ITALO SARTO CARVALHO RODRIGUES

EFEITO DE DIFERENTES CONCENTRAÇÕES DE CLOREXIDINA NA PERIODONTITE INDUZIDA EM RATOS E A INFLUÊNCIA DO CÁLCIO NA FORMAÇÃO DE BIOFILMES POR *PREVOTELLA INTERMEDIA*

EFFECT OF CHLORHEXIDINE AT MULTIPLE-DOSES AND CONCENTRATIONS ON EXPERIMENTAL PERIODONTITIS IN RATS AND IMPACT OF CALCIUM ON *PREVOTELLA INTERMEDIA* SURFACE ATTACHMENT AND BIOFILM FORMATION

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FACULDADE DE ODONTOLOGIA DE PIRACICABA

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutor em Biologia Buco-Dental, na Área de concentração em Microbiologia e Imunologia.

Thesis presented in partial fulfillment of the requirements for the degree of Doctor in Oral Biology, field of Microbiology and Immunology, in the Piracicaba Dental School, University of Campinas.

Orientador: Prof. Dr. Rafael Nobrega Stipp

Este exemplar corresponde à versão final da tese defendida por Italo Sarto Carvalho Rodrigues e orientado pelo Prof. Dr. Rafael Nobrega Stipp.

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RESUMO

O biofilme é uma população biológica com um elevado grau de organização, onde os microrganismos presentes formam uma comunidade estruturada, coordenada e funcional. O estudo do comportamento dos biofilmes é fundamental para melhorar as formas de controle, especialmente durante infecções, tais como as doenças periodontais. No primeiro capítulo, foram avaliados os efeitos da aplicação tópica e frequente do digluconato de clorexidina (CLX) em diferentes concentrações na periodontite induzida por ligadura nos primeiros molares de ratos. As ligaduras receberam 10 µl de soluções de CLX à 0,2%, 2%, 10%, 20% ou diluente, de quatro em quatro dias, em um total de quatro aplicações. Após eutanásia, a quantidade de células bacterianas no biofilme formado sobre a ligadura foi estimada por cultura e por PCR quantitativo. A reabsorção óssea foi mensurada em altura e área por fotografia digital e em volume por microtomógrafo. Depois de quatro dias a partir da última aplicação da CLX, as reduções bacterianas mantidas pelos tratamentos com CLX atingiram até 10^{-6}. O grupo que recebeu CLX a 20% teve, em média, logs bacterianos 3,3× menor ($p<0.01$, Kruskal-Wallis). Não houve diferença estatística entre os grupos em relação à reabsorção óssea por ambos os métodos testados ($p>0.05$, Kruskal-Wallis), embora 55% dos sítios apresentaram menor reabsorção óssea. No segundo capítulo, foi avaliada a influência de diversas substâncias na formação de biofilme por *Prevotella intermedia*. Os biofilmes foram formados em placas de 48 poços contendo tratamento de superfície prévio com DNA purificado, hemina, $CaCO_3$, $Ca(OH)_2$, $CaCl_2$, soro, albumina, dextrana, metionina, glicose, glutamina, KCl, complexo vitamínico, cistina ou mucina. O biofilme formado foi corado e quantificado por espectrofotometria. A arquitetura do biofilme foi visualizada por microscopia confocal de fluorescência por varredura laser. O tratamento da superfície com $CaCl_2$ a 1 mg/cm^2 permitiu a formação do biofilme em quantidade de 0,3 OD_{490nm} ($p<0.01$, ANOVA Dunnet), sendo esse valor 10× superior quando a superfície foi tratada com 2,5 mg/cm^2 ($p<0.01$, ANOVA Dunnet). As demais

substâncias tiveram pouco ou nenhum impacto sobre a formação do biofilme. A visualização por microscopia confocal revelou uma comunidade estruturada e com vitalidade em toda sua espessura. Conclusões: os dados indicam que a CLX concentrada diminui a carga bacteriana na região da periodontite induzida, que reflete em menor reabsorção óssea apenas em parte das amostras. O pré-revestimento da superfície de crescimento com cálcio promove a formação de biofilme por *P. intermedia*.

PALAVRAS-CHAVE: Cloreto de cálcio. Clorexidina. Doença periodontal. Microtomografia por Raio-X. *Prevotella intermedia*. Reação em cadeia da polimerase em tempo real.

ABSTRACT

Biofilms are biological communities with a high degree of organization, in which micro-organisms form structured, coordinated and functional population embedded in a self-created extracellular matrix. Biofilm is also associated with a high level of antimicrobial tolerance of the associated organisms. Understanding biofilm behavior is crucial to develop ways for its control during infections, such as periodontal disease. In the first chapter, topical and frequent application of various concentrations of chlorhexidine digluconate (CLX) where evaluated. Periodontitis were induced by ligature on first molars. Then, ligatures were treated with 10 µl of chlorhexidine solutions at 0.2%, 2%, 10%, 20% or diluent, every four days in a total of four applications periods. After euthanasia, bacterial loads on ligatures were estimated by both culture and qPCR. Bone resorption height and area were measured by digital photography and its volume by microtomography. Treated sites had bacterial reductions up to 10^{-6} cells. Treatment with 20% CLX showed mean of 3.3× lower bacterial levels (p<0.01, Kruskal-Wallis). There was no statistical difference between groups regarding bone resorption (p>0.05, Kruskal-Wallis), although 55% of the treated sites had some lower bone resorption. In the second chapter, substances that may enhance biofilm formation by *Prevotella intermedia* were investigated. Wells of 48-well plates were coated with DNA, hemin, $CaCO_3$, $Ca(OH)_2$, $CaCl_2$, serum, albumin, dextran, methionine, glucose, glutamine, KCl, vitamin complex, cystine or mucin. Biofilms were grown for 24 h, washed, stained and quantified by spectrophotometry. Biofilm architecture and its viability were visualized by Confocal Laser Scanning Microscopy. Surfaces treated with 1 mg/cm^2 of $CaCl_2$ enhanced biofilm amount by 0.3 OD_{490nm} (p<0.01, ANOVA Dunnet), while 2.5 mg/cm^2 yielded 10-fold more biofilm mass (p<0.01, ANOVA Dunnet). Other substances had modest or no impact in biofilm mass. Confocal microscopy images showed structured and alive biofilms with no dead areas. Conclusions: concentrated CLX reduces bacterial load, which reflects in lower

bone resorption in few sites. Surfaces pre-coated with calcium chloride enhance *P. intermedia* biofilm formation.

KEYWORDS: Chlorhexidine. Calcium chloride. Periodontal disease. *Prevotella intermedia*. Real-time polymerase chain reaction. X-ray microtomography.

SUMÁRIO

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EPÍGRAFE

"Life moves pretty fast. If you don't stop and look around once in a while, you could miss it."

Ferris Bueller's Day Off. Paramount Pictures, EUA. 1986.

LISTA DE ABREVIATURAS E SIGLAS

16S rRNA - Gene codificador do RNA ribossômico 16S / *Gene coding for 16S ribosomal RNA*

A_{550nm} - Absorbância no comprimento de onda de 550 nanômetros / *Absorbance at a wavelength of 550 nanometers*

A_{650nm} - Absorbância no comprimento de onda de 650 nanômetros / *Absorbance at a wavelength of 650 nanometers*

BHI - Infusão Cérebro e Coração / *Brain Heart Infusion*

$CaCl_2$ - Cloreto de cálcio / *Calcium chloride*

µCT - Microtomografia computadorizada por Raios X / *X-ray micro–computed tomography*

DO / ***OD*** - Densidade Óptica / O*ptical Density*

qPCR - Reação em cadeia da polimerase quantitativa / *Quantitative polymerase chain reaction*

TSB - Caldo de Soja Triptica / *Tryptic Soy Broth*

INTRODUÇÃO

Doenças periodontais e o digluconato de clorexidina

As doenças periodontais representam uma preocupação global pela alta prevalência e pelos prejuízos aos tecidos de suporte dental, sendo a principal causa de perda dentária da atualidade (Oliver et al., 1991; Varoni et al., 2012; Teles et al., 2013). No mundo, apenas a periodontite crônica atinge entre 10-30% da população mundial, com prevalência em torno de 30% da população brasileira. Nos Estados Unidos da América mais de 47% da população, o que representa 64,7 milhões de adultos diagnosticados com periodontite (Ministério da Saúde, 2010; Eke et al., 2012; Varoni et al., 2012; Marcenes et al., 2013; Richards, 2013).

A periodontite é definida como uma doença de etiologia multifatorial resultante da interação do biofilme microbiano com a resposta imunoinflamatória do hospedeiro, que podem ser modulados por fatores sistêmicos e ambientais, como o diabetes e o tabagismo (Shlossman et al., 1990; Page & Kornman, 1997; Haffajee & Socransky, 2001; Teles et al., 2013). Tal interação resulta na destruição do aparato de inserção dos dentes, que em última instância pode levar à perda do elemento dental (Löe et al., 1978; Offenbacher, 1996).

