UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

Myrella Lessio Castro

"Avaliação da terapia com dose sub-antimicrobiana de doxiciclina como um modulador da resposta imuno-inflamatória do hospedeiro em modelo de doença periodontal e os efeitos dessa terapia sobre a susceptibilidade da *Porphyromonas gingivalis.*"

Tese de Doutorado apresentada à Faculdade de Odontologia de Piracicaba da UNICAMP, para obtenção do título de Doutor em Odontologia, na área de Farmacologia, Anestesiologia e Terapêutica.

Este exemplar corresponde à versão final da Tese denfendida pela aluna, e orienada pelo Prof. Dr. Pedro Luiz Rosalen

Orientador: Prof. Dr. Pedro Luiz Rosalen
Co-Orientador: Prof. Dr. Gilson Cesar N. Franco.

Assinatura do Orientador

Piracicaba,
2012

FICHA CATALOGRÁFICA ELABORADA POR
MARILENE GIRELLO – CRB8/6159 - BIBLIOTECA DA
FACULDADE DE ODONTOLOGIA DE PIRACICABA DA UNICAMP

Castro, Myrella Lessio, 1978-
C279a Avaliação da terapia com dose sub-antimicrobiana de doxiciclina como um modulador da resposta imuno-inflamatória do hospedeiro em modelo de doença periodontal e os efeitos dessa terapia sobre a susceptibilidade da Porphyromonas gingivalis / Myrella Lessio Castro. -- Piracicaba, SP : [s.n.], 2012.

Orientador: Pedro Luiz Rosalen.
Coorientador: Gilson Cesar Nobre Franco.
Tese (doutorado) - Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Doenças periodontais. 2. Inflamação. 3. Reabsorção óssea. 4. Colágeno. I. Rosalen, Pedro Luiz, 1960- II. Franco, Gilson Cesar Nobre. III. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. IV. Título.

Informações para a Biblioteca Digital

Título em Inglês: Evaluation of therapy with sub-antimicrobial dose of doxycycline as a modulator of the immune-inflammatory response of the host model of periodontal disease and the effects of this therapy on the susceptibility of Porphyromonas gingivalis
Palavras-chave em Inglês:
Periodontal disease
Inflammation
Bone reabsorption
Collagen
Área de concentração: Farmacologia, Anestesiologia e Terapêutica
Titulação: Doutor em Odontologia
Banca examinadora:
Pedro Luiz Rosalen [Orientador]
Regiane Yatsuda
Maria Luiza Ozores Polacow
Cristiane de Cassia Bergamaschi Motta
Karina Cogo
Data da defesa: 28-02-2012
Programa de Pós-Graduação: Odontologia

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 28 de Fevereiro de 2012, considerou a candidata MYRELLA LESSIO CASTRO aprovada.

Prof. Dr. PEDRO LUIZ ROSALEN

Profa. Dra. REGIANE YATSUDA

Profa. Dra. MARIA LUIZA OZORES POLACOW

Profa. Dra. KARINA COGO

Profa. Dra. CRISTIANE DE CÁSSIA BERGAMASCHI

Agradecimentos Especiais

À Deus e à Nossa Senhora, que sempre abençoaram de graças minha vida e amparam-me nas horas difíceis.

"Tudo posso naquele que fortalece"

Agradeço à minha mãe Irene, pelo amor incondicional, a educação moldada em honestidade e humildade, pelo incentivo e pela batalha de transformar o meu sonho em
realidade.

À minha família, obrigada por terem me ajudado a suportar os desafios, a distância, os momentos de ausência e as dificuldades.

"Eu tenho tanto pra te falar, mas com palavras não sei dizer como é grande o meu amor por vocês."

Ao Caio, meu companheiro, cúmplice, amante, amigo e namorado; Agradeço pela torcida, incentivo, compreensão e principalmente pelo amor e a paciência.

"Eu sei que vou te amar, por toda minha vida eu vou te amar".

Ao prof. Dr. Pedro Luiz Rosalen, por ter aberto as portas da pós-graduação, possibilitando meu amadurecimento e crescimento profissional. Por ter se tornado uma referência de caráter e dedicação à pesquisa em minha formação. Destes 11 anos de convivência (Bolsa SAE, Iniciação Científica, Mestrado e Doutorado) levo um saldo muito positivo: amadureci, aprendi muito, fui colocada a prova diversas vezes e sei que só passei por isso porque você acreditou em mim. E também

agradeço pela amizade que fica e levo para sempre no meu coração.

"Os melhores professores da Humanidade são as vidas de grandes Homens" – (Charles H. Fowler)

Ao prof. Dr. Gilson Cesar Nobre Franco pelas inúmeras oportunidades, por aceitar co-orientar esta tese e pela forma que conduziu; pelos ensinamentos, estímulos e apoio dados durante todas as etapas deste trabalho e também por me permitir despertar a curiosidade sempre. Agradeço ao amigo Gilson Cesar Nobre pelas palavras de apoio, por sempre ter algo bom a dizer e por ser um exemplo de pessoa.

"Sábio não é quem dá as verdadeiras respostas, mas quem formula as verdadeiras questões".

À Profa. Dra Simone Duarte, amiga que me incentivou na vida acadêmica; Pela paciência, apoio, incentivo, acolhimento, amizade e força. Por todas as oportunidades que tive nesses anos de convivência e principalmente pelo ano que moramos juntas. Pela vontade de me ver crescer e conquistar o meu espaço e, principalmente, pelos ensinamentos pessoais que me acompanharão até o fim de minha vida.

"Conhecer alguém aqui e ali que pensa e sente como nós, e que embora distante, está perto em espírito, eis o que faz da Terra um jardim habitado." (Goethe)

AGRADECIMENTOS

Agradeço ao Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq pelo financiamento do projeto através das bolsas de doutorado e doutorado-sanduíche, sem a qual este trabalho definitivamente não poderia ser realizado.

À Universidade Estadual de Campinas - UNICAMP, na pessoa do Magnífico Reitor Prof. Dr. Fernando Ferreira Costa e à Faculdade de Odontologia de Piracicaba - FOP, por meio do diretor Prof. Dr. Jacks Jorge Junior.

À Profa. Dra. Renata Cunha Matheus Rodrigues Garcia, coordenadora dos cursos de Pós-Graduação da FOP/UNICAMP e a Profa. Dra. Cinthia Pereira Machado Tabchoury, Coordenadora do Programa de Pós-Graduação em Odontologia da FOP/UNICAMP.

Aos professores da Área de Farmacologia, Anestesiologia, e Terapêutica, Profa. Dra. Maria Cristina Volpato, Prof. Dr. Francisco Groppo, Prof. Dr. Eduardo Dias Andrade, Prof. Dr. José Ranali e á todos os docentes do Programa de Pós-Graduação em Odontologia de Piracicaba pelos ensinamentos, dicas e vivência passados durantes as aulas e conversas de corredores.

Ao Prof Deepak Saxena por ter aberto a oportunidade de trabalhar nos EUA.

À Helena Duarte que fez meus dias nos EUA mais felizes.

Agradeço os amigos Ramiro M. Murata e Juliana Delben, pelo empenho em me ajudar a realizar este trabalho e pela amizade que ultrapassou as barreiras do laboratório e por nossas aventuras nos EUA.

Aos Professores Prof. Dr. Marcelo Rocha Marques, Prof. Dr. Pedro Duarte Novaes, Profa. Dra. Vivian Fernandes F de Goes, membros da banca de qualificação, pelas sugestões para realização e finalização deste projeto.

Agradeço à Luciana Salles Branco de Almeida pela amizade que construímos e pelos conhecimentos que adquirimos juntas nestes anos de pós-graduação. Pela disposição em me apoiar nas horas de dificuldade e pelas risadas merecidas.

Às amigas Michelle Franz M Braga Leite, Cristiane de Cassia Bergamaschi Motta e Cristina Gibilini, Agradeço a paciência, os conselhos e os momentos felizes durante o tempo que moramos juntas em Piracicaba. Jamais vou esquecer de vocês por terem participado de um momento importante da minha vida.

Aos meus amigos e amigas da "velha-guarda" de laboratório da Farmacologia/FOP: Bruno Bueno da Silva, Karina Cogo, Luciana Aranha Berto e Regiane Yatsuda pelos momentos sempre regados com muita risada, ao carinho e por me fazer acreditar que qualquer dificuldade, por maior que pareça, torna-se insignificante quando se tem amigos.

Com a mesma intensidade agradeço aos amigos "da nova safra" da Farmacologia/FOP: Ana Paula Bentes, Camila Silva, Carina Denny, Cleiton Santos, Cristina Caldas, Daniela Baroni, Fabiana Nolasco, Lívia Galvão, Luciano Serpe, Luiz Eduardo Ferreira, Marcelo Franchin, Marcos Cunha, Patrícia Zago, Paulo Venâncio, Sônia Fernandes, Sidney Figueroba e Talita S. Graziano agradeço o apoio e bons momento que vivemos juntos nesta reta final.

