. This percentage is even higher at 70.1% for adults older than 65 years [12]. The cost associated with the treatment and prevention of this disease reached 14.3 billion dollars in 1999 [13]. In an attempt to reduce these numbers, antibiotic therapy is often recommended for patients receiving periodontitis and peri-implantitis treatment procedures [14]. According to some authors, the advantage of antibiotic use is the short

course of administration, which may contribute to patient compliance [15]. Despite the clinical relevance and the effective use of systemic antibiotics to treat numerous infectious diseases, the currently available scientific information on the use of these agents in the treatment of periodontal and peri-implant diseases is insufficient to support any official recommendations on the use of these medicines [16]. It is important to emphasize that antibiotics are antimicrobial substances that can lead to side effects of varying intensities, and their unselective use can increase selection for bacteria that are resistant to antibiotics. In 2014, a new report by the World Health Organization (WHO) revealed that antimicrobial resistance is currently a serious threat and is no longer simply a future problem. This phenomenon is occurring across many different regions of the world and can affect anyone, independent of age or country.

Although dental implants are a successful treatment modality [17], peri-implantitis is the most common cause of late failure and can occur years after osseointegration [18]. To address this issue, increased attention has been paid to non-surgical alternatives for treatment of localized infections [19]. Recently, antimicrobial photodynamic therapy (aPDT) has been considered as an adjunct treatment approach to the bacterial decontamination of teeth and implants affected by periodontal and peri-implant disease. aPDT involves exposure to a combination of a photosensitizer [20] and an appropriate wavelength of laser light, resulting in the destruction of different oral pathogens in planktonic and biofilm forms [21, 22]. *In vitro* and *in vivo* studies confirmed that a major periodontopathogenic bacterium, *Porphyromonas gingivalis*, is susceptible to aPDT [22-24]. Despite promising results, several factors should be considered in order to obtain good treatment outcomes in patients, such as the type of PS, total exposure time, wavelength, intensity of laser irradiation, and the combination

of another treatment with aPDT. Thus, we address the impact of aPDT on peri-implant disease and discuss all of the factors related to this novel therapy.

1.1 Bacterial adherence to implant surfaces – a key factor in peri-implantitis

Peri-implantitis is a complex and interesting disease in which alterations in bone and connective tissue homeostasis involve intricate interactions between bacteria and the inflammatory immune response of the host [25]. Bacteria are considered to play a principal role in initiating the host inflammatory process [14]. Increased understanding of the various factors contributing to peri-implantitis has revealed that the clinical phenotype is not simply the translation of microbial challenge into a standard host response. Strong evidence has suggested that smoking, diabetes, and susceptibility to periodontitis are powerful determinants of peri-implantitis development as well as disease severity [26, 27]. To create a strategy for treating peri-implantitis, it is crucial to understand all the factors involved in the development of the disease and its mechanisms of action.

Regarding the bacteria that are responsible for initiating host inflammatory processes and bone loss, two points should be considered: the bacterial species involved and the host immune response to the bacteria. Molecular analysis of oral microorganisms has identified approximately 700 species of bacteria inside the mouth of any individual [28-30]. Due to high diversity, it is therefore necessary that oral bacteria adhere to solid surfaces for the development of oral disease. This specificity occurs via mechanisms of adherence, i.e., several cell surface structures (especially those proteinaceous and carbohydrate molecules) of different bacterial species can identify receptors in the salivary pellicle, and these structures coat enamel and/or dental implant materials and their prosthetic components. Importantly, the chemical

composition of different materials can have a significant impact on biofilm formation [31-33], initiating gene expression and determining the bacterial profile of the species adhering to the biofilm. Recently, an *in vitro* study evaluated the effect of several implant materials in comparison to enamel on bacterial adhesion. A preference of *Streptococcus mutans* and *P. gingivalis* for the chemical composition of enamel surfaces was suggested [34], as it was not possible to detect bacteria on titanium or zirconia materials. In general, streptococci and actinomyces initially dominate the bacterial composition of the tooth surface and can recognize receptors in the salivary pellicle [35-37]. In the case of dental implant surfaces, while some findings have reported similarities in the microbiota composition between the surfaces of both healthy and infected implants and teeth [38-41], other findings have indicated that peri-implantitis may be more complex and diverse than periodontitis [40, 42]. Overall, black-pigmented *Prevotella* species, *Aggregatibacter actinomycetemcomitans,* and *P. gingivalis* are found in higher quantities in peri-implantitis lesions than in healthy control tissue and at comparable levels in periodontitis samples; however, enterobacteria and staphylococci have been identified around implants [43]. Another important factor that regulates bacterial colonization profiles and should be considered before planning treatment is the type of edentulism: either full or partial. Some findings demonstrated that 1 month after total dental extraction in individuals with periodontal disease, *A. actinomycetemcomitans* and *P. gingivalis* were undetectable in the oral cavity [44]. Similarly, *Streptococcus sanguinis, S. mutans,* and lactobacilli were visibly reduced in edentulous adults with or without standard removable dentures compared with dentate patients [45]. Therefore, the environment can be considered as the main factor that influences the microbial colonization profile.

1.2 Biofilm complexity and bacterial invasion

The persistence of dental plaque changes the dental ecosystem, and new bacterial composition appears to affect the environment, thus resulting in clinical disease. The cell-to-cell interactions involved in coaggregation are responsible for dynamic biofilm construction, which is categorized as either cooperative or competitive [46]. It has been known that bacteria of the genus *Fusobacterium* exhibit partnerships with initial, early, and late colonizers and thus serve as a bridge in the succession of genera in naturally developing dental plaque [47, 48]. The ability of *F. nucleatum* to adhere to biofilm at different stages can be explained by its two distinct types of adherence, classified based on their inhibition by either D-galactose or L-arginine. While the adherence of *F. nucleatum* to Gram-negative bacteria is galactose sensitive, its adherence to Gram-positive bacteria is mediated by arginine-inhibitable adhesins [49]. Below the gum line, the environment changes and becomes anaerobic. In this context, subgingival anaerobic bacteria dominate the environment, which has a higher overall species diversity than that of supragingival biofilms [28]. Among the anaerobic bacteria considered to be periodontopathogens, *P. gingivalis* is known to misdirect the host defense and increase tissue-destructive inflammation [50], thus influencing disease initiation and progression. Scientific evidence has shown that *P. gingivalis* is commonly found in patients with periodontitis [51, 52] and is associated with peri-implantitis [53, 54]. Additionally, interaction with early microbial colonizers, such as *Streptococcus* species, can also promote the migration of *P. gingivalis* in subgingival biofilms [55].

In addition to their interactions with other bacteria, some pathogenic species adhere to oral epithelial cells and induce interleukin production [56]. A small number of microorganisms are able to bind to and invade different types of host cells, thereby eliciting proinflammatory responses and periodontal destruction [56, 57]. *P. gingivalis*,

for example, is capable of producing a number of virulence factors such as fimbriae, lipopolysaccharide (LPS), capsules, and proteases, which can bind to and activate human epithelial cells, thus resulting in cytokine release [58, 59]. Another bacterial species involved in the stimulation of the innate immune response is *F. nucleatum*. Recently, a novel type of adhesion was identified as being involved in bacterial attachment to host epithelial cells; this type of adhesion is unique to the oral microbiota and may play an important role in *Fusobacterium* colonization in the host [60]. It has been postulated that these bacteria not only induce peptide production against periodontopathogens but also influence the immune response through the induction of cytokines and chemokines [61]. The invasion of epithelial cells [62] was also demonstrated by the Gram-negative anaerobic bacteria *Treponema denticola,* characterized as the "red complex" by Socransky et al. [63]. *T. denticola* possesses several virulence factors responsible for adherence, tissue penetration, cytotoxicity, and immunomodulation and is involved in inhibiting the complement system [64].

Gingival epithelial cells are the first human cells with which bacteria of the biofilm interact. Once bacterial proteins binds to their receptors, gingival epithelial cells produce a wide array of responses, thus increasing the abundance of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukins (ILs), which indirectly attempt to eliminate the infection [65]. Different cytokine response profiles are induced by distinct bacterial species; pathogenic species, for example, can provoke an inflammatory response, while those considered to be commensal produce an insignificant inflammatory response. In an *in vitro* study, primary human gingival epithelial cells (HGECs) were incubated with several species of dental plaque bacteria to determine the levels of specific interleukins. The results showed that the cells stimulated with live *P. gingivalis* produced high levels of IL-1b but that the same cells

stimulated with live *A. actinomycetemcomitans* produced high levels of IL-8. In contrast to pathogenic bacteria, the commensal *Streptococcus gordonii* induced low levels of pro-inflammatory cytokines [65].

Peri-implant tissue surrounding the implants provides a barrier resisting frictional forces and protecting the soft tissue against microorganisms. Thus, the penetration and injury of the epithelial layer are important steps in the pathogenesis of peri-implantitis.

1.3 Stages of peri-implant disease

When individuals lose their teeth due to periodontal disease, the pathogenic microorganism remains inside the mouth. Within almost 30 minutes of transmucosal implant placement, bacteria initiate colonization on the implant surfaces [66]. The progression of the adherent biofilm on the dental implant seems to be the driving force in the commencement and development of peri-implant disease. When signs of inflammation without loss of connective tissue are identified following initial bone remodeling around the implant during healing, it is believed that peri-implant mucositis has been established. For this stage of the disease, mechanical therapy (with or without adjunctive use of antiseptic rinses) is commonly the initial treatment of choice [67, 68]. However, during disease progression, inflammatory mediators produced by the soft tissue activate osteoclastogenesis and the subsequent loss of the marginal, supporting bone around the functioning implant [25]. At this stage, peri-implantitis becomes established. The presence of increased levels of pathogens in peri-implantitis is a serious treatment issue, as discussed below. Treatment difficulties at this point are directly related to the complexity of the biofilm, the probing depth, and the inflammatory immune response of the host.

Overall, evidence from *in vivo* studies points to a questionable theory of microbial similarity between teeth and implants [39, 69, 70]. This information has guided the treatment of peri-implantitis to be similar to that of periodontitis. Efforts to control peri-implantitis have been made with different methods of open or closed debridement, systemic or local delivery of antibiotics, aPDT, and combinations of these therapies.

1.4 Treatment options

Although peri-implantitis is modulated and mediated by the host, supportive peri-implant therapy is a critical procedure for preventing the incidence and/or for treating the disease [71-73]. One of the main challenges in the treatment of peri-implantitis is the disinfection process of dental implant surfaces to reduce inflammation and stimulate re-osseointegration. Although periodontitis and peri-implantitis share similar etiological factors, in peri-implantitis, the irregular structure of the dental implant can promote plaque accumulation when exposed to the oral cavity [74] and can interfere with the quantity and quality of the biofilm that adheres to the implants. Conventional treatment for periodontal disease involves debridement of the root surfaces with mechanical instruments. Considering that decontamination of the implant surface is much more problematic than decontamination of natural root surfaces, mechanical therapy alone could be insufficient for biofilm elimination in peri-implantitis [75]. Furthermore, titanium curettes could severely damage the implant surface, thus increasing its roughness and bacterial adherence. Consequently, plastic curettes were introduced in an attempt to reduce the damage caused by metal instruments, but plastic curettes cannot reach the macro- or micro-pores of these dental implant substrates. The ineffectiveness of these instruments results in large residual plaque areas after treatment [76]. Another

treatment option is the use of an air-abrasive device. This procedure is effective for the removal of biofilm from implant surfaces [77], but one disadvantage is the risk of emphysema after treatment [78]. Thus, to further facilitate bacterial reduction, additional approaches have been used, such as the use of systemically or locally administered antibiotics that act directly on active subgingival species in the dental plaque or in adjacent epithelial tissues lining the peri-implant pocket. It is believed that local or systemic antibiotics eliminate periodontopathogenic bacteria to a greater extent than conventional therapy. This phenomenon is explained by several findings that the short-term clinical benefits achieved with conventional methods (scaling and root planing) are frequently not sustained in the long term, especially in more progressive cases [79] and in cases associated with risk factors such as smoking [80] and diabetes [81]. However, it is imperative to highlight that antibiotics are biologically active substances that can lead to side effects of differing severity. Additionally, the WHO has questioned the current practice of indiscriminate antibiotic use, which is progressively leading to antibiotic resistance, the persistence of infections, and treatment failure (http://www.who.int/mediacentre/facsheets/fs194/en/). In an attempt to diminish the inflammatory process and reduce the potential for pathogen resistance, alternative treatment methods have been introduced. One of the most promising methods for treating peri-implant mucositis and peri-implantitis is aPDT.