Há muito tempo sabe-se que o processo patológico inicia-se por bactérias periodontopatogênicas presentes no biofilme bucal em um hospedeiro susceptível (Löe et al., 1965). Esses microrganismos atuam por meio de mecanismos diretos, como a liberação de produtos tóxicos e a secreção de enzimas líticas, que causam a destruição tecidual; e indiretos, como as reações do sistema imune do hospedeiro que podem contribuir significativamente no processo de destruição tecidual (Kinane et al., 2008).

A prevenção é dependente da adesão do indivíduo e envolve atividades que podem ser realizadas diariamente (escovação e uso do fio-dental), entretanto não atuam no biofilme subgengival já formado, dificultando o controle de

microrganismos subgengivais pela ação do indivíduo (Drisko, 2001; Hau et al., 2014).

O tratamento do quadro patológico se dá por intervenção profissional (Monteiro et al., 2011; Varoni et al., 2012). As técnicas de raspagem e de alisamento radicular (RAR), reconhecidas como padrão para o tratamento clínico periodontal, objetivam o restabelecimento da saúde periodontal pela remoção do biofilme (Haffajee & Socransky, 2005) e manutenção da microbiota bucal em níveis que sejam compatíveis em quantidade e qualidade com a homeostasia dos tecidos periodontais.

Porém, mesmo após a RAR, há o eminente risco da permanência de biofilme residual, com presença de periodontopatógenos que podem influenciar negativamente o prognóstico clínico (Teles et al., 2013). A permanência de biofilme residual pode ser favorecida pela complexa anatomia e grandes profundidades das bolsas periodontais, a presença de concavidades radiculares, as bifurcações e as trifurcações, em base do aumento de dificuldade de instrumentação pelo operador (Adriaens & Adriaens, 2004; Umeda et al., 2004). Ainda, há reservatórios remanescentes de bactérias nos túbulos dentinários e nos próprios tecidos periodontais (Takasaki et al., 2009), em cálculos remanescentes (Cobb, 2009), que também podem comprometer o sucesso a longo prazo dos sítios tratados. O uso de antimicrobianos, locais ou sistêmicos, como adjuvantes químicos tem o objetivo de suprimir ou reduzir a quantidade de microrganismos patógenos remanescentes e trazer benefícios adicionais à terapia mecânica convencional (Quirynen et al., 2002; Slots, 2002; Slots, 2002b; Varoni et al., 2012; Keestra et al., 2014; Teles et al., 2013). Dentre os antimicrobianos locais destaca-se o digluconato de clorexidina (CLX), com bons resultados na redução microbiana (Slots, 2002; Slots, 2002b; Varoni et al., 2012; Keestra et al., 2014)

O CLX é a substância referência no controle químico tópico do biofilme supragengival em Odontologia. O bochecho com CLX vem sendo utilizado sob um protocolo que admite uma frequência de administração duas vezes ao dia, quer como adjunto ao tratamento não cirúrgico ou como agente químico coadjuvante

pós-cirúrgico (Campos et al., 2012; Varoni et al., 2012). Possui amplo espectro de ação, alta substantividade e boa biocompatibilidade (Varoni et al., 2012). Face à eficácia e segurança de seu emprego, o CLX é considerado um dos antisséptico essenciais pela Organização Mundial de Saúde (WHO, 2013).

CLX é caracterizado como um detergente catiônico constituído por dois anéis de 4-clorofenol e dois grupos bisbiguanidas que estão simetricamente ligados a uma cadeia hexametilena. Estas características fornecem à molécula propriedades hidrofílicas e hidrofóbicas que contribuem para seu amplo espectro de ação. Geralmente é utilizado na forma de sal de gluconato, mas pode também ser encontrado na formulação de acetato ou hidroclorido (Barrett-Bee et al., 1994; Varoni et al., 2012).

O mecanismo de ação do CLX, quando em altas concentrações, envolve intercalar-se à membrana celular microbiana, onde provoca o aumento da permeabilidade e liberação do conteúdo intracelular, causando ainda precipitação/coagulação das proteínas microbianas (revisado em Varoni et al., 2012). Além disso, o CLX tem a capacidade de se adsorver à proteína salivar mucina, aumentando sua substantividade intra-bucal e sendo liberada lentamente. Dessa forma sua ação antimicrobiana pode durar, de forma mais branda, entre 8-12 horas (Barrett-Bee et al., 1994; Varoni et al., 2012).

O CLX apresenta baixa evidência de toxicidade sistêmica em seres humanos e não produz qualquer resistência apreciável dos microrganismos bucais (Löe, 1973; Carranza & Newman, 1997). Há evidências conflitantes sobre a toxicidade do CLX nos tecidos bucais. Embora alguns estudos demonstraram que CLX não afeta a síntese de colágeno durante a cicatrização tecidual (Brennan et al., 1986; Heitz et al., 2004; Faria et al., 2007), outros afirmam que essa droga apresentaria o efeito contrário, retardando a cicatrização (Luostarinen et al., 1977; Paunio et al., 1978; Bassetti et al., 1980; Faria et al., 2007). Em um estudo mais recente, que avaliou a citotoxicidade de soluções aquosas de CLX (0,006%; 0,125%; 0,2%; 1% e 2%) em cultura de células humanas MDPC-23, demonstrou que quanto maior a concentração de CLX e maior for o tempo de contato com as

células, mais fortes são os efeitos citotóxicos (Lessa et al., 2010). Entretanto alguns efeitos colaterais são comumente atribuídos ao uso oral prolongado, por mais de 14 dias, da CLX em colutórios bucais, como: pigmentação negro-acastanhada dos dentes, restaurações, ou dorso de língua, descamação e perda temporária da sensibilidade oral (Pratten et al., 1998; Varoni et al., 2012).

A aplicação do CLX como colutório bucal promove redução significativa nos índices de placa e sangramento gengival, contribuindo no controle do biofilme supragengival (Varoni et al., 2012). Além disso, o bochecho com 10 ml de 0,2% de CLX, durante 60 segundos, por duas vezes ao dia, mesmo na ausência de limpeza mecânica dos dentes, inibe ou atrasa o início da formação do biofilme e consequente desenvolvimento da gengivite (Löe & Schiott, 1970).

Um estudo utilizando CLX 0,2% como colutório bucal e gel de CLX 1% no dorso de língua, chegaram a resultados que indicavam o retardo na formação de biofilme e diminuição na carga bacteriana total das superfícies dentais e da saliva (Sekino et al., 2004). Contudo, após a suspensão do regime de utilização do antimicrobiano, nova estrutura de microrganismos se restabelecia num curto período de quatro dias (Sekino et al., 2004).

Com relação à terapia periodontal, a irrigação subgengival de sítios periodontais isolados com solução de CLX 0,12% foi incapaz de mostrar melhoras clínicas e microbiológicas significativas, apresentando resultados semelhantes aos sítios irrigados com solução salina estéril (Stabholz et al., 1998; Campos et al., 2012). Em outro estudo, que utiliza gel contendo CLX 1%, a irrigação das bolsas periodontais se mostrou eficaz na melhoria desses parâmetros (Vinholis et al., 2001; Campos et al., 2012). Entretanto não existe consenso sobre a melhor concentração e regime de tratamento.

Revisões sobre uso do Periochip®, uma matriz biodegradável de gelatina hidrolisada contendo 2,5 mg de CLX, que adicionada localmente no interior da bolsa periodontal funciona como um dispositivo de liberação de CLX não são conclusivos (Cosyn & Wyn, 2006; Plessas, 2014). Segundo o fabricante, a liberação do dispositivo é de cerca de 40% nas primeiras quatro horas e chega a

uma concentração média de 1,4 mg/mL de fluído crevicular, ou seja, 0,14% de CLX. O Periochip® permanece entre 7-10 dias até sua total reabsorção, e a concentração de CLX residual após as 4 e 24 h é menor (Dexcel Pharma, 2014).

O CLX aplicado subgengivalmente, em baixas concentrações (<1%), aparenta ter baixa eficácia no controle da doença periodontal, devido à dificuldade de combater efetivamente o biofilme residual no interior da bolsa (Campos et al., 2012, Varoni et al., 2012; Plessas, 2014). Ainda há necessidade de pesquisas que esclareçam a concentração ideal da substância, a sua permanência no ambiente subgengival para exercer a ação esperada, a atuação do CLX como agente irrigador nos diferentes tipos de doença periodontal, assim como sua eficácia em diferentes profundidades de bolsa.

A remoção mecânica profissional do biofilme é a forma de principal de controle do biofilme subgengival (revisado em Teles et al., 2013). O uso do CLX concentrado, associado à remoção mecânica, parece promissor, uma vez que pode ser capaz de exercer ação sobre biofilmes residuais. Considerando a inevitável remoção do agente antimicrobiano pela renovação do fluido gengival, espera-se com aplicações frequentes, mas não constantes, evitar os estágios de maturação do biofilme onde espécies periodontopatogênicas comecem a serem prevalentes, o que ocorre em torno do quarto dia de formação não perturbado (Löe et al., 1965, Teles et al., 2013).