À Profa. Ana Lia Anbinder, da UNESP –São José dos Campos pela sua importante colaboração para os procedimentos histológicos e a escrita desta tese.

À Profa. Sheila Cortelli, da Universidade de Taubaté (UNITAU), pela grande parceria.

Ao Prof. Dr. Severino Matias de Alencar, Prof. Dr. Massarahu Profa Dra. Vera Redher, Profa Dra. Mary Ann Foglio, Prof. Dr. Marcelo Rocha Marques, Profa. Dra. Ana Paula de Sousa Pardo pelos ensinamentos dados durante minha pós-gradução.

Agradeço os amigos que conquistei nos EUA que foram muito importantes para minha "sobrevivencia emocional": Amilkar Chagas Freitas Jr, Antônio Salomone Donola, Erika Almeida, Guilherme Valverde, Iriana Zanin, Jacqueline Grenn, Laura Silva, Livia Peruzi, Luna Mello Castro, Lorenzo Mello Castro, Monica Mello Castro, Nelson Silva, Sergio Castro, Renata Dias, Roberta Salomone, Vanessa Pardi, Vinicius Donala, Xi Wei; amei cada momento ao lado de voces.

Aos meus amigos e amigas de laboratório de Histologia (Luciana Souto Mafatto, Aline Planello e Mariana Martins Ribeiro), do Laboratorios de endodontia (Emmanuel Nogueira), do laboratorio de Microbiologia (Rafael Nobrega Stip, Vivian Goes) pelos apoio, carinho e atenção.

Às Amigas de graduação Mariana Moure, Marcela Machado, Simone Buzzo e Valéria Palmieri obrigada por me ensinarem o segredo da amizade, da convivência e da harmonia em grupo.

Às Amigas Claudia Nascimento e Elaine Fazzio obrigada por serem amigas especiais, e que nestes últimos anos aceitarem minha amizade em pequenas doses homeopáticas, porem sempre verdadeiras.

À Elisa Santos e Eliane Franco que com muita calma e paciência me aguentaram e ajudaram todos estes anos.

Às minhas irmãs, que amo tanto, e aos meus sobrinhos que entenderam minha ausência, pelo apoio e torcida.

À minha mais nova família Pacheco-Lopes pelo acolhimento, atenção e carinho.

A todos que de alguma forma contribuíram para a realização deste trabalho;

Meus agradecimentos!

"Aprendi que se depende sempre de tanta gente diferente e toda pessoa sempre reflete as marcas de lições diárias de outras tantas pessoas. É tão bonito quando a gente entende que a gente é tanta gente , onde quer que a gente vá; É tão bonito quando a gente sente que nunca está sozinho por mais que a pessoa pense estar..." (Gonzaguinha)

RESUMO

Periodontite é a doença multifatorial que envolvem interações entre algumas espécies bacterianas, como *Porphyromonas gingivalis* W83 e células do hospedeiro. Levando a uma resposta imuno-inflamatória que causa a destruição do tecido ósseo e gengival Neste contexto, fármacos com a habilidade de modular este processo imuno-inflamatório podem auxiliar no tratamento da doença periodontal (DP). A doxiciclina em dose subantimicrobiana (DDS), apresenta propriedades anti-inflamatórias pela sua atuação em algumas vias da inflamação. No entanto, ainda é discutido o efeito desta terapia dobre a susceptibilidade bacteriana por longo tempo. Assim, o objetivo deste trabalho foi analisar os efeitos da DDS como um modulador da resposta imuno-inflamatória do hospedeiro na DP induzidas em ratos e avaliar a susceptibilidade da *P. gingivalis* cultivadas com DDS por longo tempo. A DP foi induzida em ratos Wistar machos (SPF) submetidos à colocação de ligadura em torno dos primeiros molares inferiores foram randomizados e divididos em 3 grupos experimentais (n=10 animais/grupo/experimento): 1) grupo controle: ratos sem ligadura e sem tratamento; 2) grupo ligadura: ratos com ligadura e tratados com solução NaCl 0,9 %) e 3) grupo ligadura + DDS: ratos com ligadura e tratados com a DDS (5 mg/kg/dia). No tecido gengival, extraídos de animais tratados por 3 dias, foram avaliadas as expressões gênicas de TNF-α, IL-1β, IL-17 e PAR_2 através de RT-PCR. As mandíbulas dos ratos tratados por 15 dias foram usadas para mensuração da reabsorção óssea alveolar (coradas com Hematoxilina e Eosina) e da quantidade de fibras colágenas (coradas com Picrosirius- Vermelho). Para a análise microbiológica, a *P. gingivalis* (ATCC BAA-308) foi cultivada por 3 meses (45 gerações) em meio de cultura contendo 0,4 µg/mL de DDS e avaliada por meio de concentração inibitória mínima (CIM) para Amoxiciclina, Doxiciclina e Metronidazol. A DDS inibiu significativamente os níveis de RNAm do tecido gengival para os IL-1β, IL-17, TNF-α e PAR_2 ($P<0,05$, ANOVA, teste Tukey). Além disso, a DDS reduziu a perda óssea quando comparada ao grupo ligadura ($P<0,05$

ANOVA, teste Tukey) e manteve a porcentagem de fibras colágenas com níveis similares ao grupo controle (P>0,05). Na análise da susceptibilidade de *P. gingivalis* a DDS não apresentou resistência multi-antibiótica para esta cepa, entretanto, houve uma alteração nos valores de CIM para todos antibióticos testados com a *P. gingivalis* crescida ao longo do tempo. Em conjunto, os dados demonstram que a DDS diminuiu a resposta inflamatória, a reabsorção óssea e a degradação de colágeno no modelo utilizado de DP, indicando sua atividade como moduladora da resposta do hospedeiro na DP. A alteração microbiana com o uso contínuo e de longo período de DDS modificou a sensibilidade da *P. gingivalis,* entretanto não desenvolveu resistência antibiótica a doxiciclina.

Palavras-chave: doença periodontal, doxiciclina em dose subantimicrobiana, inflamação, modulação da resposta do hospedeiro, reabsorção óssea, colágeno, *Porphyromonas gingivalis*, resistência bacteriana.

ABSTRACT

Periodontitis is a multifactorial disease involving interactions between some bacterial species, as *Porphyromonas gingivalis* W83, and host cells. Leading to an immune-inflammatory response that causes the destruction of bone and gingival. In this context, drugs with the ability to modulate immuno-inflammatory process that may aid in the treatment of periodontal disease (PD). Doxycycline dose subantimicrobiana (DDS) has anti-inflammatory properties because of its role in some pathways of inflammation. However, it is still discussed the effect of this therapy fold the bacterial susceptibility for a long time. The objective of this study was to analyze the effects of DDS as a modulator of the immune-inflammatory response in the host DP induced in rats and to evaluate the susceptibility of *P. gingivalis* grown with DDS for a long time. The DP was induced in male Wistar rats (SPF) submitted of ligature around the first molars and divided into three experimental groups (n = 10 animals/group/experiment): 1) control group: rats without ligature and without treatment; 2) ligature group: rats with ligature and treated with 0.9% NaCl solution and 3) ligature + SDD group: rats with ligature and treated with SDD (5 mg/kg/day). In gingival tissue, extracted from animals treated for 3 days, we assessed the gene expression of TNF-α, IL-1β, IL-17 and PAR2 by RT-PCR. The jaws of rats treated for 15 days were used for measurement of alveolar bone resorption (stained with Hematoxylin and Eosin) and collagen fibers (stained with Picrosirius-Red). For microbiological analysis, *P. gingivalis* was grown for 3 months (45 generations) in culture medium containing 0.4 µg/mL of SDD and evaluated by minimum inhibitory concentration (MIC). SDD significantly inhibited mRNA levels of the gingival tissue for IL-1β, IL-17, TNF-α and PAR_2 ($p<0.05$, ANOVA, Tukey test). In addition, SDD has reduced bone loss when compared to the ligature group ($p<0.05$ ANOVA, Tukey test) and also maintained the percentage of collagen fibers at levels similar to the control group ($p> 0.05$). In the analysis of susceptibility to *P. gingivalis* SDD showed no multi-antibiotic resistance

for this strain, however, there was a change in the MIC values for all antibiotics tested with *P. gingivalis* growth to long-time. Together, these data demonstrate that SDD reduced the inflammatory response, bone resorption and collagen degradation in PD, indicating its activity as a modulator of the host response in PD. Furthermore, SDD affected the sensitivity of *P. gingivalis*, however not developing antibiotic resistance with 3 months therapy.