1.5 aPDT - definition, application, and mechanism of action

The use of light with a sensitizing agent was first described in the medical literature more than 100 years ago [82]. Interestingly, the discovery occurred incidentally after a medical student observed that paramecia, unicellular protozoa, were killed only when a dye was exposed to strong daylight. Since then, various studies have investigated the

efficacy and efficiency of this approach, mainly as a cancer therapy. The applicability of this therapy is a consequence of its mechanism of action. aPDT involves the activation of a drug using light, and at the trigger time, exposure of the drug to excitation light leads to cell death via apoptosis or necrosis. The mechanism of action, although not completely understood, involves the production of reactive oxygen species (ROS), which can damage the target cell. Regarding its effects on microorganisms, the literature has shown that aPDT is more effective in inactivating Gram-positive bacteria than Gram-negative bacteria due to the chemical structure of the cell walls [83]. The driving force of aPDT is photosensitization. For this therapy to work, the PS molecule must penetrate the cell walls of the microorganisms until it reaches its final destination and binds to the plasma membrane of the microbial cell. However, besides a pronounced antimicrobial efficacy, PS should not be toxic toward mammalian cells. Since PS play a pivotal role in aPDT therapy this substance should be effective in the selectivity for microbial cells over host mammalian cells [84]. In this context, the cytotoxicity to normal tissue are minimized due to high selective affinity of the PS to the diseased tissue and microbial cells, and by delivering the light in a spatially confined and focused manner. Increasing the selective accumulation of the PS into target cells can be explained by the strong interaction between PS with low-density lipoprotein (LDL) overexpressed on cancer cells [85]. In fact, which factors in the chemical structures of the PS are involved for maximizing the selectivity for the tumor over normal tissue and microbial cells are still not completely understood [86]. However, studies have been performed to investigate if a desired therapeutic dosage might kill microbes effectively without damaging the adjacent cells. The data found in the literature have demonstrated low toxicity against mammalian cells when PS is applied to a specific area [87, 88].

The membrane affinity of a PS is directed by its amphiphilic properties, and this is dependent on the chemical organization of hydrophobic and hydrophilic regions in its structure [89, 90]. However, the type of membrane barriers of the bacterial cell, for example, can limit the simple dissemination of a PS into the bacterial cytosol. The composition of Gram-positive bacteria differs in several key ways from their Gram-negative counterparts. Overall, the outer membrane surrounding Gram-positive bacteria becomes the cell wall of this bacterial class, and their outer membrane is more permeable to hydrophobic small molecules. This structure plays a key role in protecting Gram-negative bacteria from the environment by eliminating toxic molecules and offering an additional stabilizing layer around the cell. However, a thick layer of peptidoglycans around Gram-positive microorganisms could limit the diffusion of the PS into the bacteria. Threading through these layers of peptidoglycans are teichoic acids, which are long anionic polymers whose negative charge can attract cationic molecules [91]. The outer membrane is composed of glycolipids, principally LPS, a well-known molecule responsible for much of the toxicity of Gram-negative organisms. LPS induces the production of different mediators associated with septicemia [92]. The human innate immune system is sensitized to LPS, which is an unquestionable indicator of infection. Therefore, aPDT-mediated killing of Gram-positive bacteria is definitely much easier to accomplish than that of Gram-negative bacteria. Thus, it is more challenging to obtain a highly potent PS for mediating aPDT against Gram-negative bacteria, as their cell wall prevents the uptake of anionic and neutral PSs. This theory is corroborated by previous results presented in the scientific literature.

In this review, we discuss aPDT as an alternative method for eradicating bacteria from peri-implant pockets; however, we should be cautious considering that antimicrobial/antibacterial treatment results have revealed a CFU reduction rate of

greater than 3 log_{10}, as stated by the American Society of Microbiology (ASM) in 2010 [93]. The bactericidal effect of aPDT using methylene blue (MB) was studied in planktonic cultures of *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, *P. intermedia*, and *S. sanguinis.* Consistent with the theory described above, the data showed that Gram-negative species were more resistant to aPDT-mediated killing than Gram-positive species. *S. sanguinis* was the most susceptible strain [94]. MB belongs to the phenothiazinium family of positively charged sanguinis dyes, and the cationic molecules present in this PS may interact with anionic regions from *S. sanguinis* cell walls. The results of this study also demonstrated that the bactericidal effect of aPDT is wavelength-dependent, dose-dependent, and bacterial species-dependent. Another example for phenothiazinium dye, which has been tested for inactivation of planktonic cells and biofilm, is toluidine blue (TB). The interesting and promisors previous data obtained from *in vitro* studies [95, 96] instigated the continued use of this PS in current reports. Recently, the effectiveness of the TB on multispecies biofilm grown on bovine enamel slabs was evaluated within the oral cavity. For initially adherent oral anaerobic microorganisms, the results showed significant CFU reduction from a native *in situ* biofilm. The effect was sustained during the subsequent biofilm formation and the number of cultivable microorganisms within mature oral biofilms declined by 2.21 log_{10}. However, more important than the capacity of reducing the number of bacteria is the regular oral microflora disturbed by this therapy [97]. Remarkably, the data revealed that *F. nucleaum*, for example, could not be detected in the biofilm after the application of aPDT using TB [97]. Since this bacterium plays an important role in the establishment of anaerobes species in the periodontal pocket, *F. nucleatum* reduction could affect the survival of periodontopathogens [98]. Similar to MB, TB was initially used by the dye industry due to its affinity for nucleic acids, and therefore binds to

nuclear material of tissues with a high DNA and RNA content [99]. Those properties also conferred it negative aspects of the clinical use related to its capacity to stain hard tissues of the tooth. However, it has been reported that residual staining of teeth and gingival tissue with TB is not visible after the aPDT application, and therefore, caused no esthetic problems for the patients [100].

Other hydrophobic compounds often used in aPDT include porphyrins, chlorins, and phthalocyanines, which are structurally comparable heterocyclic macrocycles. Porphyrins, for example, are endogenous substances and Gram-positive cell wall constituents; moreover, they act as PSs and induce a lethal auto-photosensitization process that kills bacteria via an oxidative burst similar to the photodynamic inactivation of bacteria. Furthermore, the membrane affinity for PS molecules facilitates the penetration of porphyrins [101]. However, varying results were observed even when different Gram-positive bacterial species were examined. In an interesting report, researchers tested the effect of the porphyrin 5,10,15,20-tetrakis(1-methyl-4-pyridyl)-21H,23H-porphine tetra-*p*-tosylate salt (TMPyP) against *Enterococcus faecalis* monospecies biofilm and verified the inefficacy of the treatment. One explanation for this outcome is the large molecular structure of TMPyP, which may delay the penetration of this PS through the extracellular polymeric substances [99]. Additionally, electrostatic interactions between the positively charged TMPyP and negatively charged EPS could delay PS diffusion [102]. In the same report, the authors suggested that the emission of the LED light-curing unit was not ideal for excitation of TMPyP, which is another important point to consider. The wavelength of the light source excites the PS to produce free radicals and/or ROS. If the PS compound is unable to absorb laser energy, the therapy will not be efficient [94].

Limited data were obtained when aPDT was applied with a cationic chlorin-*e*6 derivative, commercially marketed as Photodithazine®, on fungal biofilms from *Candida albicans* and *Candida glabrata*. The results showed a CFU reduction of approximately 1 $\log_{10}$ [21, 103]. Similar to Gram-positive bacteria, fungi have a thick cell wall and no outer membrane, but fungi have a unique chemical composition. The *C. albicans* cell wall, for example, is primarily composed of glucan and chitin, very hydrophobic carbohydrates responsible for the mechanical strength of the cell wall, as well as mannoproteins [104]. Quite divergent data were acquired in recent and innovator investigations. A succession of *in situ* studies has shown high antimicrobial effects of aPDT with chlorine e6 (Ce6) against initial and mature in oral biofilm, reducing significantly the numbers of viable anaerobic microorganisms. The differences in the susceptibility for microorganisms presented in these studies clearly underline the wavelength-dependence, since the authors combined visible-light [96] and water-filtered infrared A (wIRA) with this cationic PS [105, 106]. Noticeably, we must consider the particularities and limitations of both studies that could have interfered with the final results, such as the time of biofilm formation, type of PS used, PS concentration, wavelength, intensity of laser irradiation, and light source. The chemical properties of the fungal cell wall reflect the limited interactions among chlorin, carbohydrates, and proteins. In another study, the investigators demonstrated that both XF-73 and TMPyP, porphyrin molecules, exposed to blue light effectively photodynamically killed *C. albicans* in suspension [107]. The positive interaction between porphyrin molecules and chitosan, a chitin derivative, can be explained by the fact that chitosan promotes greater adsorption of porphyrins on phospholipid monolayers and allows the porphyrin to stay in its monomeric form [108]. Closely associated with PS interaction, the fact of the tests have been performed against

planktonic microorganisms can also have contributed to the experimental success. The conflicting results found in the scientific literature prompt us to consider and further explore the possible reasons underlying this discrepancy. In the case of PS, for example, it was previously shown that TMPyP attaches to and has a high affinity for *C. albicans* cells. XF-73 compounds show a similar property. Although porphyrins and chlorin share similar chemical properties, the fact that Photodithazine® does not have an antimicrobial effect inspires new investigation into the particularities of each microorganismal species.

Another important and intriguing class of PSs recently introduced in aPDT is curcumin [109]. This compound has been isolated from the plant *Curcuma longa,* and because this product is natural and confers antimicrobial properties, accumulating studies have investigated its therapeutic efficacy in various inflammatory diseases [110, 111]. Among these studies, the antifungal effect of curcumin-mediated aPDT against oral candida infections caused by *Candida spp* has been evaluated. Previous findings have indicated this PS as an effective photosensitizing agent for the inactivation of *C. albicans* in both its planktonic and biofilm forms [87]. In addition to its antifungal properties, curcumin has been noted for its beneficial treatment outcomes for dentine carious lesions. Impressive results were obtained when mature, multispecies biofilms of *S. mutans* and *Lactobacillus acidophilus* were exposed to a curcumin solution for 5 minutes and were irradiated for 5 minutes with blue light, leading to a CFU reduction of more than 3 log10. However, a different outcome was observed when dentin carious lesions were exposed to this compound under the same concentration, time, and light conditions [112]. The depth of dentin could have reduced curcumin penetration due to their unique physicochemical properties. Dentin is a highly hydrophilic connective tissue, whereas curcumin is a hydrophobically derived polyphenol, and this difference

can explain the discrepant results observed in distinct experimental designs. As curcumin is a natural product, its mechanism of action is also attractive with regard to human healthcare. It has been demonstrated that curcumin is a potent inhibitor of the generation of ROS, which are mediators of inflammation. The photodynamic effect of curcumin involves hydrogen peroxide production without the generation of singlet oxygen [113], which in turn potently enhances heme oxygenase-1 (HO-1) expression. However, it was shown that the activity of HO-1 in angiogenesis upregulates the synthesis of vascular endothelial growth factor (VEGF) under both physiological and pathological conditions [114]. Thus, the benefits of this compound depend on both its dose and the chemical environment.