Biofilmes e *Prevotella intermedia*

Biofilmes são comunidades biológicas com elevados graus de organização, onde os microrganismos presentes formam comunidades estruturadas, coordenadas e funcionais. As células encontram-se ligadas umas às outras e às superfícies, embebidas em uma matriz extracelular de natureza polimérica produzida por elas próprias. A associação das células em biofilmes constitui uma forma vantajosa de desenvolvimento que permite benefícios reprodutivos, metabólicos e defensivos contra outros organismos, substâncias ou

o próprio hospedeiro (Epstein et al., 2011, Huang et al., 2011, Dentino et al., 2013).

A matriz extracelular (MEC) pode atuar como uma barreira de difusão e até como um filtro de ligação para certos antibióticos (Epstein et al., 2011). A estrutura do biofilme compreende múltiplas camadas celulares recobertas pela matriz extracelular. A composição do MEC varia muito entre espécies, mas, em geral, os seus componentes principais são exopolissacarídeos (Epstein et al., 2011; Huang et al., 2011). A persistência antibiótica no interior do biofilme pode chegar a ser 1000-1500× maior do que a de células planctônicas (Huang et al., 2011).

Nas superfícies dentais, o processo de formação dos biofilmes tem início sobre a película adquirida (uma fina camada de glicoproteínas salivares que se adsorve a superfície dental limpa). Os colonizadores iniciais, que possuem a capacidade de interagir com as proteínas da película adquirida fixam-se e iniciam a colonização (revisado em Huang et al., 2011). Posteriormente, o biofilme vai passando por estágios de maturação, com crescimento microbiano, produção de MEC e colonização continua de espécies que tem a habilidade de reconhecer e de se coagregar aos colonizadores iniciais. Essa sucessão de espécies acompanha em aumento de volume e complexidade do biofilme, culminando em alterações físicas e metabólicas na estrutura. Alterações classicamente associadas ao favorecimento de espécies patógenas oportunistas e/ou patógenos incluem, por exemplo, a diminuição da concentração de oxigênio e/ou acidificação (Huang et al., 2011; Teles et al., 2013).

As condições físicas e bioquímicas que favorecem as espécies periodontopatógenas, embora não totalmente elucidadas, são atingidas em cerca de quatro dias da formação do biofilme (Löe et al., 1965, Teles et al., 2013). Espécies associadas com quadros de saúde e doenças periodontais, foram definidas e organizadas em “Complexos Microbianos” a mais de 15 anos (Socransky et al., 1998) e recentemente revistas, com poucas alterações (Teles et al., 2013). Dentre esses complexos, o Complexo Laranja inclui as espécies

Parvimonas micra, *P. intermedia*, *Prevotella nigrescens*, *Fusobacterium nucleatum,* entre outras; e o Complexo Vermelho, constituído por *Porphyromonas gingivalis*, *Tannerella forsythia, Treponema denticola,* são reconhecidos como diretamente relacionados com o desenvolvimento de doenças periodontais, notavelmente periodontites (Socransky et al., 1998; Socransky & Haffajee, 2005; Teles et al., 2013). Os integrantes do Complexo Laranja fornecem condições adequadas para o aumento das espécies mais virulentas do Complexo Vermelho (Socransky & Haffajee, 2005; Teles et al., 2013) e assim podem ser alvos interessantes para combate do biofilme periodonto patogênico.

Dentre os componentes do Complexo Laranja, destacamos a espécie *P. intermedia. P. intermedia* é um bacilo Gram-negativo, anaeróbio estrito, fastidioso e que forma colônias pigmentadas em negro (Shah & Gharbia, 1992; Socransky et al., 1998). Assim como os demais membros do gênero *Prevotella, P. intermedia* necessita de condições especiais para o crescimento em condições laboratoriais, que incluem o enriquecimento do meio de cultivo com hemina e menadiona (Yu et al., 2007; Kuboniwa et al., 2012).

P. intermedia é associada com o aumento de profundidade de sondagem (Socransky et al., 1998). Apresenta diversos fatores de virulência que fundamentam o seu potencial patogênico para causar infecções (Shibata et al., 1993; Jansen et al., 1995; Dorn et al., 1998; Chen et al., 1999; Leung et al., 1999; Hashimoto et al., 2003, Yamanaka et al., 2011), como, por exemplo, a grande capacidade de invasão de células eucarióticas (Dorn et al., 1998). A espécie, embora associada à quadros patológicos periodontais, também pode ser encontrada em locais saudáveis (Shah & Gharbia, 1992; Gharbia et al., 1994; Mättö et al., 1996; Kuboniwa et al., 2012).

Como colonizador tardio (Socransky et al., 1998; Socransky & Haffajee, 2005; Teles et al., 2013), *P. intermedia* não tende a organizar-se em biofilmes monoespécie em condições laboratoriais. Nessa condição, ou são formados "biofilmes" extremamente frágeis, pouco aderidos e sem espessura, ou não são formados (Yamanaka et al., 2011).

A disponibilidade de nutrientes minerais específicos pode funcionar como um estímulo metabólico, provocando mudanças nas células que podem promover o desenvolvimento de biofilmes (Prakash et al., 2003; Cruz et al., 2012). Geralmente o aumento da concentração de nutrientes está, até certo ponto, diretamente relacionado com o aumento no número de células bacterianas aderidas no biofilme. Alguns nutrientes estão presentes no cultivo em concentrações quase insignificantes de se aferir, tais como íons inorgânicos (Ferro, Cálcio, Cobre, Magnésio, Potássio, Zinco) e algumas vitaminas, mas esses micronutrientes podem ser necessários para o crescimento do biofilme (Prakash et al., 2003, Cobine et al., 2013).

Todos os organismos têm de adquirir quantidades adequadas de cada um dos micronutrientes que estão envolvidos em diversas funções metabólicas e celulares, como o metabolismo de energia, metabolismo primário e secundário, a proteção de células, a regulação de genes, a transdução de sinal, a divisão celular, entre outros (Hänsch & Mendel, 2009). A ausência de alguns micronutrientes pode não afetar a vitalidade do microrganismo, mas quando suplementados podem amplificar algumas características metabólicas (Hänsch & Mendel, 2009).

Cátions bivalentes (Ca^{2+} e Mg^{2+}, por exemplo) são associados ao crescimento em biofilme em diferentes espécies bacterianas (Cruz, 2012; Cobine, 2013). Inicialmente, esses cátions podem estabilizar o envelope celular e promover uma ponte iônica entre células, e entre células e biopolímeros da matriz do biofilme (Rose, 2000; Chen et al., 2002; Cruz et al., 2012). Eles também desempenham um papel na regulação da expressão gênica de fatores de virulência e de formação de biofilme (Patrauchan et al., 2005; Sarkisova et al., 2005; Cruz et al., 2012).

Estudos com diversas espécies que envolvem a formação de biofilme em meio suplementados com alguns compostos químicos, como alguns tipos de sais que liberam íons cálcio, demonstram uma amplificação na capacidade de formação e de aderência do biofilme (Hinsa et al., 2003; Patrauchan et al., 2005;

Sarkisova et al., 2005; Sauer et al., 2002; Hänsch & Mendel, 2009; Cruz et al., 2012). A formação de biofilme inicia-se com a fixação transitória de células, seguido de ligações estáveis, uma etapa chave no processo. Em estágios subsquentes, as células aumentam em número e há a produção de matriz extracelular (Sauer et al., 2002; Hinsa et al., 2003; Sarkisova et al., 2005; Cruz et al., 2012).

Em espécies bucais como *Streptococcus mutans*, o crescimento em biofilmes monoespécie *in vitro* é estimulado pela presença e quantidade de sacarose, enquanto que em *Streptococcus sanguinis*, por superfícies tratadas com saliva (revisado em Nobbs et al., 2008). Não se conhece estímulos laboratoriais que favoreçam a formação de biofilmes monoespécie *in vitro* robustos por *P. intermedia.*

Frente ao exposto, no primeiro capítulo foram avaliados os efeitos da aplicação tópica e frequente do CLX em diferentes concentrações na periodontite induzida em ratos, através de quantificações do total de microrganismos acumulados e da perda óssea. No segundo capítulo foi avaliada a influência de diversos tratamentos de superfície no favorecimento da formação de biofilme por *P. intermedia.*

CAPÍTULO 1: EFFECT OF CHLORHEXIDINE AT MULTIPLE-DOSES AND CONCENTRATIONS ON MICROBIAL LOAD AND PERIODONTITIS IN RATS.

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Abstract

Major clinical benefits of subgengival antimicrobial irrigants during mechanical root debridement are limited and somehow conflicting. Since conventional concentrations of chlorhexidine digluconate (CHX) (<0.2%) are unlike to act in the subgingival sites, this work evaluated the effects of CHX) applied in higher frequency and concentrations. **Methods:** Periodontitis were induced by ligature on first molars of Wistar rats. Ligatures were treated with 10 µl of chlorhexidine solutions at 0.2%, 2%, 10%, 20% or diluent (control), every four days in a total of four applications periods. The animals were euthanized on the 20^{th} day of experiment and bacterial loads on ligatures were estimated by both culture and qPCR. Bone resorption height and area were measured by digital photography and its volume by microtomography. **Results:** After four days from the last application of CHX, CHX-treated sites still have bacterial reductions up to 10^{-6} cells when compared to control. Treatment with 20% CLX showed mean of 3.3× lower bacterial levels (p<0.01, Kruskal-Wallis). There was no statistical difference between groups regarding bone resorption (p>0.05, Kruskal-Wallis), although 55% of the CHX-treated sites were responsive to therapies showing improvements in bone tissue. **Conclusion:** Concentrated CHX reduces periodontal biofilm-organized bacterial load, which reflects in lower bone resorption in few sites under experimental periodontitis.