Keywords: Periodontal Disease, Subantimicrobial Dose of Doxycycline, Inflammation, Modulation of Host Response, Bone Reabsorption, Collagen, *Porphyromonas Gingivalis*, Bacterial Resistance.

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1- INTRODUÇÃO

A doença periodontal (DP) caracteriza-se como um processo inflamatório crônico, causada por infecção microbiana biofilme dependente que acomete os tecidos periodontais, levando a perda progressiva de inserção, com reabsorção óssea e migração apical do epitélio juncional (Dumitrescu *et al.*, 2004). Esta patologia se constitui como uma das principais causas de perda do elemento dentário, além de representar um fator modificador da saúde sistêmica dos pacientes (Garlet *et al.*, 2005).

Desde o clássico trabalho de Löe *et al.*(1965) intitulado "Gengivite experimental em humanos", o biofilme bacteriano foi relacionado como o fator etiológico primário da DP (Löe *et al.*, 1965). Atualmente, já foram isoladas e identificadas mais de 500 espécies, entretanto poucas destas estão associadas com a DP sendo que a bactéria *Porphyromonas gingivalis* é reconhecidamente o patógeno de maior importância no desenvolvimento da DP (Cortelli *et al.*, 2008).

A colonização por *P. gingivalis*, uma bactéria gram-negativa anaeróbia, resulta em lesão tecidual, através da produção de uma variedade de fatores de virulência, tais como os lipopolissacarídeos (LPS) e as proteases (hemaglutinina, gingipaínas) (Goulbourne & Ellen, 1991). O LPS é responsável pelo recrutamento de células inflamatórias como neutrófilos, linfócitos e macrafógos, bem como pela liberação de citocinas pro-inflamatórias como interleucina 1 (IL-1β), interleucina 17 (IL-17) e fatores de necrose tumoral (TNF-α) (Beklen *et al.,* 2007; Emingil *et al*., 2011).

Interleucina-1 (IL-1) e fator de necrose tumoral (TNF-α) são citocinas pro-inflamatórias e pluripotentes, que desempenham um importante papel na DP cuja relação apresenta-se bem consolidada na literatura em modelos *in situ*, *in vitro* e *in vivo* com animais e humanos (Ide *et al*., 2003; Kinane *et al*., 2011; Bostanci *et al*., 2011). A presença destas citocinas no tecido gengival leva ao recrutamento e ativação dos leucócitos, aumenta a permeabilidade vascular, induz a liberação de outras interleucinas (IL-6 e IL-8), aumenta a produção de proteinases de matriz

(MMPs), bem como, tem a capacidade de ativar osteoclastos que induzem a reabsorção óssea (Kotake *et al.*, 1999).

Nas últimas décadas, foi descoberto que a interleucina-17 (IL-17) apresenta efeito sinérgico potencializador associado as IL-1β e TNF-α (Chen *et al.,* 1992; Takahashi *et al.*, 2005; Vernal *et al.*, 2005). Além disso, alguns autores tem relatado que a IL-17 também está relacionada ao aumento da produção das MMPs e na indução do ligante do receptor ativador do fator nuclear kappa B (RANKL), o principal fator estimulante para a diferenciação e ativação dos osteoclastos, levando assim aos sinais clássicos da DP (Kotake *et al.*, 1999; Beklen *et al.*, 2007).

Nas ultimas décadas, a descoberta de um "novo" fator de virulência da *P. gingivalis*, as gingipaínas, tem mostrado que essa enzima podem agir diretamente sobre as células imunológicas com a produção de citocinas responsáveis pela destruição tecidual observada na DP (Nakayama, 2003; Grenier *et al.,* 2011). Além disso, foi demonstrado que gingipaínas também podem ativar as proteases ativadoras de receptor 2 (PAR_2) (Belibasakis *et al.*, 2010), que é um receptor de proteína G acoplado a uma variedade de tipos de células como: células epiteliais, endoteliais, fibroblastos, osteoblastos, neutrófilos, miócitos, neurônios e astrócitos (Abraham *et al.* 2000; Lourbakos *et al.*, 2001; Miike *et al.*, 2001; Uehara *et al.* 2003; Ossovskaya & Bunnett 2004), bem como, está relacionada a diversas doenças imune-inflamatória (Howells *et al.*, 1997; Lourbakos *et al.*, 2001, Miike *et al.*, 2001). Além disso, estudos têm demonstrado que PAR_2 pode ativar a produção de citocinas pró-inflamatórias, incluindo TNF-α e diversos subtipos de interleucinas como IL-1β, IL-6 e IL-8 (Lourbakos *et al.,* 2001; Uehara *et al.,* 2003). PAR_2 também está relacionada ao relaxamento dos vasos sanguíneos, aumento da permeabilidade vascular, infiltração de granulócitos, adesão leucocitária, dor e reabsorção óssea (Cocks & Moffatt, 2000; Vergnolle *et al.,* 2001; Coughlin & Camerer, 2003), sinais clássicos da reação inflamatória periodontal.

Desta forma, além do aspecto microbiano, a periodontia tem tentado elucidar os exatos mecanismos imunológicos envolvidos na destruição óssea e

gengival, que ocorre na evolução da DP (Taubman *et al.*, 2005). E assim, uma nova modalidade terapêutica vem sendo introduzida como coadjuvante ao tratamento mecânico para o controle da progressão da DP. Trata-se de um "novo" grupo de fármacos denominado de "modulador da resposta do hospedeiro" (MRH), justamente por controlar a resposta imuno-inflamatória local (Lee *et al.*, 2004). Como exemplo, desta abordagem terapêutica tem o uso de fármacos inibidores da formação do ácido araquidônico, com o uso de anti-inflamatórios não esteróides (AINEs) (Seymour & Heasman, 1988; Reddy *et al.*, 2003), e também com drogas que atuam na inibição do fator de necrose tumoral (TNF-α) (Delima *et al.*, 2001) e das metaloproteinases de matriz (MMPs) (Peterson, 2004).

Desde 1973, os AINEs têm sido estudados como uma alternativa no controle imuno-inflamatório, porém esse grupo de drogas não é comumente usado no tratamento e prevenção da periodontite devido os efeitos colaterais sistêmicos, que podem ser mais graves do que a DP quando prescritos sistemicamente e por longos períodos (Seymour & Heasman, 1988). Já o uso de antagonistas ao TNF-α tem sido investigado por uma redução significativa na perda de tecido conjuntivo, porém esta abordagem é nova e os efeitos sistêmicos desta terapia precisam ser investigados (Delima *et al.*, 2001; Salvi & Lang, 2005).

Neste contexto, fármacos usados principalmente para outros fins terapêuticos têm demonstrado efeito imuno-inflamatório como é o caso da fluoxetina, um medicamento usado como antidepressivo, com resultados promissores na terapia de DP por sua ação na inibição de citocinas pró-inflamatórias e também na diminuição da perda óssea em modelos animais (Branco-de-Almeida *et al.*, 2011). No entanto, estudos em humanos são necessários para que possamos considerá-lo uma alternativa ao MHR.

Outro fármaco, a doxiciclina (Dox) é um agente antibacteriano pertencente à classe das tetraciclinas. Recebe um destaque terapêutico pelo seu amplo espectro de ação e baixo efeito colateral (Roberts, 2003). Além da atividade antimicrobiana, a Dox apresenta uma importante propriedade na inativação das enzimas MMPs, em doses subs-antimicrobianas (Emingil *et al.*, 2004; 2006).

Golub *et al.* (1983) mostraram que a Dox em dose sub-antimicrobiana (DDS), é capaz de inibir colagenases (MMPs) e propôs que essa propriedade pode ser útil no tratamento da DP. Assim, este fármaco vem sendo usado na periodontia como um possível coadjuvante em pacientes em que a terapia mecânica isolada não apresentou resultados satisfatórios. Desde 1998, o Periostat® (doxiciclina, 20mg) é o único fármaco aprovado pela Food and Drug Administration/EUA (FDA) para o tratamento da DP (Lee *et al.*, 2004), sendo o uso prescrito por 2 vezes ao dia, durante um período variado de 2 semanas a 12 meses de tratamento (Caton *et al.*, 2000; Emingil *et al.*, .2004; Choi *et al.*, .2004).

Estudos publicados mostraram que além da atividade sobre as MMPs, a DDS tem atividade inibitória em várias citocinas pró-inflamatórias como as IL-1β e TNF-α (Emingil *et al*., 2011; Kinane *et al., 2011*), e também a DDS mostrou uma alta eficácia no processo de inibição da diferenciação osteoclástica utilizando-se modelos de estudo *in vitro* e *in vivo* (Franco *et al.*, 2011).