1.6 A new insight into light source for aPDT success

Antimicrobial PDT requires a set of procedures to work. The evidences presented above display the impact of the light source to improve the interaction between PS and cell compositions from different microorganisms. Most PS is activated by specific wavelength. In spite of the PS excitation is required, the degree of penetration can compromise the tissue health and cause injuries [115].

Depth of light penetration in human tissue is wavelength-dependent. Up to date, a variety of light sources have been employed for aPDT protocols, such as: nonlaser light generators (halogen or light-emitting diode [LED] lamps). The main issue of halogens lamps is the gas contained inside the tube that makes the light much brighter and can induce tissue overheating [116]. On the other hand, the intensity of light emitted by LEDs on the skin is lower, since its cells maintain a good interaction with the light. However, LEDs produce relatively limited bands of green, yellow, orange or red light and this restricted emission wavelength spectrum [117]has not provided antimicrobial

effects, so far. Thus, alternative strategies have been introduced in an attempt to combine the PS with the appropriate light source and improve the effect of aPDT to treat oral diseases.

Recent investigations, combining visible light with water-filtered infrared-A (VIS+ wIRA), have described a significant reduction of the total oral bacterial for the chronic wound treatments [118]. This potential effect has directed the use of the VIS + wIRA device to improve the efficacy of aPDT. The combination of both light and radiation is based on a natural process in which mankind has developed, i.e., the heat radiation of the sun, in moderate climatic zones, is filtered by water vapor in the atmosphere of the earth. Similar to sun heat radiation, the water-filtering allows to high penetration properties with a low thermal load to the surface of the skin, (within 780-1400 nm) [119]. The mechanism of action involves the cells and cellular structures stimulation by direct radiation effect. Some reports have shown that wavelengths within wIRA influence interactions between cells and extra- cellular matrices, increasing the amount of ATP available [120, 121], participating in wound repair processes and modulating the immune system and/or to induce necrosis/apoptosis of damaged cells and of bacteria [122]. Thus, it seems probable that VIS+wIRA could increase the desired PDT outcomes, in a number of dental procedures.

Since the concept of the aPDT involves the production of ROS, responsible to damage the target cell, the rising production of ROS and singlet oxygen would improve its antimicrobial effects. As discussed in the previous topic, effectiveness of aPDT approach using VIS+wIRA in combination with PS has been tested on *in situ* experiments [97, 105, 106]. Besides a successful outcome demonstrated by CFU reduction, viability assay enabled understanding the relevant contribution of the light source in the eradication of biofilm bacteria. Interestingly, when the authors exposed the

PS onto the oral biofilms in the absence of VIS+wIRA, the cells preserved their viability, indicating a VIS+wIRA-dependence to destroy a vast amount of microorganisms. This new insight about the impact of the light sources on aPDT efficacy could be tested onto a pathogenic environment and be directed, in future, to treat peri-implantitis.

1.7 Could the inflammatory response activated by aPDT modulate bone resorption?

Initially, aPDT was discovered because of its antimicrobial properties. However, as researchers began to understand part of its mechanism of action, this therapy was directed towards cancer treatment. Accordingly, activation of the immune response is necessary for effective tumor control [123]. aPDT activates several cell-signaling cascades and the release of cell fragments, cytokines, and inflammatory mediators, which stimulate the recruitment of neutrophils [124]. In an interesting report, the authors investigated two distinct mechanisms of neutrophil migration induced by aPDT, and they found that the early phase reaction may be regulated by TNF-α, neutrophil chemo-attractants, or IL-6. Recently, a research group evaluated inflammatory cytokine expression after aPDT application in the treatment of oral candidiasis in a murine model [125]. Consistent with the mechanism of action of this therapy [126], the results revealed high TNF-α expression; however, the expression levels of IL-1 and IL-6 in the aPDT group were lower than and similar to those in the untreated group, respectively [125]. During the delayed phase reaction, neutrophil chemo-attractants and IL-1b are the factors regulating neutrophil migration [127]. With regard to peri-implantitis, this disease involves the destruction of alveolar bone, which leads to implant loss. During the bone resorption process, different types of cells, such as neutrophils, macrophages,

dendritic cells (DCs), and T cells, participate in the immune response [128]. Furthermore, similar cytokines produced by immune cells after aPDT irradiation are released during the disease process [129]. The association among IL-1/6, TNF-α, and peri-implantitis has already been well documented [128, 130]. The main question in this field is if aPDT stimulates an inflammatory response in tumor cells, could this therapy also treat peri-implantitis and exacerbate bone loss? Clearly, we must consider several issues such as the short aPDT irradiation time and the levels of cytokines produced during treatment. We believe that the present review provides new insights into the possible connections of the immune response triggered by aPDT with peri-implantitis to ensure the safety of this therapeutic approach.

2. Final considerations

Could PDT be considered as a novel modality for treating peri-implant disease?

In this review, we have summarized the most important factors related to aPDT for peri-implantitis treatment and have focused on the outcomes of previous *in vitro* and *in vivo* studies. The selected bacteria and/or fungi used in the *in vitro* experiments demonstrate the mechanism of action of PSs within microorganisms of different classes. Although the effects of aPDT on peri-implant disease have previously been investigated, the exact mechanism of action of aPDT against peri-implantitis remains largely unknown. The insufficient results found in the scientific literature with regard to using aPDT against pathogenic biofilms have not discouraged new investigations due to the advantage of this therapy in avoiding antibiotic resistance. Accordingly, we made significant effort to describe and discuss all the contradictory results found in the literature. We have demonstrated that the microorganism selected, PS properties, wavelength, and light source play critical roles in the clinical efficacy of aPDT. Focusing on peri-implant

disease and considering that peri-implantitis is dominated by Gram-negative anaerobic bacteria, the PS composition ultimately determines the affinity and specificity of the PS between different species. LPS is a highly anionic and important pathogenic factor present in Gram-negative bacteria that extends beyond outer membrane proteins. Additionally, all carbon atoms that are not bound to nitrogen or oxygen atoms from the thin peptidoglycan layer confer a hydrophobic property. Thus, new studies should be directed towards the development of specific PSs. Indeed, a detailed understanding of the mechanisms of action of aPDT could position this therapy as the treatment of choice in selected cases and as an important adjunct to other therapies.

Conflicts of Interest: The authors declare no conflicts of interest.

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4 PUBLICAÇÃO 2*

An in vitro model for periodontopathogenic biofilm

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ABSTRACT

Statement of problem. Consistent in vitro biofilm models are required to elucidate the understanding about the interactions among species and to assess the antimicrobial effect of specific materials as well as to investigate the efficiency of different treatments.

Purpose. The goal of this study was to design a standard in vitro periodontopathogenic biofilm model to lead therapeutic approaches in future studies.

Material and Methods. *Fusobacterium nucleatum* and *Porphyromonas gingivalis* strains were growth under anaerobic conditions in single and dual bacteria species. First, bacterial biomass was evaluated at 24 and 48 hours to determine adhesion phase onto saliva coated polystyrene surfaces. Thereafter, the biofilm development was assessed overtime by crystal violet staining and the biofilm maturity was confirmed by scanning electron microscopy (SEM). An unpaired *t* test, one tailed, was applied to define the best time point to adhesion period. In case of biofilm formation, one way analysis of variance (ANOVA), with a Tukey's posthoc test, was employed to indicate the difference among the periods previously established.

Results. The data showed a significant difference in total biomass of bacteria adhered after 48 hours for *P. gingivalis* in single and dual species. For biofilm development approaches, *P. gingivalis* in single and dual species, the biomasses accumulated were substantially higher after 7 days than after 3 days of incubation; but no significant difference was obtained between 5 and 7 days growth. On the other hand, the biomass of *F. nucleatum* biofilm was higher at earlier time point and the results did not show any difference among 3, 5 and 7 days of incubation.

Conclusion. The assessment of this research were efficient in revealing the pathogenic bacterial growth periods and the establishment of mature biofilm, describing an

important sequence in the development an in vitro model of periodontopathogenic biofilm in single and dual species.

CLINICAL IMPLICATIONS

An in vitro periodontopathogenic biofilm model construction is crucial to lead a deeper understanding about the efficiency of new antimicrobial materials surfaces as well as chemical agents development to treat oral disease.

INTRODUCTION

Periodontal and periimplant diseases are triggered infections associated to complex biofilms structure, which induce an inflammatory response causing the destruction of the connective tissues.[1, 2] The prevalence of periodontitis in adults is about 47%,[3] being the 6th most prevalent oral disease,[4] while periimplantitis is present in 28% of subjects examined.[5] *Porphyromonas gingivalis (P. gingivalis)* is a red complex anaerobic Gram negative bacteria, strongly associated with the advancement of both oral infections.[6-8] The mechanisms involved in bacteria colonization on natural and artificial surfaces as well as the surround periodontal tissues consist of straight attaching themselves to saliva proteins, to epithelial and connective cells receptors and/or interacting with others intermediate and/or early bacterial colonizers.[9-12] *F. nucleatum* is also a Gram negative bacteria, regarded as a central organism for dental biofilm maturation, due to its wide ability of coaggregation to other microorganisms, as *P. gingivalis*.[13-16] These coaggregation, known as mutually beneficial, promotes a high number of virulence factors expressed by both species.[17] Consequently, those molecules may contributes to survival, presence and pathogenicity of these microorganisms in various oral niches the bacterial pathogenicity.[13, 18] Once bacteria are attached to a surface, the dynamic interactions between the host and the bacteria evolve into an organized and complex microbial community, protected from mechanical and chemical damage.[19] The development of promising strategies for fighting biofilm related infections requires in vitro models of mature biofilm, which are useful in obtaining a better understanding about the action mechanism of some drugs, for example. Such models are essential for evaluating the efficiency of therapies that aim to control and prevent oral diseases caused by periodontopathogenic biofilm.

Scientific literature has reported different in vitro biofilm representations to assess the effects of specific materials, as well as to investigate the efficiency of treatments.[20-23] However, there is limited knowledge on the adequate periods necessary for establishing a mature biofilm. Therefore, in this investigation, *P. gingivalis* and *F. nucleatum* were grown onto saliva coated surfaces with the goal of developing in vitro models of periodontopathogenic single and dual species biofilm to evaluate therapeutic approaches in future studies. Additionally, since the bacterial growth pattern[24-26] understanding is important to know the ideal concentration to initiate the biofilm development, the growth curve of both bacteria species was described.