Keywords: Alveolar bone loss. Periodontitis. Real-time polymerase chain reaction. X-ray microtomography.

Introduction

Periodontitis is a highly prevalent chronic opportunistic disease that affects soft and mineralized periodontal tissues. The disease is initiated by a synergistic and dysbiotic subgingival polymicrobial biofilm accumulated on the surfaces of teeth and adjacent structures (Hajishengallis & Lamont, 2012; Teles et al., 2013). This growth when undisturbed allows biofilm maturation over time and a shift in the bacterial communities occurs. Later colonizers and keystone species may trigger inflammatory process and downstream events that lead to degradation of tissues as well as resorption of the alveolar bone itself (Hajishengallis & Lamont, 2012; Teles et al, 2013).

The traditional therapy for periodontitis disease has been the mechanical root debridement. Although largely effective, therapies that predictably can treat the condition in all subjects are still missing, as evidenced by the existence of refractory cases in which disease continues to progress despite comprehensive periodontal treatment (Teles et al., 2013).

Following debridement, residual biofilm are likely to be left in up to 30% of areas (Slots, 2002), into dentinal tubules and either in the periodontal tissues themselves (Takasaki et al., 2009) delaying or avoiding host healing. To prevent an early microbial re-colonization, the use of subgengival antimicrobial irrigants or local drug delivery devices has been proposed (Slots, 2002; Varoni et al., 2012; Keestra et al., 2014). However, their long-term efficacy and major clinical benefits in periodontitis are limited and somehow conflicting (Cosyn & Wyn, 2006; Krayer et al., 2010; Varoni et al., 2012; Tariq et al., 2012).

As adjunctive, chlorhexidine gluconate (CHX) is one of the most promising agent, due to its broad-spectrum activity, substantivity and biocompatibility (Slots, 2002; Varoni et al., 2012). Apart of technical issues that may vary between studies, bacteria in mature biofilms and nutrient-limited biofilms are up to 1000× more tolerant to antimicrobials (Shen et al., 2011; Varoni et al., 2012; Teles et al., 2013). Hence, the desired antimicrobial effects with single episodes of subgingival

irrigation are not possible (Slots, 2002). Moreover, 0.2% CHX is usually not efficacious for subgingival irrigation and causes minimal change in the subgingival microbiota (Slots, 2002). Using 1.5% or 2% CHX provide a more effective may for killing residual subgingival biofilm (Southard et al., 1989; Slots, 2002; Chauhan et al., 2013).

In this work we theorize that if the maturation of the disease-associated subgingival climax community can be delayed, initiation of periodontal tissue destruction could be averted.

Materials and method

Animals. Sixty 3-week-old male specific pathogen free (SPF) Wistar rats (CEMIB/UNICAMP) were included in this study. Animals were acclimatized for five days at 12 h day/night cycles and constant temperature (21°C). Standard rat chow pellets and water were available *ad libitum* (Marques et al., 2005). Procedures and experimental protocol were approved by the University of Campinas Institutional Animal Care and Use Committee (#2948-1/2012).

Experimental design. General anesthesia was obtained by intramuscular administration of 10 mg/kg of ketamine hydrochloride (Dopalen; Vetbrands, SP, Brazil) and 5 mg/kg of xylazine hydrochloride (Anasedan; Vetbrands) (Marques et al., 2005). To induce experimental periodontitis, a ligature was placed bilaterally around mandibular first molars in a submarginal position (Marques et al., 2005). Ligature placement was accomplished by one trained operator (MLGC). The animals were randomly assigned to of one of the following experimental groups:

- G1: Ligated tooth with water treatment (disease-positive, sham-treatment);

- G2: Ligated tooth with 0.2% CHX (Varoni et al., 2012) treatment (n=10);

- G3: Ligated tooth with 2% CHX (Southard et al., 1989; Chauhan et al., 2013) treatment (n=10);

- G4: Ligated tooth with 10% CHX treatment (n=10);

- G5: Ligated tooth with 20% CHX treatment (n=10);

- G6: No ligature and no treatment (no-disease control) (n=10).

Animals belonging to the same group were housed together (5 per cage). On the 4^{th}, 8^{th}, 12^{th} and 16^{th} days, animals from G1 to G5 were anesthetized and bilaterally treated with 10 µL per teeth, applied with micropipette, of the nominated group's liquid, during 1 min. CHX solution (20% in H_2O) was purchased from Sigma (#C9394; Sigma-Aldrich, Saint Louis, MO, USA), and diluted accordingly. Animals from G6 received sham procedures as handling and anesthesia only.

Sampling. Rats were euthanized on the 20^{th} day and mandibles were excised. Cotton ligatures were carefully removed and transferred individually to microtubes containing 450 µl of reduced transport fluid solution (RTF) (Syed & Loesche, 1972). Right and left hemimandibles were cleaned and fixed in 10 mL of 4% paraformaldehyde in 0.1 M phosphate buffer for 48h at 4-8°C (Williaws et al., 1989).

Bacterial quantification by culture. Microtubes containing ligature were vigorously vortexed for 2 min to dislodge biofilm and free bacterial cells. Bacterial dispersions were serially diluted 10-fold in RTF. Dilutions ranging from 10^{-6} to 10^{-14} were plated in triplicate (Westergren & Krasse, 1978) onto complete medium with menadione and hemin (Shelburne et al., 2008) containing 5% defibrinated sheep blood and 1.6% agar (blood agar). The plates were incubated anaerobically (80% N_2, 10% H_2, 10% CO_2) at 37°C for 4 days for total viable colony formation units (CFU) estimation. Microtubes were then stored at -20ºC until use.

Bacterial quantification by qPCR. Microtubes containing 400µl of bacterial dispersions were mixed with 0.16 g of 0.1mm diameter Zirconium Beads (Biospec). Mechanical disruption of cells was carried out with a Mini-beadbeater (Biospec) at maximum power for 60 seconds. Microtubes were centrifuged (10 min, >13000g, 4°C) and supernatants were transferred to new tubes. qPCR was performed in

StepOne™ Real-Time PCR System (Life Technologies) with supernatants (1µl), 300 nM of each primer, 1× Power SYBR® Green PCR Master Mix (Lifetech) and a supplement of 1U of Taq Polimerase recombinant (Lifetech) in a total volume of 10µl. Prime-pair forward 5'-TCCTACGGGAGGCAGCAGT-3' and reverse 5'-GGACTACCAGGGTATCTAATCCTGTT-3' targeting 16S *rRNA* gene from the Domain Bacteria was used to estimate total bacterial load as previous described (Nadkarni et al., 2002). For each run, known genomic bacterial DNA concentrations (300 ng to 0.0003 ng in 10-fold serial dilutions) were used to generate standard curves for absolute quantification of DNA levels. Assays were performed in duplicate. Absences of reaction's inhibitors were confirmed by systematic tests with internal amplification controls (Nolte, 2004) and absence of primer dimers were evaluated by melting curves. Statistical analysis was performed using Kruskal-Wallis.

3D-Measurement of alveolar bone loss thru X-ray µCT images and reconstruction. Each hemimandible was individually scanned with an X-ray micro-computed tomography scanner (SkyScan1174 Compact Micro-CT, Bruker, Belgium). Specimens were placed with the inferior first molar occlusal surface parallel to the sample holding device and imagined using the following configuration: rotation step 0.6°, exposure 3800ms, pixel size 1304x1024, resolution 9.5µm. Acquired images were reconstructed by Cross-Section (SkyScan NRecon Reconstruction v1.69, Bruker) according to default settings. 3D-bone loss analysis was conduced in inferiors' first molars furcation area. With the SkyScan software package (CTan v1.13, CVol v2.2 and DataViewer v1.5.0) and renderized 3D models, a VOI (volume of interest) was delimited within bottom of the pulp chamber, the lower limit of the reabsorption (i.e. appearance of continuous, intact trabecular bone) and a imaginary cylinder that fits the enclosed area formed between the three roots. Software package can differentiate between bone and empty space, so lesions' volumes were accurately measured and compared with

linear measurement obtained from digital photographs of hemimandibles stained with the standard method.