Baseado nesse mecanismo de ação reconhecido, vários estudos clínicos em pacientes portadores de diferentes estágios de periodontite, têm demonstrado que a terapia coadjuvante com dose sub-antimicrobiana da Dox promoveu uma melhora significativa nos parâmetros clínicos quando comparada a terapia mecânica isolada (Gürkan *et al.*, 2008; Novak *et al.*, 2008). Alguns estudos mostraram uma significativa redução da DP com 3 meses de uso diário (Emingil *et al.*, 2006; Haffajee *et al.*, 2008) e outros estudos mostraram melhorias nos parâmetros clínicos com o uso diário por um período de 9 meses de uso sistêmico da DDS (Caton *et al.*, 2000; Novak *et al.*, 2002; Preshaw *et al.*, 2004; Walker *et al.*, 2007).

Em decorrência dos resultados clínicos satisfatórios obtidos com a Dox, torna-se plausível a hipótese de que este fármaco também possa atuar em outras vias relacionadas com a resposta imuno-inflamatória na DP. Assim, o presente estudo contribui com a continuidade ao processo de verificação da existência de propriedades moduladoras da dose sub-antimicrobiana de Dox em outras vias da resposta imuno-inflamatória observada na DP.

Apesar do grande número de relatos na literatura, a investigação sobre esta terapia no tratamento periodontal é ainda recente e, portanto, outros estudos também são necessários para estabelecer seu real benefício clínico e microbiológico, principalmente em relação à manutenção dos resultados obtidos à longo prazo, para que se possa estabelecer a sua correta indicação. Isto se torna importante quando vemos um aumento do número de prescrições nos EUA (mais de 1,5 milhões) com o uso diário e prolongado da sub-dose de Dox (Lee *et al.*, 2004).

Em acréscimo, há uma importante controvérsia na literatura, pois alguns estudos mostram que a Dox, nesta sub-dose, não apresenta atividade antimicrobiana e como consequência, não promove a resistência bacteriana, mesmo com o uso diário e prolongado (Caton *et al.*, 2001; Preshaw *et al.*, 2004; Walker *et al.*, 2007; Haffajee *et al.*, 2008). Entretanto, outros estudos mostram uma relativa atividade antimicrobiana e sugerem um possível desenvolvimento de resistência bacteriana a este fármaco mesmo em dose subantimicrobiana (Feres *et al.*, 1999a; 1999b).

Com isso, é importante avaliar a influência deste fármaco, em sub-dose, sobre a susceptibilidade *da P. gingivalis*, a própria dose e a outros antimicrobianos, uma vez que a *P. gingivalis* está diretamente relacionada com a DP e a sua permanência em um foco infeccioso é potencialmente perigosa a saúde do hospedeiro, pois prolonga a resposta imuno-inflamatória e pode lavar ao desenvolvimento da DP (Li *et al*., 2002).

2. PROPOSIÇÃO

O presente trabalho teve como objetivo geral analisar os efeitos *in vivo* da doxiciclina em dose sub-antimicrobiana sobre as principais vias de modulação da resposta imuno-inflamatória do hospedeiro em modelo de doença periodontal e avaliar a susceptibilidade ou a resistência bacteriana de *P. gingivalis* em dose sub-antimicrobiana de Dox por um período de até 3 meses de terapia.

Os objetivos específicos do presente estudo foram:

a) Avaliou-se os níveis de expressão do RNA mensageiro presente no tecido gengival de ratos com doença periodontal induzida por ligadura e tratados com Dox em dose sub-antimicrobiana por 3 dias, para as seguintes citocinas : IL-1β, IL-17, TNF-α e PAR_2;
b) Determinou-se a área de reabsorção alveolar óssea e a porcentagem de degradação de fibras colágenas na região do primeiro molar dos dentes de ratos com ligaduras e tratados com Dox, por 15 dias, em sub-dose;
c) Determinou-se as concentrações inibitórias mínimas (CIM) da Amoxiciclina (Amox), Metronidazol (Met) e Dox em culturas de *P. gingivalis* W83 previamente exposta a terapia de DDS durante um período de 3 meses contínuos (45 gerações).

3. PREÂMBULO DOS CAPÍTULOS

Esta tese está baseada na Informação CCPG/002/06/UNICAMP, que regulamenta o formato alternativo para a tese de Doutorado e permite a inserção de artigos científicos de autoria ou co-autoria do candidato (Anexo 1).

Dessa forma, esta tese apresenta um capítulo, composto pelo estudo que encontra-se em fase de submissão, cujo título está descrito abaixo:

Capítulo 1

Título: *Subantimicrobial Dose of Doxycycline in Periodontal Disease: Immunological, Histological and Microbiological Aspects.*

Autores: Myrella L. Castro[1], Gilson C. N. Franco[2], Luciana S. Branco-de-Almeida[1], Ana Lia Anbinder[3], Sheila C. Cortelli[2], Simone Duarte[4], Deepak Saxena[4], Pedro L. Rosalen[1]*

Este estudo está em processo de submissão ao European Journal of Pharmacology (fator de impacto = 2.150).

4. **Capítulo 1**

Subantimicrobial Dose of Doxycycline in Periodontal Disease: Immunological, Histological and Microbiological Aspects.

Myrella L. Castro[1], Gilson C. N. Franco[2], Luciana S. Branco-de-Almeida[1], Ana Lia Anbinder[3], Sheila C. Cortelli[2], Simone Duarte[4], Deepak Saxena[4], Pedro L. Rosalen[1]*

[1]Department of Physiological Sciences, Piracicaba Dental School, State University of Campinas, SP, Brazil;
[2]Department of Oral Biology, University of Taubaté, SP, Brazil;
[3]Department of Bioscience and Oral Diagnosis, São José dos Campos School of Dentistry, Universidade Estadual Paulista-UNESP, SP, Brazil;
[4]Department of Basic Science and Craniofacial Biology, College of Dentistry, New York University, New York, USA

***Corresponding Author:**
Dr. Pedro Luiz Rosalen, State University of Campinas, Piracicaba Dental School, Department of Physiological Sciences, Avenida Limeira, 901, Areão, Caixa Postal 52, 13414-903, Piracicaba, SP, Brazil. E-mail: rosalen@fop.unicamp.br. Telephone: +55 19 2106 5313, Fax +55 19 2106 5308.

ABSTRACT

Background: Specific products of *Porphyromonas gingivalis* activates the immune-inflammatory response which releases interleukins (IL)-1β and IL-17, tumor necrosis factor (TNF)-α, matrix metalloproteinases (MMPs) and protease-activated receptor-2 (PAR_2). Subantimicrobial dose doxycycline (SDD) has been used as an adjunct in periodontal therapy by reducing the immune response. However, it is still

questionable whether possible changes may occur in the susceptibility of *P. gingivalis* with its long-term use. In this context, the aim of this investigation was to evaluate the molecular and histological effects of SDD as a modulator of the host response (MHR) in the ligature-induced periodontitis in rats. Additionally, *in vitro* susceptibility of *P. gingivalis* in long-term treatment with SDD was analyzed. Methods: Male Wistar rats were randomly assigned into three groups (n=10 animals/group/experiment): 1) control group: rats without ligature and without treatment; 2) ligature group: rats with ligature and treated with 0.9% NaCl solution and 3) ligature + SDD group: rats with ligature and treated with SDD (5 mg/kg/day). The animals were treated with SDD for 3 days and reverse transcriptase-polymerase chain reaction (RT-PCR) were performed to analyze the mRNA expression of interleukin IL-1β, IL-17, TNF-α and PAR_2 activity, respectively, in gingival tissue samples. Histological analyses were performed on the furcation region and mesial of mandibular first molars of rats sacrificed at 15 days after the ligature-induced PD. For the microbiological analysis, *P. gingivalis* was cultivated for 3 months (45 generations) in medium cultures containing 0.4 μg/mL of SDD and evaluated the antimicrobial susceptibility by minimum inhibitory concentration (MIC) test. Results: Compared to the ligature group, alveolar bone loss was reduced in the SDD group ($p<0.05$), and the amount of collagen fibers in the gingival tissue was maintained. Moreover, in the gingival tissue sampled 3 days after ligature attachment, SDD administration reduced IL-17 and PAR_2 of mRNA expression ($p<0.05$). SDD down-regulated IL-1 and TNF-α activity mRNA expression induced by ligature, compared to the ligature group ($p<0.05$). These data suggested that SDD suppresses pro-inflammatory responses induced by ligature. SDD used long-term did not cause resistance of the *P. gingivalis* strain, but caused a change in the antimicrobial values, showing that SDD may interfere with the bacterial culture. Conclusions: SDD reduced the bone resorption and collagen destruction, for his role in IL-1β, IL-17, TNF-α and PAR_2. The microbial alteration with continuous use of SDD over a long period changed the *P. gingivalis* susceptibility to antibiotics, though it did not develop resistance to doxycycline.