MATERIAL AND METHODS

Human saliva samples from three healthy adult male volunteers were collected under the approval of the Ethics Committee for Research in Humans (CAAE 26142014.0.0000.5416) (ANEXO A) and after informed consent obtaining. None of the participants had been treated for oral diseases or had taken any prescription medication during the three months previous to the study.[27] The saliva preparation was performed as described in the previous studies.[28] Before its use, the supernatant obtained after centrifugation was purified with a 0.22 μm membrane filter (Millipore), and stored at -80°C.[29, 30]

The pathogenic bacteria strains selected to this study were *P. gingivalis* ATCC 32277 and *F. nucleatum* NCTC 11326. The microorganisms stored at -80ºC were seeded onto brucella agar (Himedia) prepared with sheep blood 5% (Microlab) and kept at 37°C, inside the anaerobic environmental chamber oxygen free atmosphere (85% N_2, 10% de H_2, 5% de CO_2) (Don Whitley – Inglaterra). After 48 hours incubation, the microorganisms colonies were transferred to 10 mL of Brain Heart Infuse (BHI – Difco)

broth medium, supplemented with hemin (10 mg/mL), menadione (5 mg/mL) and maintained at 37°C under anaerobic conditions, for 24 hours. In sequence, 500 µL of bacterial cells was dispensed in 9,5 mL of fresh BHI medium and the tubes incubated in the same conditions described above until the mid exponential growth phase: 5 hours for *F. nucleatum* and 15 hours for *P. gingivalis* (Fig. 1A and Fig 1B, respectively). Bacterial cell concentrations were estimated determining the OD_{600nm} (Spectrophotometer Spectrum – SP 2000 UV). When the mid log phase was reached, the inoculums were diluted to obtain a final concentration of 1×10^7 CFU/mL in BHI fresh media for bacteria adhesion and subsequent biofilms formation assays.

The initial step involved acquired pellicle formation. For that, fifty µL of saliva was placed to each 96 well plate (TPP tissue culture, Switzerland) and maintained at 37°C, in an orbital shaker (75 rpm).[31] After 4 hours incubation, saliva excess was removed and the wells were rinsed twice with 100 µL of sterile phosphate buffered saline PBS (100 mM NaCl, 100 mM NaH_2PO_4, pH 7.2). Next, the best time point to adhesion of bacterial cells was defined at 24 and 48 h incubation. One hundred fifty µL of the mid exponential phase bacterial concentrations (1×10^7 CFU/mL for both *P. gingivalis* and/or *F. nucleatum)* were added into each 96 well plate and then incubated at 37°C, under anaerobic conditions. After adhesion incubation period, the medium was removed, the wells were washed gently twice with 200 µL of PBS, to eliminate unattached bacteria, and 150 µL of fresh supplemented BHI medium was added to the biofilm formation assay. Biofilm maturation was evaluated after 3, 5 and 7 days, corresponding to the respective experimental times for biomass accumulation (Fig. 2). Importantly, the culture medium was changed every 24 h.

The biomass of bacteria adhered and biofilm accumulated on the polystyrene plates was determined by crystal violet stain. After the established periods, the culture

medium was removed and then each well received 50 µL of 0.1% crystal violet solution. After 15 minutes at room temperature, the solution was removed and each well was carefully washed twice with 350 µL of PBS to remove the excess of dye. In sequence, two hundred µL of 99% ethanol was pipetted to each well and the plate was maintained for 15 minutes at room temperature. The solution containing the eluted crystal violet staining was transferred onto a new micro plate for overall biomass estimation. The experiment was performed in triplicate with four repetitions to ensure methodological and biological reproducibility.

Samples for maturation assessment of biofilm were cultured on sterile polystyrene discs on a 24 well plate (TPP tissue culture, Switzerland) in single and dual species. After 3, 5 and 7 days of incubation, the discs were rinsed twice with 1 mL of sterile 0.89% sodium chloride (NaCl) and prepared for SEM analyzes. A solution of 2.5% glutaraldehyde (pH 7.4) was used to fix the samples at room temperature for 1h followed by a standard graded series of ethanol solutions sequence for dehydrating of specimens: 70% and 90% ethanol for 60 minutes per step, ending with 5 changes within 30s of 100% ethanol. Prior visualization, the discs were kept under vacuum to guarantee moisture free samples and after 7 days stored, the polystyrene discs were sputter coated with gold. Images at high magnification, × 3500, were taken from different areas of the discs with a SEM (JEOL JSM-6610LV). The SEM approach was performed in two samples of single and dual biofilms for each time point in two different occasions.

An unpaired *t* test, one tailed, was applied to define the best time point to adhesion period. In case of biofilm formation, one way analysis of variance (ANOVA), with a Tukey's posthoc test, was employed to indicate the difference among the periods previously established. Prior statistical procedures, D'Agostino Pearson normality tests

were applied to check the data distribution (alpha=0.05). Once normal distribution was not confirmed for *P. gingivalis* in single species, the Kruskal Wallis test followed by Dunn's multiple comparison was required.

RESULTS

Prior the experiments, the growth curves of both pathogenic bacteria were constructed to standard the bacteria concentration and the exponential phase was defined as representative of the cellular proliferation period.

For adhesion phase period, both bacteria were cultured in single and dual species setups in two different time points. Crystal violet assay revealed a statistical difference in overall bacterial attachment for *F. nucleatum* single species, with 1.6 times more biomass after 24 hours (vs. 48 hours), which may correspond to a highly proliferative phase of adhesion development. Since no difference was observed after 24 and 48 hours for single species *P. gingivalis* and for the dual species setup, the first time point was determined as the adhesion phase for both *P. gingivalis* and *F. nucleatum* (Fig. 3).

Afterward, the growth of *F. nucleatum* and *P. gingivalis* in single and dual biofilms was examined over time. In single species *P. gingivalis* and dual species biofilms the biomasses accumulated were considerably higher after 7 days than after 3 days of incubation; but the statistical tests show no difference between 5 and 7 days for *P. gingivalis* in single species biofilm (Fig. 4A). Contrarily, the proliferative phase of *F. nucleatum* biofilm is higher at earlier time point (Fig. 4B), consistent with the bacterial biomass referring to adhesion step. When *F. nucleatum* and *P. gingivalis* bacteria were grown in dual biofilm species the results demonstrated higher amount of biomass in comparison to single biofilm, revealing the close interaction between both bacterial species (Fig. 4C). The outcomes acquired using crystal violet staining were consistent

with SEM analyzes. *P. gingivalis* alone formed early biofilm showing well spaced microcolonies cells, but without a complex structure after 3 days. In contrast, *F. nucleatum* single species and dual species biofilms exhibited high and dense conglomerates of bacterial cells and more coverage area. Moreover, extracellular matrix could be seen enmeshing the cells. For all of the single and dual species biofilms, the amount of bacterial cells on the polystyrene discs increased over time, and the increase in the biomass was more evident after 5 days of incubation (Fig. 5).

DISCUSSION

A variety of in vitro biofilm models have been employed to test the effects of different antimicrobial treatments. However, experimental success depends primarily on the appropriate methodology to construct biofilms that respond better to preventive and therapeutic strategies. The goal of this research was to provide and validate a robust in vitro periodontopathogenic biofilm model system that attaches to saliva coated surfaces. The use of human saliva is relevant to clinical contexts and is probably an important factor for the initial bacterial attachment during the adhesion phase of biofilm development. Substantial data have evidenced *P. gingivalis* and *F. nucleatum* as keystone bacteria species involved in the periodontal disease progression.[2, 7, 8, 11] Therefore, this research studied the stages of bacterial growth in the planktonic state and then defined the adhesion phase period and subsequent biofilm development steps on saliva coated surfaces, considering different time points.

The bacteria concentration used for in vitro experiments must be standardized according to the growth curve. The bacterial growth period selected for experimental studies can hiding or interfering with the real outcomes. To better understanding, a truthful growth curve comprehends five critical phases of development: lag,

exponential, stationary, death and long term stationary.[25, 26] The duration of each one is directed by factors involving, mainly, the quality of growth culture medium, which can affect the metabolism conditions. In the case of in vitro determination of bacterial susceptibility to antimicrobial agents, for example, the variability of the bacterial growth phase should be further evaluated and standardized for quantitative testing.[24] In general, exponential phase is preferred to experimental investigation since this period characterizes an increased metabolic activity and cell proliferation. Here, growth was carefully monitored, using absorbance measurements of each bacterium proposed before designing the biofilm model. Additionally, the amount of CFU/mL at the mid log phase was also determined. The data collected were coherent in showing that *F. nucleatum* grew earlier when compared with *P. gingivalis,* At 5 hours of incubation in broth medium, the *F. nucleatum* bacterium had already reached the exponential phase, whereas *P. gingivalis* required 15 hours to obtain the same result.

After that, the adhesion phases for both bacteria in single and dual species setups were investigated by culturing the bacteria at the concentration found in the exponential phase onto saliva coated polystyrene well plates. The amount of biomass of bacteria that deposited onto the surfaces showed no difference between 24 and 48 hours of incubation for both single *P. gingivalis* and dual bacteria species. However, the biomass of *F. nucleatum* in single species setups was significantly higher at the earlier time point. Thus, 48 h of incubation lead to decreased biomass of attached *F. nucleatum*, indicating that there was no or slow growth and possibly cell death, as previously discussed.[17] This behavior can be explained by the fast consumption of nutrients by *F. nucleatum* considering the peak of growth found in the first 24 hours.

In the oral cavity and in vitro models, bacterial cells irreversibly interact to the natural and/or artificial substrates, or to each other, and initiate the biofilm formation

with extracellular polymeric matrix production. Here, independent approaches demonstrated differences in biofilm development when *P. gingivalis* and *F. nucleatum* grown in single species. *P. gingivalis* exhibited slower growth and the biomass of biofilm disclosed early stage of development only after 3 days of incubation. The quantitative data was also supported by microscopic images, which showed spread out and high density cells condensed in specifics areas and not covering the entire surface. On the other hand, *F. nucleatum* produced intricate networks after 3 days, which increased after 5 days of incubation and revealed mature biofilm at this stage. Furthermore, incubation for 5 and 7 days did not present any differences in biomass or 3D architecture and the same situation was identified in the cases of dual species biofilms. *F. nucleatum* facilitate *P. gingivalis* biofilm growth based on a synergistic interaction, when compared with single specie biofilms growth.[12, 15, 16]

CONCLUSION

The methodologies used in this study successful demonstrated the biofilm growth sequence of two pathogenic bacteria involved in oral diseases and in describing an important sequence for developing an in vitro model for single and dual species periodontopathogenic biofilm. The current model may be employed to evaluate not only antimicrobial treatments but also to understand how *P. gingivalis* and *F. nucleatum* grown together express virulence factors, and colonize and form biofilm onto distinct dental material surfaces.

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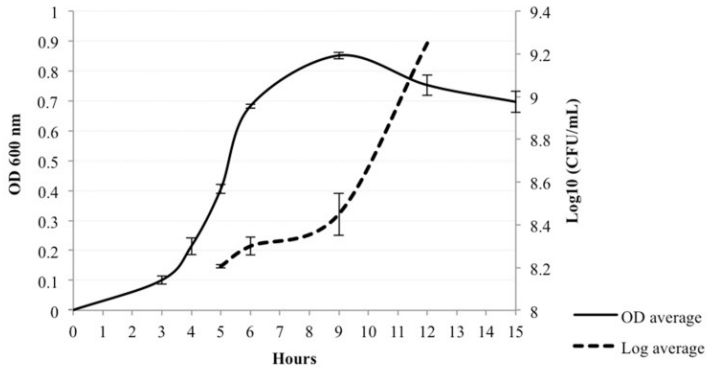
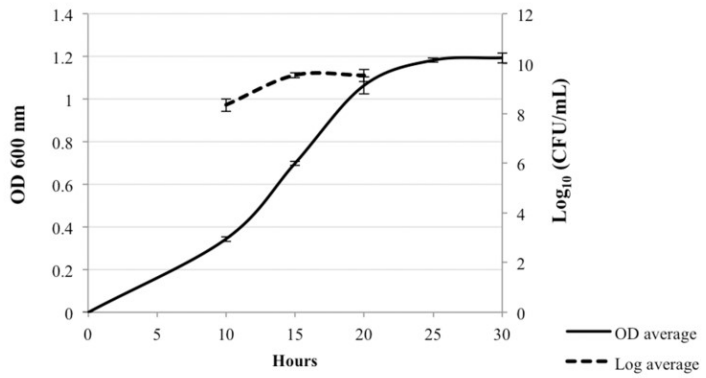
FIGURES

Fig. 1. Growth curves represented by $OD_{600\ nm}$ and CFU/mL for *F. nucleatum* NCTC 11326 (OD 0.4 ± 0.01; 8.2 ± 0.007 CFU/mL) (A) and *P. gingivalis* ATCC 33277 (OD 0.7 ± 0.01; 9.5 ± 0.1 CFU/mL) (B) at mid log phase.