2D and area measurements of alveolar bone loss thru digital photographs. After µCT analysis, the hemimandibles were bleached with hydrogen peroxide 3% for 16 hours, rinsed with deionized water and then stained with 1% aqueous methylene blue for 1 min. After drying, samples were placed under a stereomicroscope (Zeiss Stemi DV4; Carl Zeiss, Oberkochen, Germany) in standardized positions and digital images were acquired at 32× magnification. The alveolar bone height was measured using ImageJ (v1.6.0; NIH, Bethesda, MD) by recording the distance from the cemento-enamel junction to the alveolar bone crest (Toker *et al.* 2012). Measurements were taken at three points on both the buccal and lingual sides and a mean value for each was calculated. The morphometric measurement was performed by a single examiner (ISCR).

Statistical Analyses. Sample size was calculated using statistical software. A sample size of 10 per group was required for detection of a significant difference (80% power, 5% significance level). Values of bacteria load obtained from plate counting were correlated with qPCR data using Spearman multiple comparisons tests (α=0,01). Quantification of alveolar bone loss by the standard histometric method was correlated with the data obtained by microtomography using Spearman multiple comparisons tests (α=0,05). Alveolar bone loss and bacterial load were initially assessed for homogeneity by Shappiro-Wilk test. Due to the heterogeneity detected in these parameters, Kruskal-Wallis analysis with subsequent comparisons by Dunn's method for intergroup analysis were performed (α=0,05).

Results

Quantification of bacteria load. Total amounts of bacteria in ligatures were evaluated by culture (colony forming units counting) and molecular methods (qPCR). Results of these measurements were crossed and the data were correlated statistically ($p=0.02$, $R^2=0.38$ Spearman).

Quantification of bone loss. Measurements of alveolar bone loss by 3D-volume quantification correlates with linear parameters obtained in conventional 1D and 2D-linear measurements obtained thru digital photographs. 3D-volume quantification correlates with the: (1) total area of exposed root from facial surface ($p=0.0097$, $R^2=0.31$ Spearman); (2) height of exposed root from facial surface ($p = 0.0025$, $R^2=0.31$ Spearman); (3) total area of exposed root from lingual surface ($p = 0.0005$, $R^2=0.35$ Spearman) and (4) height of exposed root from lingual surface ($p = 0.0963$, $R^2=0.26$ Spearman).

CHX therapy. The estimated bacterial reduction per group is shown in Table 1. Mean values of bacterial DNA in sites from group with disease and no treatment (G1) was set to 1, and sites from treated animals relatively expressed. CHX treatments ≤10% archived numeric reductions of <2-fold in bacterial quantity and raw values up to order of 10^5 in some sites. Significant results were found in G5 (mean 3.3-fold) raw values up to order of 10^6 in some sites. Two sites from each all treated groups unnoticed treatments and showed no reduction in bacterial load. After four days from the last application of CHX, bacterial levels was lower only in 20% CHX group ($p<0.01$, Kruskal-Wallis) (Figure 1).

Table 1. Estimated bacterial reduction per group.

Groups	Mean fold reduction in bacterial counts (log ± SD)	Site with maximum reduction (DNA copies)	Site with minimum reduction
CLX 0.2%	1.8 (±2.4)	$3.4x10^5$	NR (2/18)
CLX 2%	1.4 (±1.1)	$1.8x10^5$	NR (2/18)
CLX 10%	1.2 (±1.2)	$5.0x10^5$	NR (2/16)
CLX 20%	*3.3 (±7.8)	$13.0x10^5$	NR (2/18)

NR: no reduction.
Significant difference is indicated by asterisk (* = p<0.01, Kruskal-Wallis).

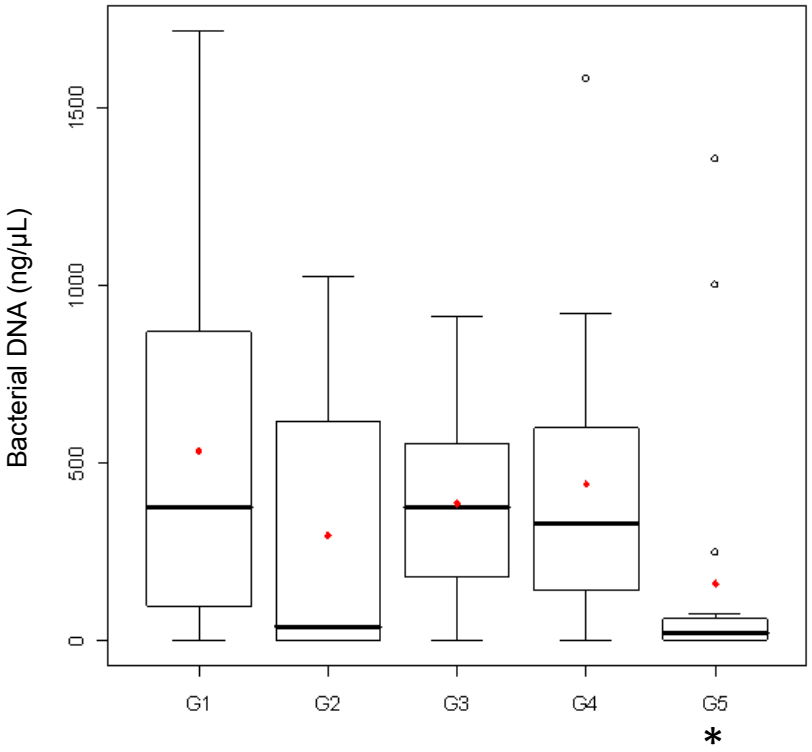

Fig 1. Mean of bacterial DNA (ng/µL) quantified with qPCR for each treatment group. Significant difference is indicated by asterisk (* = p<0.01, Kruskal-Wallis).

Regarding bone loss, no statistical difference was found between the groups when compared to animals from G1 ($p>0.05$, Kruskal-Wallis) (Table 2). Beside this, when comparing the best site from group G1 (i.e. site with the lowest bone loss), about half of evaluated sites in groups were responsive to therapy with improvements in bone tissue (Table 2, 2nd column). Moreover, in best responsive sites, the bone tissue loss was not as pronounced as group G1, the spared tissue had shown a range from 30 to 45% greater bone.

Table 2. Bone loss per group.

Groups	Responsive sites	Min. loss	Non-responsive molars
CLX 0.2%	7/18	- 40.6%	NR 11/18
CLX 2%	8/18	- 45.6%	NR 10/18
CLX 10%	10/16	- 29.4%	NR 6/16
CLX 20%	14/18	- 37.1%	NR 4/18

NR: no reduction.

Discussion

Periodontal diseases are caused by mixed infections with the subgingival microbiota. While hundreds of different bacterial species can colonize the oral cavity, it is generally accepted that specific microorganisms are found more frequently in periodontal lesions (Teles et al., 2013). Treatment by mechanical means such as mechanical root debridement generally results in significant clinical improvement but may not arrest the progress or recurrence of disease. The infecting bacterial species are susceptible to killing by several antibiotics including, among others, tetracycline-class drugs, amoxicillin and metronidazole as well as by local exposure to CHX (Page, 2004; Varoni et al., 2012).

The additional use of an antimicrobial agent to mechanical plaque control could enhance the effect of therapy and result in a late subgingival recolonization (Slots, 2002; Varoni et al., 2012). The substantivity of a drug in the periodontal pocket is an important factor determining its effect on the subgingival microbiota. Several attempts have been made to develop local delivery devices for the subgingival application of antiseptic, including crylic strips, ethyl-cellulose compounds, custom-fabricated trays, bioabsorbable chips have been tested for this purpose (Slots, 2002).

Reviews about Periochip® usage, a biodegradable matrix of hydrolysed gelatin containing 2.5mg of CHX, are inconclusive (Cosyn & Wyn, 2006; Plessas, 2014). According to the manufacturer, device can release about 40% in the first four hours and arrive at an average concentration of 1.4mg per ml of crevicular fluid, which means a concentration of only CLX 0.14% (Dexcel Pharma, 2014). CHX at low concentrations (<1%) have a low efficacy against biofilms and hence to periodontal disease (Slots, 2002; Varoni et al., 2012; Plessas, 2014).

The duration of the high concentration CHX stood even four days later, indicating great substantivity effect. A great variability was found between animals, which might hazy the results, despite adequate quantity of animals were used. As an example, when outliers from G5 were ignored from analysis, bacterial loads were from 70% to 95% lower, when compared to sites in G1 (data not shown). Considering all results, the only group with a significant difference was the G5 (CHX 20%), which shows approximately 70% of reduction. Considering the absence of any other form of biofilm control in animals except CHX, and besides the last application of CHX on the molars was performed four days before animals being euthanatized, the effect of CHX could still be observed.

Regarding CHX as a preventive bone loss, treated groups had a tendency to exhibit less bone loss (Table 2), but no statistical difference between the groups was found. Again, some variability was found between animals. Other studies demonstrated the ability of CHX to reduce bone loss (Leonard et al., 1980; Queiroz et al., 2012).

Bone losses were measured by μCT, a non-destructive imaging approach that provides 3D details at the micron level. A statistic correlation between conventional histomorphometry and μCT was found. Although a combination with other techniques may be preferred to obtain conclusions (Gielkens et al., 2008), in our study both techniques arrived at almost same results. Defect and graft measurements with μCT are reliable when strict criteria are applied.