However, despite the positives results immuno-inflammatory over the long term, antimicrobial studies are needed to consider it a safe modulator of the host response.

Key-words: Subantimicrobial Dose of Doxycycline, Periodontal Disease, Host Response Modulation, Inflammation, PAR_2, Interleukins-17, Bone Reabsorption, Collagen Fibers.

INTRODUCTION

Periodontitis (PD) is a chronic inflammatory disease that is results of the interaction between a complex biofilm and protective immunological mechanisms (Caton *et al., 2011;* Shaddox *et al.*, 2011; Kinane *et al.*, 2011). Among the different microbial species involved in PD, *Porphyromonas gingivalis* is a Gram-negative bacterium that plays a key role in the initiation and progression of chronic periodontitis due to its ability to produce different proteases, such as gingipains (Nakayama, 2003; Grenier *et al.*, 2011). Gingipains can act directly on the immune cells with the cytokines production responsible for tissue destruction observed in PD (Nakayama, 2003; Grenier *et al.*, 2011).

Recently, it was demonstrated that gingipains can also activate Proteinase-Actived Receptor-2 (PAR_2) (Belibasakis *et al.*, 2010) which is a seven-transmembrane G-protein-coupled receptor expressed in oral epithelial and non-epithelial cells (neutrophils, gingival fibroblasts, osteoblasts and others) and activated by proteases present during inflammation (mast cell tryptase and factor Xa) (Abraham *et al.*, 2000; Howells *et al.*, 1997; Lourbakos *et al.*, 2001; Miike *et al.*, 2001). Studies have demonstrated that PAR_2 is overexpressed in patients with PD (Holzhausen *et al.*, 2010) and it is associated with bone resorption. In addition, PAR_2 may stimulate the production of proinflammatory mediators, including tumor necrosis factor (TNF)-α and interleukin 1 (IL)-1(Amiable *et al.*, 2009).

Besides gingipains, the colonization by *P. gingivalis* produces the release of lipopolysaccharide (LPS) that is responsible for the recruitment of inflammatory cells and release of proinflammatory cytokines such as in IL-1β, IL-17 and TNF-α (Kotake *et al.,* 1999; Vernal *et al.,* 2005; Takahashi *et al.,* 2005). These cytokines are related to chronic PD signs, since their presence in gingival tissues are able to: a) increase vascular permeability, b) induce the release of other interleukins (IL-6 and IL-8), c) increase the production of proteinases (MMPs), and d) activate osteoclasts to induce bone resorption (Vernal *et al.,* 2005; Takahashi *et al.,* 2005; Beklen *et al.,* 2007).

Due to this better understanding of the pathogenesis of periodontal disease, systemic therapies involving the modulation of the immuno-inflammatory host response (MHR) were suggested as complementary to conventional mechanical/chemical procedures in the treatment of PD (Kinane *et al.,* 2011; Sgolastra *et al.,* 2011). Nowadays, doxycycline (Dox), an antimicrobial agent of the tetracycline group, is the major MHR drug used in PD (Kinane *et al.,* 2011; Sgolastra *et al.,* 2011).

Sub-antimicrobial dose of Dox (SDD) is able to inhibit the activity of matrix metalloproteinases (MMPs) and to reduce the degradation of macromolecules in the periodontal tissue such as collagens, fibronectin and elastin (Golub *et al.,* 1983). In 1998, the Food and Drug Administration (FDA) approved the use of SDD (20 mg, twice daily) as an adjunct in the treatment of PD based on its mechanism of action and on the clinical results of decreased pocket depth levels, attachment level and bleeding (Sgolastra *et al.,* 2011). In addition to this property, our group demonstrated that Dox can also inhibit osteoclast differentiation/activation using both *in vitro* and *in vivo* models (Franco *et al.*, 2011).

In spite of its efficacy, a main concern regarding the clinical use of SDD is the possibility of selection of microbial resistance during long period treatment as indicated in dentistry and its impact on the treatment of other infectious disease (Pallasch, 2004).

In this context, the aim of this investigation was to evaluate the molecular

and histological effects of Dox as a MHR in the ligature-induced periodontitis in rats. Additionally, *in vitro* susceptibility of *P. gingivalis* to long-term treatment of Dox and other non-tetracycline antibiotics was analyzed

MATERIAL AND METHODS

In vivo Study

Animals and periodontitis induction:

The experimental protocol of the present study was approved by the Ethical Committee on Animal Research (Protocol # 1591-1) at the State University of Campinas. Sixty male rats (Wistar-Specific Pathogen Free, weighting from 250 to 300 g) were obtained from CEMIB (Multidisciplinary Center for Biological Research, State University of Campinas/SP - Brazil), housed in temperature, humidity and light-dark cycle controlled rooms and received food and water *ad libitum*.

PD was induced in rats using gingival bilateral ligatures in the 1st molars (Rodini *et al.*, 2008). To obtain this result, the rats were anesthetized with an intramuscular injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). The animals were randomly allocated into the following three or fifteen days experimental groups (n=10 animals/group/experiment) (Holzhausen *et al.* 2002; Rodini *et al.* 2008): 1) control group: rats without ligature and without treatment; 2) ligature group: rats with ligature and treated with 0.9% NaCl solution and 3) ligature + SDD group: rats with ligature and treated with SDD (5 mg / kg / day). (Golub *et al.*, 1994; Budunel*i et al.*, 2004). Doxycycline hyclate was obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in water. All treatments (Dox or NaCl 0.9%) were given by gavage 1 hour before the PD induction and daily during experimental periods. The behavior and physical appearance of the animals were noted daily and their weight were assessed at the beginning and end of each experimental period.

RNA isolation, cDNA synthesis and RT-PCR.

Thirty rats were sacrificed after 3 days and the gingival tissues of the first molar area were removed. The total RNA from these tissues were extracted using TRIzol® reagent (Invitrogen®, Carlsbad, CA) according to the manufacturer's guidelines. The RNA pellet was resuspended in ultrapure DNase/RNase free water and stored at -80°C. Total RNA concentration and quality were determined using a Nanodrop Spectrophotometer (ND-3300, NanoDrop Technologies, USA). DNase I® (Invitrogen ®) was used to eliminate DNA contamination. Isolated total RNA (0.5 µg) was reverse transcribed with the SuperScript synthesis system in the presence of random primers (Invitrogen®). The subsequent complementary DNA was amplified by PCR with Taq DNA polymerase (Invitrogen®) as described by the manufacturer. Amplification of cDNA was performed using specific primers shown in Table 1. PCR conditions were 30–35 cycles of 94°C for 30 s; 55–60°C for 30 s; 72°C for 1 min. The size of the PCR products was determined by comparing with the 100 bp ladder (Invitrogen®). The agarose gels containing the amplified products were scanned and analyzed by imaging software (ImageJ®, NIH), through comparison of gel band intensity allowed a semi-quantitative comparison between target genes and the internal control gene [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] (Branco-de-Almeida *et al*., 2011).

Histological Analysis

Thirty rats were sacrificed after 15 days of oral treatment and their left hemimandibles were removed, immediately fixed with 10% neutral buffered formalin and then decalcified with 10% EDTA aqueous solution for 60 days. The specimens underwent routine histological preparation. Paraffin semi-serial sections (5 µm) were obtained in a mesiodistal direction and stained with Hematoxylin and Eosin (H&E) for measurement of bone loss or with picrosirius red for collagen content evaluation (Branco-de-Almeida *et al*., 2011).

Histological images of sections stained with H&E were scanned at a magnification of x50. Periodontal bone loss was assessed histometrically by a single examiner, blind and using an imaging software (ImageJ®, NIH) to measure

the area (mm^2) of bone loss in the furcation area, indicating destruction of the periodontal ligament and/or bone loss area of the furcation to the top of the alveolar bone crest (Nociti *et al.*, 2000).

The images of the sections stained with picrosirius red were obtained by polarization microscopy and the areas of connective tissue immediately above the bone crest, in the area corresponding to the mesial of first molar, were digitized at x400 of magnification. The percentage of collagen fibers was assessed using both the ImageJ (ImageJ®, NIH) and Adobe Photoshop® 7.0.1 (Adobe Systems Incorporated, San Jose, CA, USA) programs. First, the images of red hue collagen fibers were selected with the aid of the Adobe Photoshop 7.0.1 image-processing software. The selected images were binarized, and the percentage of area filled by collagen fibers was calculated (Rich & Whittaker, 2005).