A



B

Fig. 2. Schematic illustrating experimental sequence performed.

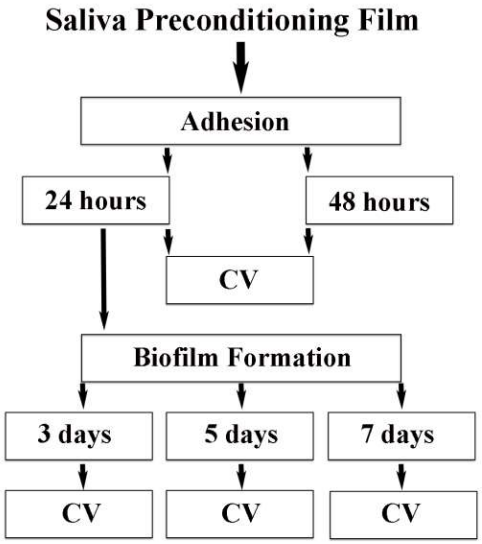


Fig. 3. Adhesion phase was evaluated via quantitative measurement of crystal violet staining as indicator of biomass accumulation after incubation for 24 hours (white bar) in comparison to 48 hours (gray bar). Unpaired t test, one tailed, indicated statistically significant higher biomass of *F. nucleatum* bacterial attachment after 24 hours, p=0.0011. The values are shown as mean ± SD. *indicates statistically significant difference ($p < 0.05$).

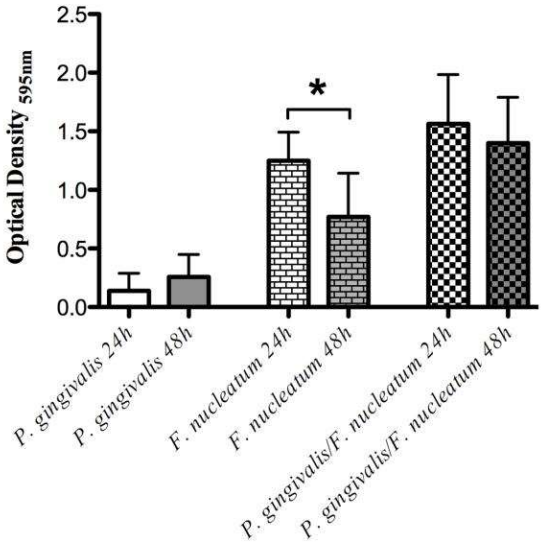


Fig. 4. Biofilm formation was evaluated via quantitative measurement of crystal violet staining as indicator of biomass accumulation after incubation for 3 days (white bar) in comparison to 5 (gray bar) and 7 days (dark gray bar). Statistical comparisons were performed using one-way analysis of variance (ANOVA) with Tukey's posthoc test, except for *P. gingivalis.* In this case, Kruskal was employed with Dunn's multiple comparison using Graph-Pad Prism version 5.0c. The values are shown as mean ± SD. *,# indicates statistically significant difference ($p < 0.05$).

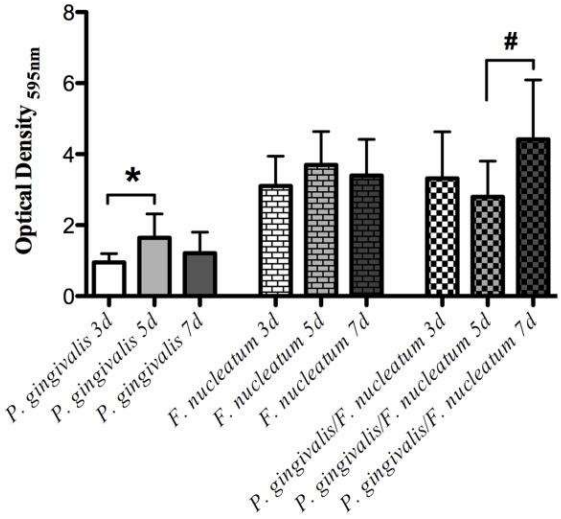
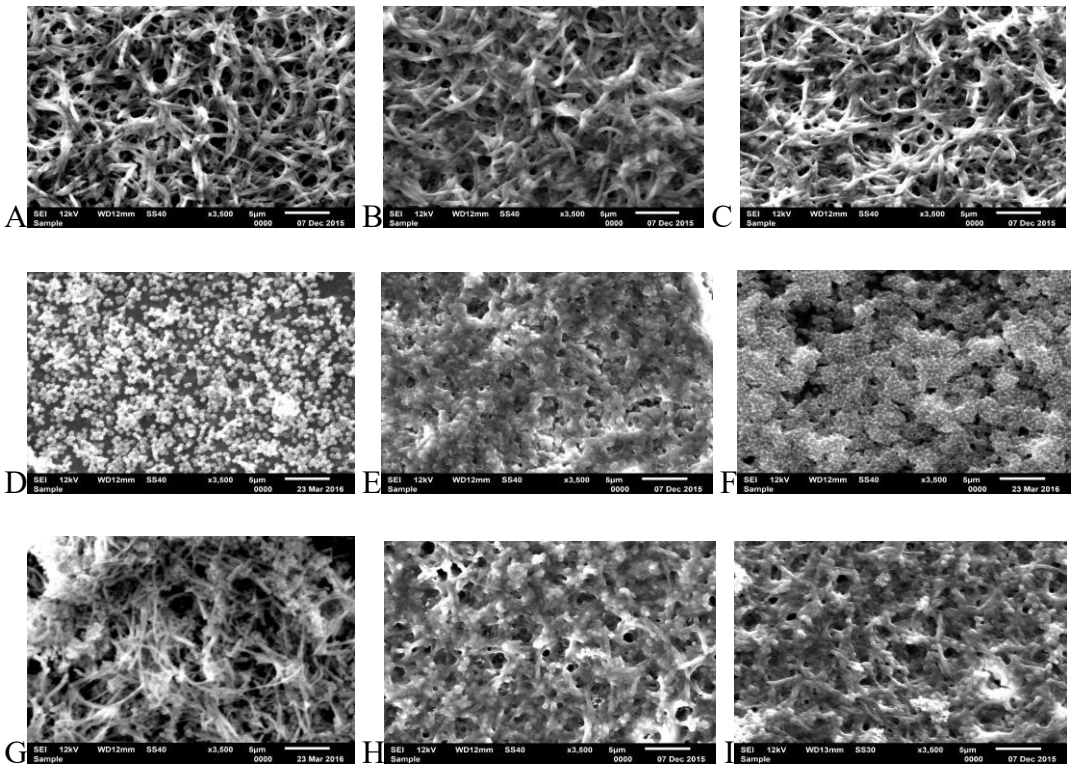


Fig. 5. SEM images of single and dual biofilm species: *F. nucleatum* (A) 3 days, (B) 5 days, (C) 7 days, *P. gingivalis* (D) 3 days, (E) 5 days, (F) 7 days, and dual species (G) 3 days, (H) 5 days, (I) 7 days.



5 PUBLICAÇÃO 3*

Effect of antimicrobial photodynamic therapy alone or in combination with antibiotic local administration on *Porphyromonas gingivalis* and *Fusobacterium nucleatum* mature biofilm

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Highlights

. aPDT has an effect on periodontal pathogens

. Biofilms are more resistant to aPDT

. aPDT associated with antibiotics has potentiated effect

Abstract

The aim of this study was to evaluate the efficiency of antimicrobial photodynamic therapy (aPDT) associated to metronidazole (MTZ) on periodontopathogenic biofilms of *Fusobacterium nucleatum* (NCTC11326) and *Porphyromonas gingivalis* (ATCC33277). Suspensions of standard cells from each species were inoculated onto polystyrene plates and the biofilms were grown for 5 days under anaerobic conditions at 37°C. The aPDT was performed applying Photodithazine (PDZ) at concentrations of 50, 75 and 100 mg/L and exposed to 50 J/cm^2 of LED light (660 nm). The minimum inhibitory concentration (MIC) of MTZ was defined for each specie. Then, MTZ was applied at concentrations of 1x; 50x and 100x MIC. Additional groups were treated with light; only aPDT and MTZ with and without light. Untreated control samples were not submitted to light, PDZ or MTZ. Cell viability was determined by colony counts (CFU/mL). The results demonstrated that aPDT 75 mg/mL associated with MTZ100x and aPDT 100 mg/L associated with MTZ at concentrations of 50x and 100x reduced significantly the CFU/mL number, 2.99; 2.9 and 3.94 Log_{10} respectively, of *F. nucleatum* biofilm. For *P. gingivalis*, the highest reduction in CFU/mL was obtained when aPDT 100 mg/L and MTZ100x was performed, resulting in 5 log_{10} reduction. Additionally, there was significant reduction in groups that were only exposed to light or to higher antibiotic concentration, 1.71 and 3.07 Log_{10}, in comparison to the untreated group. The treatment effect of aPDT associated to MTZ was potentiated when compared to isolated treatments.

Key words: Photodynamic therapy, Metronidazole, Combinated therapy, Fusobacterium nucleatum, Porphyromonas gingivalis

1. Introduction

The ability of microbial cells to interact with abiotic and biotic surfaces enables the formation of a complex microbial community surrounded by extracellular matrix of polysaccharides onto them.[1, 2] This organized structure, defined as biofilm, promotes an imbalance between microorganisms of the normal flora and opportunistic pathogens and is considered the precursor to initiate the inflammatory response from the host.[3] Among bacteria involved in this process, species belonging to the Gram-negative group are outlined due to the virulence factors directly related to several oral aggressive infections, such as periodontal and peri-implant disease.[4, 5]

Recent findings have recognized the microbiota harboring periodontal and peri-implant pockets and *P. gingivalis* appears as a crucial pathogenic species directly related to both disease progression.[6] Besides numerous toxic enzymes produced by most of *P. gingivalis* strain,[7] this dreaded bacterium specie is also capable to communicate with host cells and trigger a strong inflammatory response.[8, 9] *F. nucleatum* is another critical organism, strongly associated with periodontal and peri-implant diseases, due to its ability of congregating with other species by the high number of multivalent adhesins onto cell surfaces.[10-12] In addition, this bacterium is also capable to generate a capnophilic environment, which contributes by supporting the growth of anaerobic pathogenic bacteria including *P. gingivalis*.[13-19] Taking into consideration bacteria aggressiveness and possible sequels provoked by disease progression (teeth/implant loss), biofilm elimination and/or its inactivation from substrates are mandatory procedures to inflammatory response controlling.