In our study, we extrapolated the traditional concentration of CHX in our treatment groups up to one hundred times, without any other form of mechanical interference in the biofilm, as a way to demonstrate the possibility of its use as irrigant against the forming biofilm in periodontal sites. We could detect responsive and non-responsive sites when treated with CLX, which may have some clinical significance.

Mechanical removal of biofilm is essential and the use of CLX associated with it seems promising if used in concentrated form, since it can act on biofilms, as well in tissues (e.g. dentinal tubules). Moreover, frequent applications may be desirable, considering time and maturation stages of subgengival biofilm. We suggest, however, development of further protocols starting from lower concentrations when evaluating in clinical investigations as adjunctive to mechanical root debridement. Together, improved appreciation of such therapeutic strategies may ultimately lead to a more individualized targeted treatment for a disease.

Conclusion

Concentrated CHX reduces periodontal biofilm-organized bacterial load, which reflects in lower bone resorption in few sites under experimental periodontitis.

Conflicts of interest

This is to state that there is no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations that could have inappropriately influenced the results of the study.

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CAPÍTULO 2: CALCIUM INCREASES *PREVOTELLA INTERMEDIA* BIOFILM SURFACE ATTACHMENT AND FORMATION.

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Abstract

Prevotella intermedia is a human oral pathogenic bacterium that forms biofilms inside periodontal pockets. In this work, the effect of calcium on the production of *P. intermedia* biofilm was evaluated under *in vitro* conditions. Biofilm architecture and its viability were visualized by Confocal Laser Scanning Microscopy. Surfaces treated with 1 mg/cm^2 of $CaCl_2$ enhanced biofilm amount by 0.3 OD_{490nm} (p<0.01, ANOVA Dunnet), while 2.5 mg/cm^2 yielded 10-fold more biofilm mass (p<0.01, ANOVA Dunnet). Other substances had modest or no impact in biofilm mass. Confocal microscopy images showed structured and alive biofilms with no dead areas. Surfaces pre-coated with calcium chloride enhance *P. intermedia* biofilm formation.

Keywords: Calcium chloride. Micronutrients. Periodontitis. *Prevotella intermedia.*

Introduction

The attachment of cell and formation of biofilm are determinants for host infection by most pathogenic bacteria (Romantschuk, 1992). Direct observations of a wide variety of natural ecosystems have established that the vast majority of bacteria in nature grow within matrix-enclosed biofilms (Costerton et al., 1994). Bacterial biofilms provide protection to the community of cells from antimicrobial compounds and dehydration, promote cell-to-cell signaling interactions, and help to optimize nutrient uptake (Hopkins & Purcell, 2002).

Considering bacteria that lives inside a host, factors such as the availability of water and nutrients can limit the formation of biofilms (Ramey et al., 2004). Usually in the same host, different environments vary in their concentrations of solutes, therefore, bacteria have evolved mechanisms for regulating cellular nutrient concentrations (Snyder & Champness, 2003). For instance, bacterial biofilms have been thought to improve nutrient acquisition through the formation of

exopolysaccharides (EPSs), which have a high capacity for ion retention (Costerton et al., 1995).

The EPS has many functions varying from protective barrier, nutrient source, stock exchange market of genetic information, storage of excess energy to the promotion of adhesion and cohesion of the biofilm. The extracellular matrix mostly consists of water (90%), polymeric substances, proteins, extracellular DNA (eDNA) and its lipids (van Der Waal & van der Sluis, 2012). eDNA is formed by release of bacterial genomic DNA mostly by cell lysis (Flemming & Wingender, 2010). eDNA of the biofilms facilitates the initial stage of adhesion, forms the structural backbone and acts as glue that promotes biofilm aggregation (Whitchurch, 2002; Izano, 2008; Pammi 2013).

In the oral environment, *Prevotella intermedia* is a gram-negative black-pigmented anaerobe that plays important roles in the initiation and development of periodontitis by stimulating host cells to release proinflammatory cytokines and proteinases (Kuboniwa, 2012). Being an etiologic agent of chronic adult periodontitis and part of pathogenic biofilm in the subgingival plaque (Loesch, 1999).

Chronic periodontitis is an infectious disease resulting in inflammation within the supporting tissues of the teeth, progressive attachment and bone loss and is characterized by pocket formation and/or recession of the gingiva. It is recognized as the most frequently occurring form of periodontitis. The disease is usually associated with the presence of plaque and calculus. Progression of attachment loss usually occurs slowly, but periods of rapid progression can occur. Associated with a variable microbial pattern (AAP, 2001). Among the numerous bacterial species isolated from biofilm associated with periodontitis, the constituent species of the group Gram-negative anaerobic rods producing black pigment shown to be strongly associated with destructive periodontal infections (Kuboniwa, 2012).

Micro- and macronutrients are required for multiple functions within prokaryotic and eukaryotic cells (Hansch & Mendel, 2009; Maathuis, 2009). These essential minerals are under tight homeostatic control because, although required,

they are also toxic at high concentrations. The availability of minerals and trace elements affects host-pathogen interactions, especially pathogen survival, the expression of virulence traits, and host physiology (Cruz, 2012).

Many other micronutrients seem to influence the development of biofilm. Calcium levels modulate biofilm structures in *Vibrio cholerae*, the expression of the type III secretion apparatus and effector proteins in *Yersinia pestis* and *Pseudomonas aeruginosa*, and the production of hydrolytic enzymes (polygalacturonase and pectate lyase) in *Pectobacterium carotovorum*, all of them considered virulence determinants. In addition, in animal and plant hosts, calcium modulates defense responses, based on regulatory systems that rely on this metal as a secondary messenger (Cruz, 2012; Cobine, 2013).

In this study we tested diverse substances and observed its influence on biofilm formation and planktonic growth of *P. intermedia*. From all those substances, calcium was the one with best response in biofilm formation.

Materials and method

Bacterial strains and culture conditions. *P. intermedia* strain ATCC 49046 was used in this study. The bacteria was grown anaerobically at 37°C in TSB-BHI blood agar as solid medium (TSB, BHI, hemin, menadione, yeast extract, 5% defibrinated sheep blood and 1.6% agar) and TSB-BHI broth medium (TSB, BHI, hemin, menadione, yeast extract), as described in (Shelburn et al., 2008).

Planktonic growth influenced by metal, proteins and others. First, *P. intermedia* was grown in 4ml of TSB-BHI broth medium (18-24h, 37°C, anaerobic chamber). After growth, the starter inoculation rate 0.3 was determined with the use of a spectrophotometer (optical density at 550 nm) and inoculated in supplemented TSB-BHI broth medium. For each tube of supplemented medium was a mixture of TSB-BHI and one of the following substances or concentrations: $CaCl_2$ (1mg/ml, 10mg/ml, 15mg/ml, 20mg/ml, 25mg/ml); KCl (1mg/ml, 10mg/ml);

$CaCO_3$ (10mg/ml); $Ca(OH)_2$ (10mg/ml); $MgCl_2$ (10mg/ml); riboflavin (10mg/ml); cysteine (10mg/ml); glutamine (10mg/ml); aspartic acid (10mg/ml); glucose (10mg/ml); methionine (10mg/ml); hemin (10mg/ml); dextran (10mg/ml); mucin (10mg/ml); albumin (10mg/ml); vitamin solution (800ul); horse plasma (800ul). Then, the tube with 4ml mixture was incubated (24h, 37°C, anaerobic chamber). Three tubes per substance were cultivated on each experiment.

Plate coating. In short, the process for coating the 48-well plates was performed by filling well with 100µl of substances' solution. The homogenized solution was distributed covering the whole bottom of well. Then, the solution volume was dried in incubator (24h, 60°C). The resultant mass distribution by surface in the wells ranged from 0.005 mg/cm^2 to 2.5 mg/cm^2. Sterile polystyrene 48-well plates (Costar 3548, Corning Life Sciences, Acton, MA) were coated with: $CaCl_2$ (0.1-2.5 mg/cm^2); KCl (0.1-1 mg/cm^2); $CaCo_3$ (1 mg/cm^2); $Ca(OH)_2$ (1 mg/cm^2); $MgCL_2$ (1 mg/cm^2); riboflavin (1 mg/cm^2); cystine (1 mg/cm^2); glutamine (1 mg/cm^2); aspartic acid (1 mg/cm^2); glucose (1 mg/cm^2); methionine (1 mg/cm^2); hemin (1 mg/cm^2); dextran (1 mg/cm^2); mucin (1 mg/cm^2); albumin (1 mg/cm^2); vitamin solution (100µl, Cat# B6891, Sigma); protein-rich media (100µl, skim-milk); LPS (0.005 mg/cm^2, Cat# L2630, Sigma); DNA (100µg/cm^2); horse plasma (100µl); sheep blood (100µl). Additionally, some mixtures were performed with $CaCl_2$: amino acids, vitamin, mucin and fragmented DNA (mix II: amino acids (sigma) 50µl + Ca 30µl; mix III: vitamin B 60µl + Ca 150µl; mix IV: mucin 60µl + Ca 150µl; mix V: fragmented DNA 60µl + Ca 150 µl), following the same conditions as previously described.