In Vitro Study

Microbial Analyses

An aliquot (100 µL) of *Porphyromonas gingivalis* (strain ATCC BAA-308 / W83) was reactivated and an aliquot of 4.9 mL of Fastidious Anaerobe Broth (FAB) medium for 48 h and another 10 µL was reactivated in Fastidious Anaerobe Agar (FAA) plates, cultured for 4 days. Both were incubated under anaerobic conditions (80% N_2, 10% H_2 and 10% CO_2) (Cogo *et al.* 2008). After bacterial growth, this was considerated 1^{st} generation, with an inoculum 0.7 at 660 nm in a spectrophotometer (equivalent to 2 x 10^9 cfu/mL). Three groups were used for antimicrobial susceptibility determination: 1) subantimicrobial dose Dox (0.4 µg/mL); 2) antimicrobial dose Dox (4 µg/mL), and 3) control containing culture medium without Dox (Caton, 1999; Haffajjee *et al.*, 2008). Under anaerobic conditions, an aliquot (100 µL) of the first tube was transferred to a new tube containing fresh culture medium with or without Dox. This was considered to be the 2^{nd} generation. Triplicate tubes were made of the testing and control. We follow this procedure for 45 generations, equivalent to 3 months of therapy with sub-

antimicrobial dose of Dox (Emingil *et al.*, 2004, Choi *et al.*, 2004, Haffajee *et al.*, 2008).

For each generation, an inoculum was prepared to evaluate the susceptibility or the resistance of *P. gingivalis* through the Minimum Inhibitory Concentration (MIC) test it was considered the change in bacterial susceptibility when MIC values exceeded the known values in the first generation and the known values in the literature of antimicrobial activity for two or more drugs, following the guidelines of the CLSI, M11-A4 (1997). The determination of MIC was performed using a method described by CLSI (1997).

The FAB medium was inoculated with bacterial suspension, as described above, in the 1:20 ratio, to obtain a bacterial concentration of about 1 x 10^7 cfu/mL. Antimicrobial doses of Dox 100 mg, Amoxicillin (Amox) 500 mg and Metronidazole (Metr) 750 mg (Sigma-Aldrich; St. Louis, MO) (Thomas *et al., 2000;* Walker *et al., 2007;* Haffajee *et al.,* 2008). Each antibiotic was tested in concentration increasing two-fold from 0.05 to 16 µg/mL following the serum concentration of antibiotics and the recommendations of CLSI (1997). MIC was determined at the lowest concentration that could inhibit bacterial growth. Three repetitions were made for each antimicrobial drug.

STATISTICAL ANALYSIS

Data from assays are presented as means ± standard deviation (SD). The results were subjected to one-way analysis of variance (ANOVA), and statistical differences among the three groups were analyzed using the Tukey test at a significance level of 5%. Data were analyzed using statistical software BioEstat 5.0 (Sociedade Civil Mamirauá, Belém-PA, Brazil).

RESULTS

Gene expression data in the ligature induced gingival tissues and control are presented in relation to GAPDH values. Systemic administration of SDD affected the levels of mRNA expression in the 4 genes analyzed in RT-PCR ($p<0.05$).

Data analysis showed that the SDD group had significantly reduction levels of PAR_2 mRNA expression in the gingival tissue (Fig. 1) compared to the ligature group ($p<0.05$). Similarly, statistical analysis revealed a higher difference in the expression of IL-17 gene, showing results very close to the control group (Fig.1). To confirm the effect of SDD on the reduction of proinflammatory cytokines mRNA levels of TNF-α, IL-1β were tested. The Figure 1 shows a significant difference between the SDD and ligature groups ($P<0.05$).

Experimental periodontitis model was confirmed, since there was an increase in bone loss during the period of 15 days after placement of the ligature. SDD therapy reduced the total area of bone loss in the furcation of first molar when compared with the NaCl 0.9% group ($p<0.05$, Fig.2). After the connective tissue collagen assessment, it was found less picrosirius red staining pattern in the ligature group compared to either control or the ligature + SDD ($P<0.05$, Fig. 2).

The sensitivity of *P. gingivalis* W83 for the groups SDD and control are listed in Table 2. Furthermore, it was illustrated the patterns of susceptibility recommended by CLSI for anaerobic microorganisms to the antibiotics studied: Dox, Amox and Metr. The reactivation of *P. gingivalis* was considered the first generation, it was sensitive to most antimicrobial agents tests with MIC values of 0.2 µg/mL to Dox. After 3 months, *P. gingivalis* cultured without Dox showed MIC values of 0.4µg/mL, and their growth with Dox had MIC values of 0.8 µg/mL. These values of MIC indicate no resistance to *P. gingivalis* under CLSI guidelines for of sensitivity anaerobic (CLSI, 1997).

DISCUSSION

In the present study, Dox was able to reduce the gene expression of different proinflammatory mediators along with a reduction of bone resorption and collagen degradation. In special, to the author's knowledge, this is the first paper to show a diminished PAR_2 gene expression in the animal model of periodontitis treated with Dox. This result can indicate a new mechanism of action of this drug on the modulation of the immuno-inflammatory host response (MHR) in PD.

PAR family is composed of four members (PAR_1, PAR_2, PAR_3 and PAR_4) distributed by several types of body cells. Nowadays, PAR_2 is the most studied due to its direct association with chronic inflammatory diseases, including arthritis and PD (Holzhausen *et al.*, 2005; Kelso *et al.*, 2006). Holzhausen *et al.* (2005) demonstrated that topical application at the mesial gingival sulcus of the mandibular first molar of PAR_2 agonist (SLIGRL-NH2) induced significant alveolar bone loss and gingival granulocyte infiltration in rats. In another study, it was demonstrated that PAR_2-deficient mice ($PAR_2^{-/-}$) had less bone loss after *P. gingivalis* subcutaneous inoculation when compared to wild-type (WT) mice ($PAR_2^{+/+}$) (Holzhausen *et al.*, 2006).

Since PAR_2 is activated by proteases present in inflammation sites and Dox is considered a strong protease inhibitor, this drug can modulate PAR_2 by a dual mode, downregulating the gene expression and decreasing its posterior activation by proteases. These effects can enhance the efficacy of Dox in diseases involving PAR_2.

PD is characterized by an increase in the expression of proinflammatory cytokines (Shaddox *et al.*, 2011). Our study showed that Dox downregulated the expressions of TNF-α, IL-1β and IL-17. Although TNF-α and IL-1β are well-documented cytokines involved in the breakdown of tooth-supporting tissues in PD, IL-17 was just recently associated with PD (Oda *et al.*, 2003; Johnson *et al.*, 2004; Emingil *et al., 2011*). IL-17, a proinflammatory cytokine, is mainly produced by active CD4+ T-cells/Th17 (Aarvak *et al.*, 1999) and it induces RANKL production by osteoblast and also stimulates fibroblast to produce other inflammatory mediators such as TNF-α and IL-1β (Takahashi *et al.*, 2005; Beklen *et al.,* 2007). Yi and collaborators, (2011) using a rat model of experimental auto-immune neuritis (EAN), demonstrated that IL-17 is related to the progression of EAN and the treatment of Dox, in high dose (40 mg/kg/day), was able to reduce the levels of this cytokine, contributing to attenuate the severity of EAN. In periodontitis model, the present study was the first demonstration of a reduction of IL-17 gene expression by a low dose of Dox (5 mg/kg/day).

These results taken together can support the microscopical finds of the present study which were represented by a reduction of bone alveolar loss and lower collagen degradation in the Dox group.

It is crucial for the success or failure of SDD therapy to consider changes in bacterial susceptibility (Thomas *et al*., 2000). Treatment with SDD over time was tested for resistance to antibiotics and was measured by a change in the Dox MICs and different classes of antibiotics. Pharmacokinetic studies in humans showed that 20 mg of Dox, produces blood serum concentrations of 0.4 μg/mL free Dox (CollaGenex Pharmaceuticals, 1996). These values are lower than 3 to 4 μg/mL in blood with antibiotics produced by doses of 100-200mg (Walker *et al.* 2000). The SDD level of free Dox in plasma is considerably below the MIC determined *in vitro* for the vast majority of bacteria isolated from normal human flora (Sutter *et al.,* 1983).

Our results for the first generation of *P. gingivalis* are in agreement with literature data, which shows similar values of MIC for various strains of *P. gingivalis* with the antibiotics tested Amox, Dox and Met (0.3, 0.1 and 0.2 μg/mL, respectively) (Aldridge *et al.,* 2001; Haffajee *et al*., 2008), However, we found 0.2 μg/mL in the MIC for Dox, and thus the concentration of 0.4 μg/mL was two times higher than MIC found in this study for *P. gingivalis,* showing that the 0.4 μg/mL is antimicrobial dose contradicts the literature (Thomas *et al.*, 2000; Walker *et al.*, 2007; Haffajee *et al.*, 2008). The results obtained from the groups with SDD and control (no-dox) for the three drugs tested showed a slight change in the sensitivity of the *P. gingivalis* that can be observed from the 38th generation (about 2.5 months) and this MIC value remained for up to 3 months or 45 generations. The results showed a change in MIC intervals, however remained in the range intermediate classification according to the CLSI (1997). Thus, we can understand that over 3 months *P. gingivalis* may not develop resistance to any of the antibiotics tested, but was able to change the sensitivity of the *P. gingivalis* with the antimicrobials tested. Interesting to note that the presence of SDD decreased sensitivity of *P. gingivalis* to metronidazole, as shown in Table 2, three months

after the MIC increased from 0.1 μg/mL to 0.8 μg/mL. Further studies are needed to discover the mechanisms involved in this finding because metronidazole is an antibiotic commonly indicated for the treatment of PD (Silva *et al.,* 2011).