From a clinical point of view, non-surgical treatment directed by mechanical removal of subgingival biofilm is limited mainly by depth of periodontal/peri-implant pocket and surfaces irregularities.[20] In addition to the limited effect of mechanical

debridement,[21] further benefits of antibiotic therapy clash with over antibiotic resistance growth.[22] Antibiotic resistance crisis, attributed to the systemic overuse and misuse of these medications, has pressured researches to investigate new strategies to avoid dental or implant mutilation, as consequence of infection progression.[22-24] In face of this issue, antimicrobial photodynamic therapy (aPDT) has been introduced as a potential alternative approach for the bacterial tooth or implants surface decontamination.[25, 26]

The success of the aPDT requires photosensitizer (PS) absorption by microorganism cell wall and its subsequent activation by light irradiation. As a desired action mechanism, single oxygen and free radical produced by PS and biological system interaction can damage the plasma membrane/DNA and lead to death of target cell.[27, 28] Therefore, PS undertakes as a protagonist for the effecting of the therapy. Among commercially available PSs, Photodithazine (PDZ) based on chlorin e6, has drawn researchers attention as a second-generation PS due to its prospective antimicrobial effect against pathogenic microorganisms and low toxicity in appropriate concentrations. Our previous *in vitro*[29, 30] and *in vivo*[31, 32] outcomes have demonstrated successful inactivation of *Candida* spp biofilms and supported continue investigation against pathogenic species. Herein, we moved forward and evaluated the effect of PDZ-mediated aPDT on mature biofilms developed with *F. nucleatum* and *P. gingivalis.* Further experiments were carried in association with antibiotic in attempt to explore the potential antimicrobial substance effect as targeted delivery.

2. Materials and Methods

2.1 Human saliva preparation

Human saliva collection was carried out after approval of the Ethics Committee for Research in humans of the Araraquara School of Dentistry/UNESP (CAAE 26142014.0.0000.5416) (ANEXO A). Unstimulated saliva was obtained from three healthy male adults aged between 25 and 30 years. Subjects' inclusion followed the criteria: no active caries or periodontal disease, no systemic disease and no antibiotic-related therapy for at least 3 months before the study. After collection, saliva was mixed and clarified by centrifuging at 10000g for 15 minutes at 4°C.[33] Immediately after centrifugation, the supernatant was sterilized with membrane pore size 0.22 μm (Millipore) and stored at -80°C until use [34, 35].

2.2 Bacterial strains and inoculum preparation

F. nucleatum (NCTC 11326) and *P. gingivalis* (ATCC 32277) were grown on brucella agar (Himedia) with 5% defibrinated sheep blood (Microlab) at 37°C under anaerobic condition (85% N2, 10% de H2, 5% de CO_2) (Don Whitley – Inglaterra). After 48 hours of incubation, bacterial colonies were transferred to 10 mL of Brain Heart Infuse (BHI – Difco) broth medium, supplemented with hemin (10 mg/mL), menadione (5mg/mL) and yeast extract (6g/L), and maintained into anaerobic chamber, at 37°C, for 24 hours. Subsequently, 500 μL of bacterial cells were dispensed in 9,5 mL of fresh BHI broth medium and incubated under anaerobic condition, until reaching the mid-exponential phase according to the growth curves, previously established: 15 hours for *P. gingivalis* and 5 hours for *F. nucleatum*. Finally, the final bacterial cells concentrations were set at 10^7 CFU/mL to each species, with a spectrophotometer (Spectrum – SP 2000 UV) at 600 nm of wavelehngth.

2.3 Antibacterial susceptibility testing

Planktonic susceptibility tests were performed following the Clinical and Laboratory Standards Institute guidelines in document M07-A9, 2012. Briefly, 95 µL of metronidazole (MTZ - Sigma Chemical Co.) was serially diluted from 32 to 0,015 µg/mL in BHI broth medium in a 96-well plate. Then, 5 µL of each bacterial species was added at 1×10^7 CFU/mL into each well completing a final volume of 100 µL after the inoculation. Bacteria in the same previous concentration were also inoculated directly into the polystyrene plate to serve as growth controls. Plates were incubated under anaerobic conditions at 37 °C for 24 h and the lowest drug concentrations was measured at 595 nm with a spectrophotometer (Spectrum – SP 2000 UV). The minimum inhibitory concentration was considered the lowest capable to inhibit at least 100% of bacterial growth.

2.4 Mature biofilm formation

A conditioning saliva-derived film was developed to attachment of the initial pathogenic biofilm. Fifty µL of sterilized saliva was added into each well of a 96-well plate and kept in an orbital shaker (75rpm) at 37°C.[36] After 4 hours of incubation, saliva was removed and the wells were washed gently twice with 100 µL of sterile phosphate buffered saline (PBS). One hundred fifty µL of each bacteria species at $1x10^7$ cells/mL was individually inoculated onto the acquired salivary pellicle and the plates incubated at 37°C, under anaerobic condition. Following 24 hours to adhesion phase, the unbound cells were removed by gentle washing with 200 µL of PBS, and 150 µL of fresh BHI supplemented broth medium was added to promote the biofilm formation. Plates were statically incubated at 37°C under anaerobic conditions for 5 days and the medium was replaced every 24 hours. Bacterial species individually cultured into the

polystyrene plate under their in vitro conditions served as positive controls for biofilm formation. For all experiments, at the end of the incubation period, the wells were washed twice with 200 µL of PBS prior to further analyses.

2.5 PDZ -mediated aPDT: photosensitizer and light source properties

The photosensitizer used in this study was Photoditazine **(**PDZ), a chlorin e6 derivative (VETAGRAND, Co, Russia). Stock solutions of PDZ diluted in saline solution were prepared in different concentrations: 50, 75 and 100 mg/L and stored in darkness at room temperature until use. Red light irradiation was used to complete the photodynamic therapy with a constant dose of 50 J/cm^2, at 660 nm of excitation wavelengths and power density of 71.7 mW/cm² for 28 minutes.

2.6 Effect of PDZ -mediated aPDT on mature biofilm

In order to understand the effect of PDZ on bacteria cells viability, *F. nucleatum* and *P. gingivalis* biofilm samples were individually incubated in sterile 96-well plates with PDZ in three different concentrations. PDZ activity against single specie biofilms was compared to PDZ-mediated aPDT. Biofilm incubated with PBS in the presence (L^+) and absence of LED light (L^-) represented the control groups.

After incubation period for mature biofilm formation, the wells were washed twice with PBS and aliquots of 200 µL of PDZ or PBS were added to the respective samples: P^+L^+, P^+L^-, P^-L^+ and P^-L^-. All samples were incubated at room temperature, for 10 min in darkness, as a pre-irradiation time. In sequence, 96-well plate contained P^+L^+ and P^-L^+ groups was submitted to LED irradiation, for 28 min, whereas the other was kept in dark room. After the treatments, bacterial cells were harvested from the polystyrene wells by scraping with a sterile pipette tip in PBS and 25 µL of ten-folds

serially diluted samples were plated on Brucella agar. Plates were then incubated at 37°C under anaerobic conditions and the colony counts obtained with a digital colony counter, after 7 days. Microbiology experiments were conducted in triplicate with three repetitions.

2.7 Effect of PDZ -mediated aPDT on mature biofilm in comparison with MTZ

The effect of aPDT against biofilms was compared with MTZ, a standard antibiotic used against pathogenic oral bacteria. For each experiment, mature biofilm samples were formed in two different 96-well plates, corresponding to the irradiated and non-irradiated groups. To PDZ-mediated aPDT groups, the procedures were performed as previously described above (see 2.6). In case of antibiotic treated groups, PBS-washed samples were incubated with 200 μL of MTZ in three different concentrations (MIC, 50x MIC and 100x MIC) for 24 hours. Thereafter, 25 μL of PBS serially diluted samples were plated on Brucella agar and the plates incubated at 37°C to obtain the CFU/mL values after 7 days. The experiments were performed in triplicated and in three different occasions.

2.8 Potential effect of combination therapy on mature biofilm

To gain a better understanding about the potential antimicrobial effect of the treatment, aPDT and MTZ were applied individually against biofilm and the results of viable colonies were compared with the CFU/mL from combination therapy between aPDT and MTZ. Briefly, after aPDT application (see topic 2.6) samples were incubated with 200 μL of MTZ, in three different concentrations: MIC, 50x MIC, 100x MIC, for 24 hours. Additionally, biofilm samples submitted to PDZ-mediated aPDT or treated with MTZ as well as bacterial cells concentrations inoculated into the polystyrene well

served as controls. To each experimental occasion, biofilm samples were assessed in two different plates corresponding to LED light exposition (aPDT) and darkness room. Plates incubated in the absence of the light served as background controls. At the end of incubation period, biofilm was harvest in PBS and 25 µL of serially diluted cultures was plated on Brucella agar to obtain viable colonies after 7 days. All experiments were performed in triplicate with three repetitions to ensure biological reproducibility.

2.10 Statistical data analysis

All experiments were performed in triplicate and repeated three times for each bacteria species (n = 9). Except for confocal analyzes that it was performed in duplicate and repeated two times to ensure the reproducibility of the experiment. Visual inspection of the normality distribution was confirmed by the D'Agostino-Pearson omnibus test. Once data were collected, normal statistical comparisons were performed using one-way analysis of variance (ANOVA) with Tukey's pos-hoc test ($p < 0.05$). The non-parametric data was analyzed by the Kruskal-Wallis test, followed by Dunn's multiple comparison tests. The data presented were plotted as mean ± standard deviation (SD) using a Graph- Pad Prism version 5.0c; $p < 0.05$ was considered statistically significant.

3. Results

3.1 Minimum inhibitory concentration

MIC was considered the lowest concentration of MTZ that completely inhibits 100% of bacterial growth after 24 hours. The optimal concentration was assessed using absorbance reading. MIC values of MTZ were 1 µg/mL and 0.125 µg/mL, for *F. nucleatum* and *P. gingivalis,* respectively.

3.2 PDZ-mediated aPDT on mature biofilm

In order to confirm the effect of photosensibilizer on bacterial cells reduction, the number of viable colonies from single species biofilm after PDZ incubation was compared to PDZ followed by aPDT application. The data showed clearly that to *F. nucleatum* specie, it is crucial to combine PDZ in the highest concentrations (75 and 100 μg/mL) with LED light irradiation to reduce the number of bacteria cells (Fig. 1a). Different outcomes were observed to *P. gingivalis* biofilm since LED light was capable to affect cell viability (P^-L+ group) even in the absence of PDZ. However, CFU/mL reduction was higher when 100 mg/L of PDZ was combined with LED light (aPDT 100 group) (Fig. 1b).

3.3 PDZ-mediated aPDT on mature biofilm when in comparison to antibiotics (MTZ)

Following experiments compared the CFU/mL reduction after PDZ-mediated aPDT on biofilm with the number of sustainable colonies before MTZ treatment. *F. nucleatum* biofilm samples submitted to aPDT at 75 (aPDT75 group) and 100 mg/L (aPDT100 group) revealed significant reduction in the CFU/mL (around 1 Log_{10} reduction). Light irradiation did not affect MTZ action on *F. nucleatum* biofilms. Regardless of the presence of light, highest concentrations of antibiotic reduced significantly the log of CFU/mL, 2.22 and 2.14 Log_{10} to L^-MTZ100x and L^+MTZ100x groups, respectively, when in comparison to the controls group (P^-L^- group) (Fig. 2a).