Biofilme growth influenced by metal, proteins and DNA coated into wells. First, *P. intermedia* was grown in 4ml of TSB-BHI broth medium (18-24h, 37°C, anaerobic chamber). After growth, the starter inoculation rate 0.3 was determined with the use of a spectrophotometer (optical density at 550 nm). After measured, the bacteria were inoculated in 500µl final volume of TSB-BHI broth

medium inside the coated well plate and incubated (24h, 37°C, anaerobic chamber). Next, the formed biofilm was analyzed.

Biofilm quantification in 96-well plates. The biofilm formation by *P. intermedia* strain was assessed according to methods described previously (Davey, 2006), with some modifications. Briefly, *P. intermedia* cells cultured overnight on TSB-BHI plates were scraped from the plates and suspended in TSB-BHI medium. After 24 hour, sterile coated polystyrene 48-well plates containing 500 µl TSB-BHI per well were inoculated with 0.3 (starter inoculation rate, optical density at 550 nm) of cell suspension. After overnight incubation planktonic growth (cells in suspension) and biofilm growth (cells adhered to the substrate) were quantified. The medium was aspirated from plates. Then, plates were dried (30 minutes, 40°C) and biofilm growth was quantified by detaching the adhered biofilm cells. The biofilm adhered in the original 48-well plate was prepared: first it was rinsed with saline, after stained with 200 µl 0.1% safranin for 1 min and washed with distilled water; dissolved in ethanol 95% and 10% SDS; transferred to 96-well plate to quantify biofilm growth by ELISA (490 nm) (Davey, 2006).

Calcium quantification using Arsenazo III. In this experiment, a sterile polystyrene 48-wells plate was coated with ranged concentrations of EGTA (chelating agent), $CaCl_2$ (source of calcium ions), and the combination of both mixed. After 24 hours from the coating process, overnight cultures of *P. intermedia* grown in biofilm media (TSB-BHI) were diluted to an OD_{600} of 0.3 in fresh TSB-BHI, and 500 µl of each culture was transferred to the coated well plates. After 12 hours of growth, 400 ul were transferred to a sterile polystyrene 96-wells plate filled with 300 µl of a mix solution (arsenazo III, TSB-BHI and sterile water) for well. Calcium scale (8 solutions, starting with 0.4mM and then diluted by half until 0.003125mM) was used as a measurement parameter. Then the samples and controls were handled as described (Leitão et al., 2012) and read by ELISA (650 nm).

Assessment of biofilm structure by confocal laser scanning microscopy. Overnight cultures of *P. intermedia* grown in biofilm media (TSB-BHI) were diluted to an OD_{600} of 0.3 in fresh TSB-BHI, and 1000 ml of each culture was transferred to coated well plates (1.1 mg/cm^2, Labtek 4 wells plate, Cat#155383, Nalge Nunc International, USA) and incubated for 24 h at 37°C. The next day, the wells were gently washed three times with 0.85% (wt./vol.) NaCl, followed by staining with mixture of SYTO-9 and propidium iodide diluted in 0.85% (wt./vol.) NaCl (Cat# L7012, Invitrogen, Carlsbad, CA) for 10 min in dark. After removing the stain, the wells were gently washed once with 0.85% (wt./vol.) NaCl. Biofilm images were collected by confocal laser scanning inverted microscope TCS SP5 AOBS (Leica-microsystems, USA). SYTO 9 (green, live cells) fluorescence was detected by excitation at 488 nm, and emission was collected with a 500- to 530-nm band pass filter. Propidium Iodide (red; dead cells) fluorescence detected by excitation at 488 nm and emission collected with a 565-615 nm band pass filter. All z-sections were collected at 0.5 µm intervals, 20x/1W dry objective lens. Image acquisition and processing was performed by using ImageJ software, version 1.47 (National Institute of Health, http://imagej.nih.gov/ij, USA).

Results

Influence of coated substances on biofilm formation. The biofilm formation was stimulated with the many substances and concentrations selected and previously coated on each well's bottom. The most relevant results were showed in Fig. 1. Other substances have no influence in the development of biofilm (data not shown).

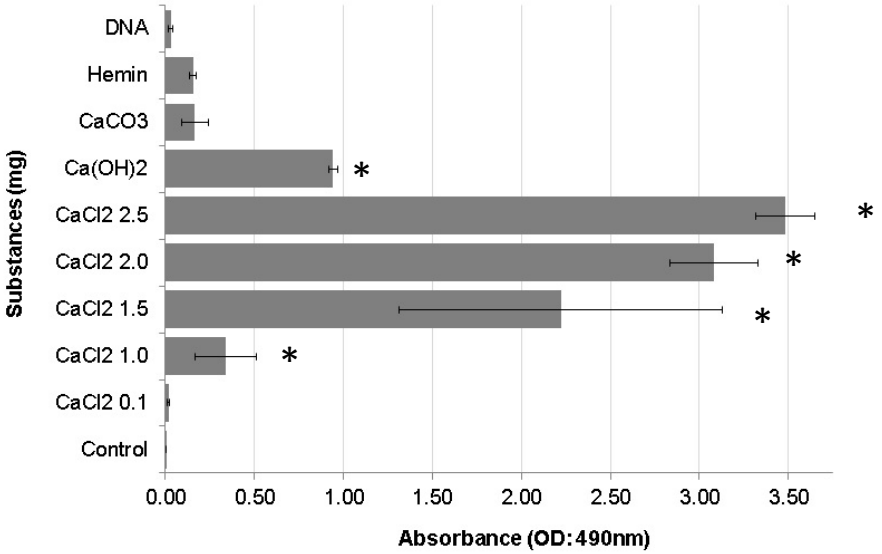

Fig. 1. Influence of substances on biofilm formation. Biofilm formation was measured by staining. Substances without or with negligible biofilm formation were not shown (*=p<0.01, ANOVA Dunnet).

Influence of supplemented substances on planktonic growth. The influence of each specific test substances in the planktonic growth was determined. The control (TSB-BHI only) and the diverse substances tested diluted in culture medium for planktonic growth: $CaCl_2$ (0.1, 1, 1.5, 2, 2.5 mg/ml); KCl (0.1-1 mg/ml); for all other substances 10 mg/ml (Fig. 2). Planktonic growth was measured with spectrophotometer (OD: 550nm). Substances without or with negligible influence were not shown.

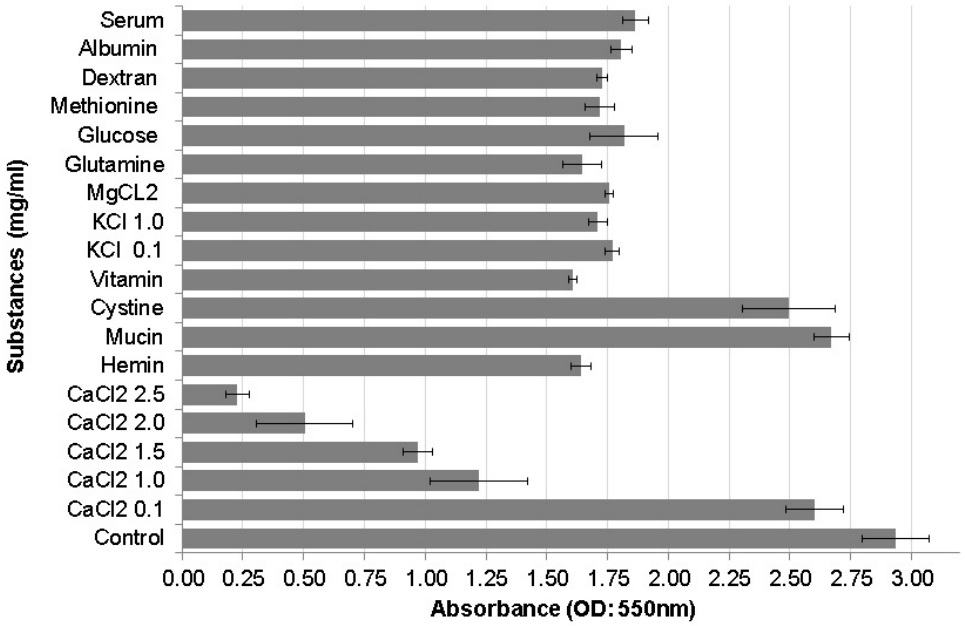

Fig. 2. Influence of substances on the planktonic growth. Planktonic growth was measured with spectrophotometer.

Calcium influence in biofilm and planktonic development. The direct effect of diverse concentrations of calcium chloride in planktonic growth and biofilm formation was shown on Fig. 3.