Generally, our results are consistent with the literature as in several clinical studies on SDD treatment over long periods which shows that the SDD causes no resistance to the oral biofilm bacteria, intestinal and vaginal flora (Thomas *et al.*, 2000; Walker *et al.*, 2005; Haffajee *et al.*, 2008). However, many of these studies show that while SDD does not develop bacterial resistance, it causes changes in the number of bacteria and also reduction in the sensitivity to diverse classes of drugs with MIC values slightly higher when compared to placebo groups (Feres et al., 1999a; 1999b). These results are similar to those found by our research group.

CONCLUSION

SDD may be beneficial in periodontal treatment by reducing the alveolar bone resorption and maintaining the amount of collagen fibers probably by acting at inhibition of the expression IL-1β, IL-17, TNF-α and PAR_2. However we need more studies with respect to bacterial susceptibility with a long-term use of SDD, since resistance was not found for *P. gingivalis* but a marked alteration of the antimicrobial values. Thus it shows that SDD interfere in some way in the bacterial culture susceptibility to Doxycycline and Metronidazole.

ACKNOWLEDGMENTS

The authors thank the Brazilian Government Agencies CNPq for fellowships to M.L.C. (142559/2008-3 and 201815/2009-5).

Table 1. Primers sequences and amplicon length for each gene used for RT-PCR amplification

Gene	Sequence gene (5'→3')	Lenght (bp)
IL-1β	ForwardTCCATGAGCTTTGTACAAGG	237 bp
	Reserve GGTGCTGATGTACCAGTTGG	
PAR $_2$	ForwardGCGTGGCTGCTGGGAGGTATC	441 bp
	Reserve GGAACAGAAAGACTCCAATG	
TNF-α	ForwardTACTGAACTTCGGGGTGATTGGTCC	295 bp
	Reserve CAGCCTTGTCCCTTGAAGAGAACC	
IL-17	Forward CTTCACCCTGGACTCTGAGC	286 bp
	Reserve TGGCGGACAATAGAGGAAAC	
GAPDH	Forward ACCACAGTCCATGCCATCAC	450 bp
	Reserve TCCACCACCCTGTTGCTGTA	

Table 2. Minimum inhibitory concentrations (MICs) and values established by the CSLI for the classification of bacteria.

Antimicrobial agent	1st G	45G without Dox	45G with Dox	Susceptible (CLSI)	Intermediate (CLSI)	Resistant (CLSI)
Amoxicillin*	0.4	1	1	≤ 0.2	> 0.2 to < 8	≥ 8
Doxycycline	0.2	0.4	0.8	≤ 0.2	> 0.2 to < 8	≥ 8
Metronidazole	0.1	0.2	0.8	≤ 0.5	> 0.5 to <16	≥ 16

G= generations

The values is expressed in µg/mL.

Each antibiotic was tested in increasing two-fold concentration ranging from 0.05 to 16 µg/mL

* Amoxicillin is considered to have an MIC similar to ampicillin.

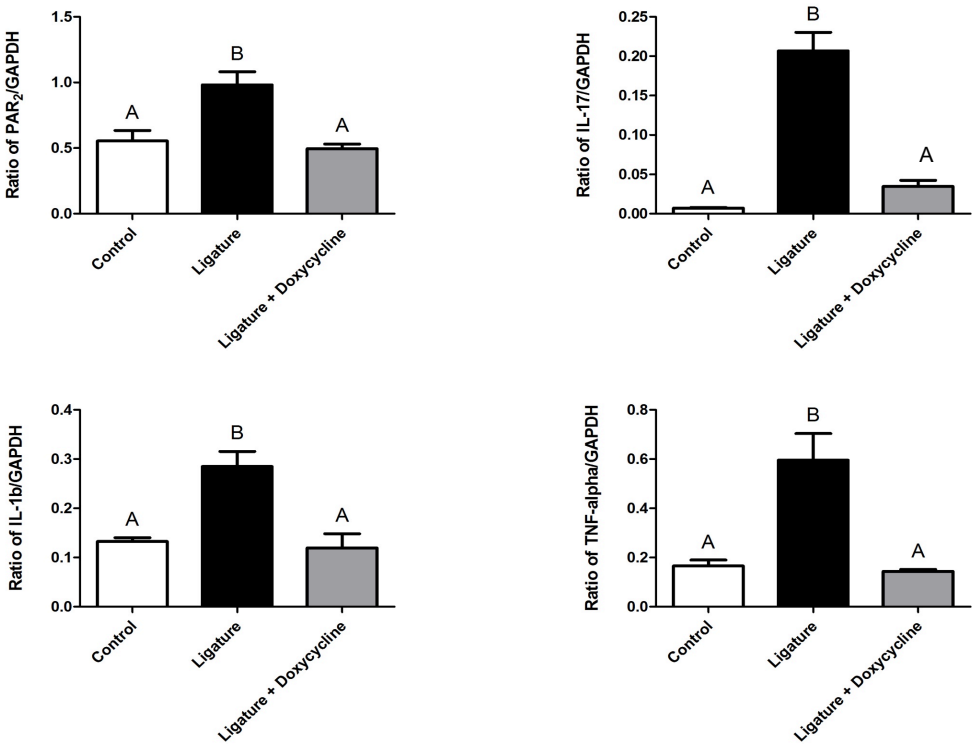

Figure 1. Effects of subantimicrobial dose of doxycycline (SDD) treatment on expressions of PAR_2, IL-17, IL-1β and TNF-α mRNA levels monitored in gingival tissues, presented by ratio: mRNA expression level/ GAPDH.

mRNA expression levels (ratio of PCR products for target gene/GAPDH measured in agarose gel) of respective genes, PAR2, IL-17, IL-1β and TNF-α in the gingival tissues were calculated. Data represents the mean ± standard deviation (SD) of 10 rats for each group. Data were collected from the samples isolated from the gingival tissues of 1) control, 2) ligature and 3) ligature + SDD on Day-3. Different letters on the top of each bar indicate statistical differences (PAR_2: A vs. B, p<0.05; IL-17: A vs. B, p< 0.01; IL-1β: A vs. B, p< 0.05; TNF-α: A vs. B, p< 0.05; ANOVA followed by Tukey test).