For *P. gingivalis* biofilm, no difference in expressing log reduction was observed between aPDT at 100 mg/L and MTZ 100x MIC without light (L^-MTZ100x), in relation

to the control: 2.59 log_{10} and 2.53 Log_{10}, respectively. Significant antimicrobial effect was only achieved when MTZ 100x MIC was associated with LED light (3.12 Log_{10}) (Fig. 2b).

3.4 PDZ-mediated aPDT in association with MTZ on mature biofilm

Since substantial reduction in the biofilm viability was observed to either MTZ in its highest concentration or aPDT (P^+L^+), a potential effect of their association was also investigated. Further understanding was achieved after all the independent variables, submitted at the same conditions, were compared to each other.

For *F. nucleatum* biofilm, the results demonstrated that aPDT 75 mg/mL associated with MTZ 100x (aPDT75+MTZ100x) and aPDT 100 mg/L associated with MTZ at concentrations of 50x and 100x (aPDT100+MTZ50x and aPDT100+MTZ100x groups) reduced significantly the log CFU/mL to 2.99, 2.9 and 3.94 Log_{10} respectively. Antimicrobial activity with more than 3 Log_{10} reduction of CFU, in comparison to the control (P^-L^-), was observed when MTZ 100x was applied in combination with aPDT 100 mg/L (aPDT100+100x group) (Fig. 3a).

In case of *P. gingivalis* biofilm, aPDT promoted significant reduction in cell viability when the therapy was applied with MTZ 100x, independent on the PDZ concentration. However, expressive log reduction of CFU/mL, with 5 Log_{10}, was obtained to aPDT 100 mg/L in association with MTZ 100x (aPDT+MTZ100). In fact, light irradiation acts as an important coadjutant against *P. gingivalis* biofilm, with data disclosing less evident viable colonies to light exposition (L^+) than L^- groups (Fig. 3b).

4. Discussion

Antimicrobial PDT has previously been demonstrated to promoting killing of planktonic periodontal pathogens.[29, 37-39] However, microorganisms' susceptibility to aPDT is considerably reduced when they are organized in biofilms. In order to gain new insight into effect of PDZ-mediated aPDT on anaerobic bacteria, we set out to investigate the impact of different PDZ concentrations, either with or without light, on *F. nucleatum* and *P. gingivalis* mature biofilms. Although, we have succeeded in showing a significant viability reduction of both bacteria after therapy application, a potentiated antimicrobial effect was only achieved after PDZ-mediated aPDT combined with local antibiotic administration.

Bacteria organized in biofilms are highly resistant to conventional antimicrobial treatments.[40] With regards to bacterial profile, strong evidences indicate *F. nucleatum* and *P. gingivalis* as two dreaded anaerobic species involved in the initiation and progression of periodontal and peri-implant disease.[41] The possibility of teeth and implant loss as a consequence of disease progression inspires the searching for new strategies to disrupt the pathogenic biofilm and collaborate with disease treatment. Taken into consideration the well-succeed preliminary data obtained from PDZ mediated-aPDT by our research group,[31, 32, 42] we moved forward and evaluated the effect of each aPDT component against periodontopathogenic mature biofilms. In case of *F. nucleatum*, the CFU/mL reduction was directly related to the association between the highest PDZ concentrations and light. Our data revealed that 75 mg/mL and 100 mg/mL of PDZ mediated-aPDT promoted 0.97 and 1.12 Log_{10} reduction in CFU/mL, respectively. Contrary to our outcomes, an interesting study, led in 2016, demonstrated significant effect against *F. nucleatum* bacterial specie, with more than 3 Log10 reductions when aPDT using visible light (vis) and water infiltrated infrared A (wIRA)

was combined with chlorine e6 (Ce6).[37] Expected antimicrobial effect of aPDT against *F. nucleatum* culture was also found in another recent study, but only after extend time of light irradiation. Remarkably, the authors indicated a slight reduced *F. nucleatum* cells even after light irradiation alone, consistent to our fluorescence information.[43] However, in both studies reported,[37, 43] the treatment was tested on planktonic and single bacteria species, in contrast to our work, in which we developed a complex and mature biofilm in single and dual species. This can explain our data is much lower than the effect demonstrated by those both studies[37, 43] in question.

For *P. gingivalis*, the effectiveness of using PDZ as a PS for aPDT was obtained to 100 mg/L, which resulted in 2.66 Log_{10} CFU/mL bacterial reduction. In spite of the efficacy of aPDT against biofilm viability, a required antimicrobial effect was not higher than 3 Log_{10} CFU reduction to ensure antibacterial properties. In contrast, a significant antimicrobial effect of aPDT using visible light and water infiltrated infrared A in combination with chlorine e6 was recently reported against different periodontal pathogens in subgingival oral biofilms.[37] Substantial difference in terms of CFU reduction bacteria when in comparison with our data can be explained by different methodologies applied. In fact, in this study,[37] the authors developed a real oral biofilm on *in situ* devices, but then, subgingival biofilm samples were pooled, centrifuged and resuspend in saline solution. Therefore, the treatment was performed against planktonic bacteria cells' arising from biofilm, in contrast to our methodology, in which aPDT was applied directly against a complex and organized pathogenic structure.

In the absence of PDZ, exposure to light irradiation affected *P. gingivalis* cells viability.[44] This can be explained by the fact of *P. gingivalis* being capable to synthetize endogenous porphyrins in their cell wall, which acts as a natural PS.[45-48]

Susceptibility of determined oral black-pigmented bacteria species to light irradiation in the absence of PS has already been demonstrated in laboratory research and *in vivo* experiments. However, as well as a PS, porphyrin are chemically excited by light of specific wavelengths[49] and promote generation of ROS capable of reacting and affecting biological systems.[50] It has been reported that *P. gingivalis* viability, in culture medium, is easily reduced over 90% by exposure to 70 mW/cm² at a broadband light ranging of 380-520 nm in case of blue light.[51] By a similar power density of 71.7 mW/cm², our results indicated a CFU/mL reduction of 1.33 Log_{10} to *P. gingivalis* after 28 minutes of red light exposition at 660 nm. The notable difference in overall bacteria viability observed in our study is justified by the fact of our outcomes have been obtained from a mature and organized biofilm, which can directly interferes on the light diffusion across extracellular matrix.

Although PDZ-mediated aPDT significantly reduced the number of cultivable bacteria within mature biofilms, the effect of this therapy still is far under the killing rates required to be considered as antimicrobial approach. To date, a major limitation of aPDT involves the inadequate uptake of PS. The idea in combining aPDT and local antibiotics administration was undertaken in an attempt to widen the possibility of therapy application against pathogenic biofilms.[54-56] Systemic antibiotic prescription to fight periodontal and peri-implant disease still is strongly discussed.[57] Antibiotic resistance and development of super infection as potential risks associated with antibiotic therapy confront with the threatening possibility of bone resorption and subsequently, teeth and/or dental implants loss.[58] Taken into account that drug still plays as an important protagonist to treat infection diseases, antibiotic topic administration has emerged as a possible coadjutant in reducing microbial resistance and controlling inflammation from sick periodontal sites.[59-63] Based on this

principle, we first investigated the upshot of different MTZ concentrations on anaerobic biofilm. In agreement with the scientific literature, we found low concentration values of MTZ as sufficient to inhibit *P. gingivalis* and *F. nucleatum* growth in planktonic cells: 0.125 μg/mL and 1 μg/mL, respectively. However, a different picture is expected when MTZ is applied against biofilm. The findings arising from controlled experiments have reported biofilms as being up to 1000 times more resistant to antimicrobials treatments than planktonic cells [64-66]. In our study, MTZ MIC, MTZ 50x or MTZ 100x showed no antimicrobial activity against either *F. nucleatum* neither *P. gingivali*s.

At this point, we demonstrated that although aPDT did not disclose antimicrobial activity, the treatment reduced significantly the number of pathogenic bacteria inside biofilm structure. In this regard, we based on our previous results to support the idea in combining aPDT with antibiotic. Antimicrobial PDT seems to make biofilm cells more sensitive to antibiotic penetration, [55, 56] and our next information confirmed the potentiated effect of this association. One hundred mg/L of PDZ-mediated aPDT associated with MTZ in the highest concentrations resulted in a significant reduction in bacterial load for *F. nucleatum* biofilm by 3.94 $\log_{10}$ CFU/mL. Comparable antimicrobial activity was demonstrated for *P. gingivalis* biofilm since PDZ-mediated aPDT in the presence of 100x MIC antibiotic concentration promoted more than 3 Log10 reductions in CFU, regardless of the PDZ concentration used. The remarkable effect by 5 $\log_{10}$ bacteria reduction was attained to 100 mg/L of PDZ mediated-aPDT combined with MTZ 100x. Underlining the influence of light on porphyrins from *P. gingivalis* cell wall, the higher efficacy of MTZ 100x in the biofilm state was only obtained after light exposure, disclosing 3.07 $\log_{10}$ CFU/mL reduction in bacterial viability. However, the desired antimicrobial properties were achieved when MTZ was applied as an adjunctive therapy to inactivate periodontopathogenic bacteria.

Indeed, aPDT is still moving towards development. The questions raised by our outcomes highlighted the limitation of the therapy alone against mature biofilm and the antibiotic contribution to succeed as antimicrobial approach. Undoubtedly, our study looked at the aPDT effect on anaerobic biofilm developed on polystyrene surfaces. Although we had been prudent in defining a robust biofilm model with human saliva to simulate an oral enviroment, polystyrene substrates act as a positive control to bacteria growth evaluation and therefore the results obtained do not reflect clinical setting. Furthermore, considering our study as the first to investigate aPDT against anaerobic biofilm, we opted for growing bacteria in single specie to reduce the number of variables and gain a better understanding about aPDT benefitis against resistant infections. Further *in vitro* experiments, involving multispecies biofilm and teeth/implant substrates are necessary to recognize the worth of aPDT-antibiotic combination.

Conflicts of interest

The authors declare no conflicts of interest.

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FIGURES

Fig. 1. (A) One-way analysis of variance (ANOVA) was employed with a Tukey's posthoc test. The result showed that the light irradiation associated with the high PDZ concentration reduced the number of viable *P. gingivalis* biofilm colonies. **(B)** Kruskal-Wallis test was employed with a Dunn's Multiple Comparison Test. The result showed that PDT associated with PDZ at concentrations of 75 and 100 mg/L reduced the number of viable *F. nucleatum* biofilm colonies. Data are shown as the mean ± SD (n=9), by using Graph- Pad Prism version 5.0c. $p < 0.05$ was considered statistically significant.