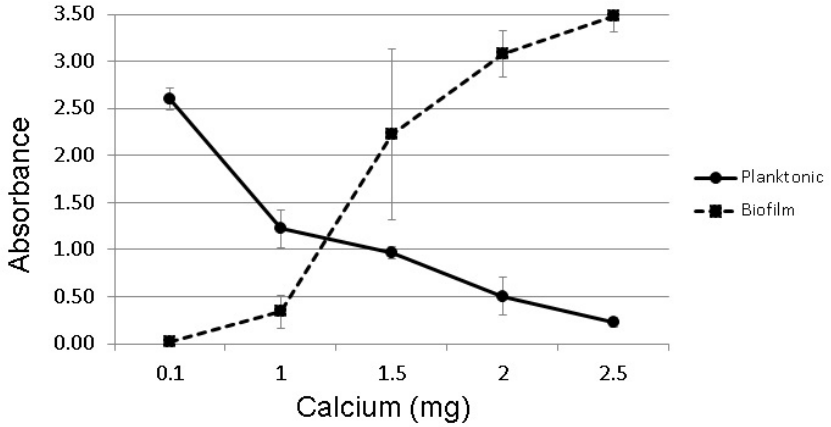

Fig. 3. Influence of CaCl2 in planktonic and biofilm growth.

The dotted line shows the influence on biofilme formation with several calcium concentrations. While the solid line shows the action on planktonic growth with the rise of calcium concentration, analyzed by spectrophotometer. The biofilm development was favored, being directly proportional to the rising concentrations of calcium chloride. Meanwhile, the planktonic growth was inversely proportional to the increase concentration of calcium chloride. Biofilm viability analyzed by confocal microscopy (Fig.4).

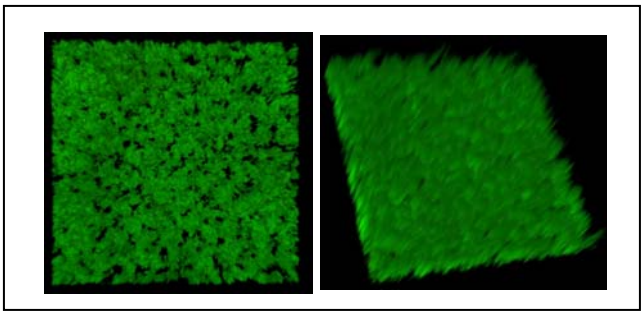

Fig. 4. Confocal visualization of biofilm. Biofilm height can reach up to 100μm. The presence of living cells of *P. intermedia* is shown in the images.

Available calcium assay. Free available calcium assay. To enable this analysis, this study used two substances with special features: EGTA and arsenazo III. EGTA is a colorless chelator that is highly selective for calcium ions, biding to them. While arsenazo III is a substance suitable for the colorimetric determination of calcium in micromolar amounts, darkening a medium when reacts with free calcium. Those substances can be used for measuring the presence of calcium in the medium and, consequently, the eventual consumption of calcium by the bacteria during the biofilm formation (Leitão et al., 2012).

Using a known scale of calcium concentration (8 concentrations, divide by 2 from 0.4mM to 0.003125mM) and the arsenazo III properties, we could evaluate the fluctuation of free calcium in the medium and biofilm in the tested solutions. Ca, Ca+EGTA, Ca'+EGTA, EGTA. Ca (0.8, 0.6, 0.4, 0.2, 0.1 mM). EGTA (0.8, 0.6, 0.4,

0.2, 0.1 mM). Ca' (0.8mM). After that, the concentration of free Ca could be estimated. Results are shown on Fig. 4.

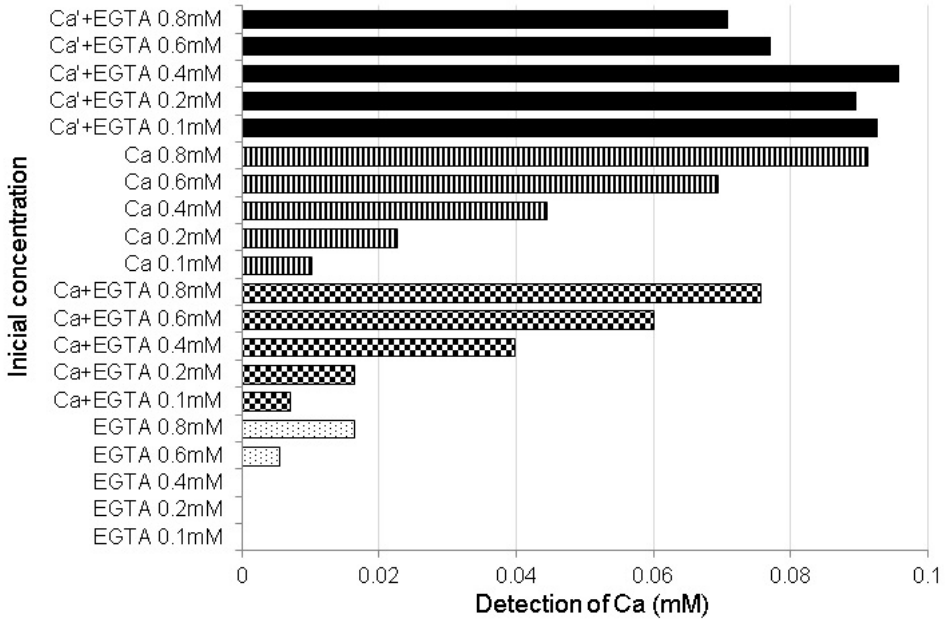

Fig. 4. Free available Ca assay. Wells were coated with Ca and/or EGTA (y axis) and biofilm was formed as described in M&M. Free Ca was measured in supernatants.

Discussion

In this study, we supplemented the planktonic growth and biofilm formation of *P. intermedia* with different substances and concentrations. The availability of some mineral nutrients can act as a stimulus, eliciting changes in bacteria that can promote the establishment and development of biofilms (Cruz et al., 2012; Cobine et al., 2013).

Various environmental factors, other than those strictly related to the inflammatory response of the host, may change during the progression of periodontitis. Such factors include, for example, pH, the concentration of iron and hemin, and the presence of various host hormones (Pöllänen et al., 2013).

The increase in biofilm formation occurred with variations between the substance or concentration tested. Calcium (Ca^{2+}) has an important structural role

in guaranteeing the integrity of the outer lipopolysaccharide layer and cell walls of other bacterial cells (Cruz et al., 2012; Cobine et al., 2013; Das et al., 2014), what might explain the action of $CaCl_2$, $Ca(OH)_2$, $CaCO_3$. Another explanation, could be the implication of divalent cations, including Ca and Mg, in the accumulation of biofilms by different bacteria. Besides acting as cross-bridging molecules that increase the stability of the biofilm matrix, those cations might also play a role in the regulation of bacterial gene expression related to virulence factors and biofilm formation (Cruz et al., 2012).

Other substances are known to have the opposite effect, like horse serum and albumin had been reported to cause a significant decrease in biofilm development (Hammond et al., 2010). Other studies have also reported that some species like *Streptococcus mutans* and *Streptococcus sobrinus* exhibited low levels of proteolytic activity against the protein substrate (Homer & Beighton, 1992).

Planktonic growth seems to not be affected by the many substances tested, being most of them similar to the control sample (Fig. 2). However, with the rise in calcium concentrations the planktonic growth levels was reduced. The direct influence of calcium in the planktonic growth and biofilm formation can be observed in fig. 3. The biofilm development was favored, what is evident by the contrast between the directly proportional and inversely proportional lines responding to the increase concentration of calcium chloride. This confirms the results of previous studies (Cruz et al., 2012; Cobine et al., 2013; Das et al., 2014), showing that calcium have a hole on the biofilm formation.

Considering the oral cavity sites, calcium is a constituent part of the dental tissue and is present in saliva, and, as seen, it plays an important role in scaffolding biofilm being incorporated in the structure of its extracellular matrix.

The Ca^{2+} can be captured from the medium by chelation, a process whereby chemicals form soluble, complex molecules with certain metal ions, inactivating the ions so that they cannot normally react with other elements or ions to produce

precipitates or scale (van Der Waal & van der Sluis 2012). For this, we used EGTA, which is a colorless chelator, that is highly selective for calcium ions.

We also detected free calcium on the non-supplemented medium growth (TSB-BHI), confirming the effect of supplemented calcium in the growth is not due to lack of calcium in regular TSB-BHI medium. In the presence of EGTA the biofilm formation suffered reduction, confirming its ability to react and trap calcium.

Conclusion

Calcium supplementation favored the force of adhesion to the substrate and enhanced the biofilm formation.

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CONCLUSÃO

A aplicação frequente do digluconato de clorexidina concentrado diminui a carga bacteriana no biofilme periodontal e a perda óssea alveolar em apenas alguns sítios.

O revestimento da superfície de crescimento com $CaCl_2$ favorece o desenvolvimento do biofilme de *P. intermedia*, sendo este efeito dose-depedente.

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* De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors. Abreviatura dos periódicos em conformidade com o Medline.

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Anexo 1

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
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C E R T I F I C A D O

Certificamos que o projeto **"Estudo de diferentes concentrações de clorexidina na microbiota do biofilme periodonto patogênico e na perda óssea alveolar induzida em ratos"** (protocolo nº **2948-1**), sob a responsabilidade de **Prof. Dr. Rafael Nobrega Stipp / Italo Sarto Carvalho Rodrigues**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **17 de dezembro de 2012**.

Campinas, 17 de dezembro de 2012.

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