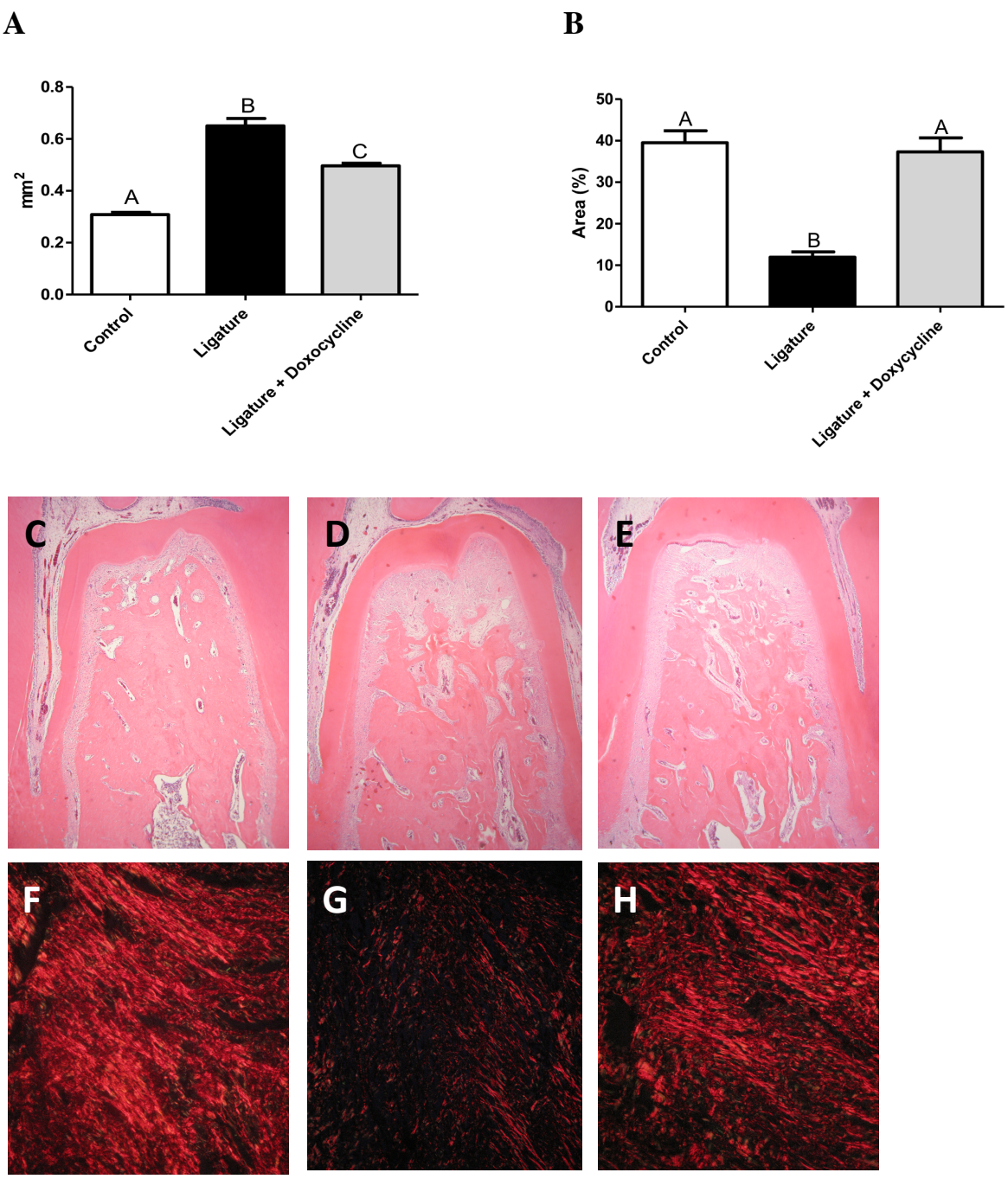

Figure 2.Effects of subantimicrobial dose of doxycycline (SDD) treatment on alveolar bone loss and collagen content in a rat model of ligature induced periodontitis.

Data represents the mean ± standard deviation (SD) of 10 rats for each group. A) Measurement of bone loss (mm^2) in the furcation region of first molars of control, ligature and ligature + SDD groups after 15 days of periodontal disease induction. B) Quantitative analysis of red-stained collagen fibers (% area) in the connective tissue immediately above the bone crest in

the mesial of the mandibular first molars of control, ligature and ligature + SDD groups after 15 days of periodontal disease induction. SDD reduced alveolar bone loss as compared to ligature group ($p<0.05$) and maintained collagen fiber levels similar to control group ($p>0.05$). Different letters on the top of each bar indicate statistical differences (Figure 1 A: A vs. B, $P< 0.01$; A vs. C, $p<0.05$; Bvs C, $P<0.01$; Figure 1 B: A vs. B, $P< 0.01$; ANOVA followed by Tukeytest). C, D, and E are the images of H&E staining at the furcation region of control, ligature, and ligature + SDD groups, respectively (Hematoxylin and Eosin staining, magnification of x50). F, G, and H are images of the collagen fibers stained with picrosirius-polarization microscopy in the connective tissue immediately above the bone crest in the mesial of mandibular first molars of control, ligature, and ligature + SDD groups, respectively (picrosirius red stain, magnification of x400).

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5. CONCLUSÕES GERAIS

A doxiciclina em dose subantimicrobiana foi capaz de modular a resposta do hospedeiro na DP, pois reduziu os níveis de mediadores inflamatórios como PAR_2 e IL-17, bem como, diminuiu outras vias da resposta inflamatória, como a reabsorção óssea e a degradação de colágeno na DP. A DDS modificou o crescimento da *P. gingivalis,* entretanto não desenvolveu resistência antibiótica a multidrogas ao longo de 3 meses.

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ANEXO 1: Resolução do formato alternativo para a defesa da tese de Doutorado.

INFORMAÇÃO CCPG/002/06

Tendo em vista a necessidade de revisão da regulamentação das normas sobre o formato e a impressão das dissertações de mestrado e teses de doutorado e com base no entendimento exarado no Parecer PG nº 1985/96, que trata da possibilidade do formato alternativo ao já estabelecido, a CCPG resolve:

Artigo 1º - O formato padrão das dissertações e teses de mestrado e doutorado da UNICAMP deverão obrigatoriamente conter:

I. Capa com formato único ou em formato alternativo que deverá conter informações relativas ao nível (mestrado ou doutorado) e à Unidade de defesa, fazendo referência à Universidade Estadual de Campinas, sendo o projeto gráfico das capas definido pela PRPG.
II. Primeira folha interna dando visibilidade à Universidade, a Unidade de defesa, ao nome do autor, ao título do trabalho, ao número de volumes (quando houver mais de um), ao nível (mestrado ou doutorado), a área de concentração, ao nome do orientador e co-orientador, ao local (cidade) e ao ano de depósito. No seu verso deve constar a ficha catalográfica.
III. Folha de aprovação, dando visibilidade à Comissão Julgadora com as respectivas assinaturas.
IV. Resumo em português e em inglês (ambos com no máximo 500 palavras).
V. Sumário.
VI. Corpo da dissertação ou tese dividido em tópicos estruturados de modo característico à área de conhecimento.
VII. Referências, formatadas segundo normas de referenciamento definidas pela CPG da Unidade ou por critério do orientador.
VIII. Todas as páginas deverão, obrigatoriamente, ser numeradas, inclusive páginas iniciais, divisões de capítulos, encartes, anexos, etc... As páginas iniciais poderão ser numeradas utilizando-se algarismos romanos em sua forma minúscula.
IX. Todas as páginas com numeração "impar" serão impressas como "frente" e todas as páginas com numeração "par" serão impressas como "verso".

§ 1º - A critério do autor e do orientador poderão ser incluídos: dedicatória; agradecimento; epígrafe; lista de: ilustrações, tabelas, abreviaturas e siglas, símbolos; glossário; apêndice; anexos.

§ 2º - A dissertação ou tese deverá ser apresentada na língua portuguesa, com exceção da possibilidade permitida no artigo 2º desta Informação.

§ 3º - As dissertações e teses cujo conteúdo versar sobre pesquisa envolvendo seres humanos, animais ou biossegurança, deverão apresentar anexos os respectivos documentos de aprovação.

Artigo 2º - A critério do orientador e com aprovação da CPG da Unidade, os capítulos e os apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

ANEXO 2: Certificado de aprovação do Comitê de Ética na experimentação animal CEEA/UNICAMP

UNICAMP

CEEA/Unicamp

Comissão de Ética na Experimentação Animal
CEEA/Unicamp

CERTIFICADO

Certificamos que o Protocolo nº **1591-1**, sobre **"Avaliação *in vivo* de dose sub-antimicrobiana de doxiciclina sobre a modulação da resposta imuno-inflamatória do hospedeiro em modelo de doença periodontal induzida em ratos"**, sob a responsabilidade de **Prof. Dr. Pedro Luiz Rosalen / Prof. Dr. Gilson César Franco / Myrella Lessio Castro**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em **15 de setembro de 2008**.

CERTIFICATE

We certify that the protocol nº **1591-1**, entitled **"*In vivo* evaluation of the activity of sub dose doxycycline in host response in periodontal disease"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **September 15, 2008**.

Campinas, 25 de setembro de 2008.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

CEEA – Unicamp
Caixa Postal 6109
13083-970 Campinas, SP – Brasil

Telefone: (19) 3521-6359
E-mail: comisib@unicamp.br
http://www.ib.unicamp.br/ceea/

ANEXO 3: Comprovante de submissão à revista

---------- Mensagem Original ----------
Assunto: Submission Confirmation for Subantimicrobial dose of doxycycline in periodontal disease: Immunological, Histological and Microbiological Aspects.
De: "The European Journal of Pharmacology" <ejp-office@pharm.uu.nl>
Data: Sab, Fevereiro 25, 2012 4:10 pm
Para: rosalen@fop.unicamp.br

Dear Dr Pedro L Rosalen,

Your submission entitled "Subantimicrobial dose of doxycycline in periodontal disease: Immunological, Histological and Microbiological Aspects." has been received by journal European Journal of Pharmacology

You will be able to check on the progress of your paper by logging on to Elsevier Editorial System as an author. The URL is http://ees.elsevier.com/ejp/.

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ANEXO 4: Comprovante de correçãoo da Língua Inglesa presente no Capitulo 1.

Campinas, April 11, 2012.

European Journal of Pharmacology

Dear Editors

This is to certify that the paper: "Subantimicrobial Dose of Doxycycline in Periodontal Disease: Immunological, Histological and Microbiological Aspects" has been duly reviewed by language professionals.

Cynthia Sonetti Valim de Oliveira
Matrícula nº 169838
Redator de Textos Técnicos
Espaço da Escrita
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