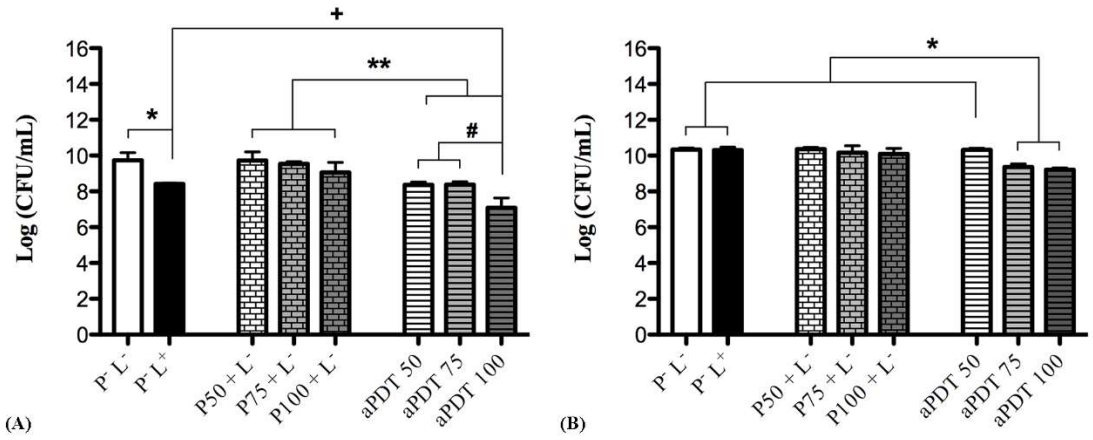


Fig. 2. (A) One-way analysis of variance (ANOVA) was employed with a Tukey's posthoc test. PDT at 100 mg/L reduced significant the number of viable *P. gingivalis* biofilm colonies and light irradiation associated with MTZ PDT at 100 mg/L showed antimicrobial activity on *P. gingivalis* biofilm. **(B)** One way analysis of variance (ANOVA) was employed with a Tukey's posthoc test. Significant reduction in the number of viable *F. nucleatum* biofilm colonies was observed with MTZ was applied in the maximum concentration (100x) independent on the light presence. Data are shown as the mean ± SD (n=9), by using Graph- Pad Prism version 5.0c. $p < 0.05$ was considered statistically significant.

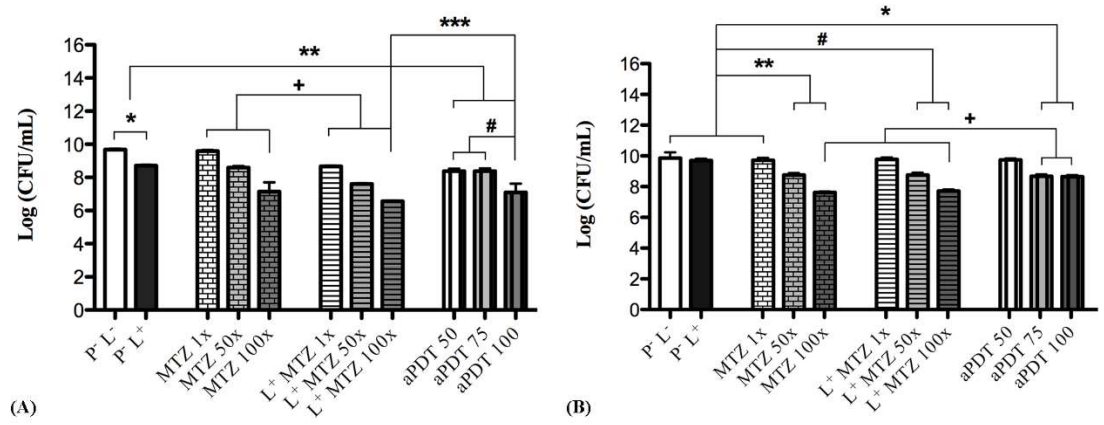
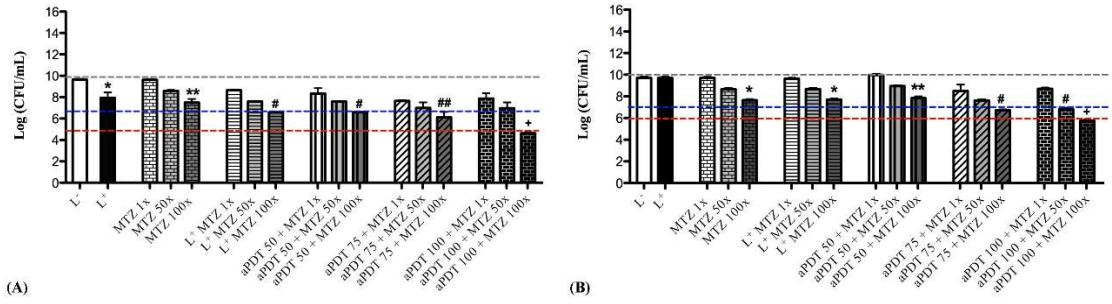


Fig. 3. (A). One way analysis of variance (ANOVA) was employed with a Tukey's posthoc test. Light irradiation associated with MTZ 100x showed antimicrobial activity on *P. gingivalis* biofilm independent on PDT/PDZ concentration. **(B)** One way analysis of variance (ANOVA) was employed with a Tukey's posthoc. PDT at 75 mg/L associated with MTZ 100x and PDT 100 mg/L associated with MTZ at concentrations of 50x and 100x showed antimicrobial activity on *F. nucleatum* biofilm. Data are shown as the mean ± SD (n=9), by using Graph- Pad Prism version 5.0c. $p < 0.05$ was considered statistically significant.



6 CONSIDERAÇÕES FINAIS

A matriz extracelular em biofilmes atua como barreira protetora que limita a penetração de agentes antimicrobianos e, consequentemente, reduz a ação de qualquer tipo de tratamento (Stewart, Franklin[38], 2008). Diante disso, estratégias que aumentam a susceptibilidade dos microrganismos aos tratamentos têm sido avaliadas. Dessa forma, no presente estudo foi avaliado a eficácia da aPDT mediada por PDZ em associação com o antibiótico MTZ em biofilmes patogênicos formados por *F. nucleatum* e *P. gingivalis*.

A reação fotodinâmica ocorre quando o fotossensibilizador é excitado pela luz a um comprimento de onda específico e na presença de oxigênio. Essa reação promove a produção de oxigênio singlete e radicais livres, que podem danificar componentes essenciais das células, como membrana plasmática e DNA, ou modificar atividades metabólicas de forma irreversível, o que causa a morte de microrganismos (Lima et al.[23], 2009; Gursoy et al.[15], 2013). Os resultados mostraram que a aplicação da aPDT na maior concentração de PDZ promoveu uma redução significativamente na viabilidade celular dos microrganismos avaliados (1 e 2.66 Log_{10} de redução, para *F. nucleatum* e *P. gingivalis*, respectivamente). No entanto, apenas a aplicação da aPDT não foi suficiente para a erradicação dos biofilmes para ambas as espécies. Portanto, uma terapia adjuvante antimicrobiana foi avaliada com objetivo de potencializar a eficácia bactericida da aPDT.

O antibiótico metronidazol foi escolhido para este estudo devido ao alto espectro de ação em microrganismos anaeróbios e, por este motivo, tem sido frequentemente utilizado no tratamento da periodontite (Müller[29], 1983). Neste estudo, foi observado através do MIC que baixas concentrações de MTZ foram suficientes para a inibição do

crescimento bacteriano em culturas planctônicas de *P. gingivalis e F. nucleatum*. Por outro lado, mesmo usando uma concentração de cem vezes o MIC (100x MIC), não houve redução bacteriana satisfatória após o tratamento com o MTZ (2 e 2.53 Log_{10} de redução para *F. nucleatum* e *P. gingivalis*, respectivamente).

Os resultados obtidos demonstraram que o efeito das terapias aplicadas aPDT/MTZ, foram potencializadas em associação. Os resultados demonstraram que a aPDT (P100+L+) isoladamente promoveu redução ao redor de 1 log_{10} para o *F. nucleatum*, por outro lado, quando a aPDT foi aplicada em associação com o antibiótico MTZ (aPDT100+MTZ100x) a redução foi de 3.94 Log_{10}. Comportamento semelhante foi observado para a *P. gingivalis*, uma vez que redução de 2.59 Log_{10} foi observada quando a aPDT (P100+L+) foi aplicada isoladamente, por outro lado, se associada ao MTZ (aPDT100+MTZ100x), a redução observada foi de 5 log_{10}. A aplicação de aPDT previamente ao antibiótico pode levar à destruição ou pelo menos danos significativos à estrutura do biofilme promovido por espécies reativas de oxigênio (ROS), o que possivelmente aumentaria a penetração e difusão e, consequentemente, a ação do antibiótico (Barra et al.[1], 2015) .

Apesar das limitações deste estudo, os resultados demonstraram que o efeito da aPDT associado ao MTZ foi potenciado quando comparado com tratamentos isolados. Com isso, esta estratégia mostrou potencial como uma nova abordagem para tratamento de biofilmes periodontopatogênicos, entretanto, estudos futuros devem ser realizados para validar a eficácia do protocolo proposto.

7 CONCLUSÃO

De acordo com as condições experimentais avaliadas, os resultados obtidos permitiram concluir que:

- O período de 24 horas de adesão seguido de 5 dias de formação de biofilme é satisfatório para a formação de biofilme maduro mono-espécie de *F. nucleatum* e *P. gingivalis;*
- A aPDT (PDZ 100 mg/L) associada ao MTZ100x promoveu uma redução significativa de UFC/mL para *F. nucleatum* e *P. gingivalis*, com redução de 3.94 e 5 Log_{10}, respectivamente;
- A aplicação de aPDT associada ao MTZ foi potencializada quando comparada aos tratamentos isoladamente.

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ANEXO A

FACULDADE DE ODONTOLOGIA DE ARARAQUARA - UNESP

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Eficácia da Terapia Fotodinâmica associada ao Metronidazol na inativação de biofilmes de Porphyromonas gingivalis e Fusobacterium nucleatum: um estudo in vitro.

Pesquisador: Ana Cláudia Pavarina

Área Temática:

Versão: 2

CAAE: 26142014.0.0000.5416

Instituição Proponente: Faculdade de Odontologia de Araraquara - UNESP

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 628.588

Data da Relatoria: 29/04/2014

Apresentação do Projeto:

Eficácia da Terapia Fotodinâmica associada ao Metronidazol na inativação de biofilmes de Porphyromonas gingivalis e Fusobacterium nucleatum: um estudo in vitro.

Objetivo da Pesquisa:

Avaliar in vitro a eficácia da Terapia Fotodinâmica Antimicrobiana e da aplicação de Metronidazol, isolados e associados, na inativação de biofilme de Fusobacterium nucleatum (ATCC 25586) e Porphyromonas gingivalis(ATCC 32277).

Avaliação dos Riscos e Benefícios:

RISCOS:Neste estudo será realizada somente a coleta de saliva dos indivíduos.Serão tomados todos os cuidados relativos a biossegurança inerentes a situação para a proteção do indivíduo e do pesquisador.

BENEFÍCIOS: Com a resistência dos micro-organismos a medicamentos tópicos e sistêmicos, este estudo tem como objetivo fazer combinações de terapias,associação de terapia fotodinâmica e antibioticoterapia,buscando a eliminação destes micro-organismos e minimizar a aquisição de resistência.

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Continuação do Parecer: 626.588

Comentários e Considerações sobre a Pesquisa:
O ESTUDO TRARÁ CONTRIBUIÇÃO À ÁREA AO QUAL SE APLICA.

Considerações sobre os Termos de apresentação obrigatória:
TODOS OS TERMOS FORAM APRESENTADOS

Conclusões ou Pendências e Lista de Inadequações:
O PROJETO ESTÁ APTO PARA SER EXECUTADO

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

Considerações Finais a critério do CEP:
Atendida pendência de reunião, considero APROVADO o projeto. Encaminhe-se ao pesquisador para dar início à pesquisa.

ARARAQUARA, 28 de Abril de 2014

Assinador por:
Maurício Meirelles Nagle
(Coordenador)

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ANEXO B

Érica Dorigatti de Avila
sex 23/02, 16:04
Você

Responder

Em Sexta-feira, 23 de Fevereiro de 2018 13:46, Permissions Helpdesk <permissionshelpdesk@elsevier.com> escreveu:

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Best of luck with your thesis and best regards,
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Araraquara, 16 de março de 2017.

LÍVIA JACOVASSI TAVARES