UNESP - Universidade Estadual Paulista

"Júlio de Mesquita Filho"

Faculdade de Odontologia de Araraquara

ANDRESSA VILAS BOAS NOGUEIRA

CONTRIBUIÇÃO DAS FORÇAS BIOMECÂNICAS NA DESTRUIÇÃO PERIODONTAL. ESTUDOS IN VITRO E IN VIVO.

ARARAQUARA
2014

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Orientador: Prof. Dr. Joni Augusto Cirelli

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''Eu sou aquela mulher a quem o tempo muito ensinou.

Ensinou a amar a vida.

Não desistir da luta.

Recomeçar na derrota.

Renunciar as palavras e pensamentos negativos.

Acreditar nos valores humanos.

Ser otimista.''

Cora Coralina

RESUMO

Nogueira AVB. Contribuição das forças biomecânicas na destruição periodontal. Estudos in vitro e in vivo [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2014.

Resumo

Diversos estudos têm sido conduzidos para se entender os efeitos da doença periodontal e do movimento ortodôntico nos tecidos periodontais de forma isolada; porém, os efeitos da presença concomitante de ambos os processos ainda não foram totalmente elucidados. O objetivo destes estudos in vitro e in vivo foi investigar as interações de sinais microbianos e biomecânicos em modelos que mimetizam a influência de forças mecânicas ortodônticas na progressão da doença periodontal e destruição tecidual. Nos estudos in vitro, as células do ligamento periodontal humano (hPDL) foram tratadas de acordo com o grupo experimental I: células sem nenhum estímulo; II: células estimuladas com a bactéria inativada *Fusobacterium nucleatum* ATCC 25586; III e IV: células submetidas à força mecânica tensional baixa (III) e alta (IV); V e VI: células estimuladas com *F. nucleatum* ATCC 25586 e submetidas à força mecânica tensional baixa (V) e alta (VI). O estudo in vivo foi composto por 144 ratos que foram divididos nos grupos: 1-controle (C), 2- doença periodontal experimental induzida por ligaduras (P), 3- movimento ortodôntico (OM), 4- P seguida de OM (OMP). A doença periodontal foi induzida 5 dias antes da colocação dos aparelhos ortodônticos e os períodos de sacrifício foram de 1, 3, 7 e 15 dias após indução do OM. Expressão de citocinas inflamatórias e seus receptores foi avaliada por PCR array e seis citocinas com maior expressão tiveram seus resultados confirmados por PCR em tempo real. Blocos de hemimaxilas foram utilizados para análise de perda óssea por microtomografia computadorizada. Os resultados dos estudos in vitro demonstraram que as células hPDL são capazes de produzir visfatina e que *F.*

nucleatum aumentou significantemente essa produção. Após estímulo microbiano e mecânico houve redução significante na expressão e síntese de visfatina e aumento significante na expressão e produção de COX2 e PGE2, além de aumento na razão RANKL/OPG por redução na expressão de OPG. No estudo in vivo foi observado que a força ortodôntica aplicada em situação de doença aumentou significantemente a expressão de mediadores inflamatórios nos tecidos gengivais de ratos, como: CCL2, CCR2, ITGAM, IL-1β, TNF-α e IL6. Além disso, foi constatada maior reabsorção do osso alveolar. Portanto, a força mecânica pode agravar a destruição dos tecidos periodontais durante a progressão da doença periodontal por meio da exacerbação do processo inflamatório, caracterizado pela maior produção de mediadores inflamatórios.

Palavras-chave: Doenças periodontais; movimentação dentária; perda óssea alveolar; inflamação.

ABSTRACT

Nogueira AVB. Contribution of biomechanical forces to periodontal destruction in vitro and in vivo [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2014.

Abstract

Several studies have been conducted to understand the effects of periodontal disease and orthodontic tooth movement on periodontal tissues, but the effects of both processes present together have not been fully elucidated. The aim of these in vitro and in vivo studies was to investigate the interactions of microbial and biomechanical signals in models that mimic the influence of orthodontic mechanical forces on the progression of periodontal disease and tissue destruction. In the in vitro study, human periodontal ligament cells (hPDL) were treated according to the following groups: Group I- cells without stimulation, II: cells stimulated with inactivated bacterium *Fusobacterium nucleatum* ATCC 25586, III and IV: cells subjected to cyclic tensile strain (CTS) of low (III) and high (IV) magnitudes, V and VI: cells stimulated with *F. nucleatum* ATCC 25586 and subjected CTS of low (V) and high (VI) magnitudes. The in vivo study was composed of 144 rats that were divided into four groups: 1- control group (C), 2- ligature-induced periodontal disease (P), 3- orthodontic movement (OM), and 4- P followed by OM (OMP). Periodontal disease was induced 5 days before orthodontic appliances placement and after 1, 3, 7, and 15 days of OM induction, animals were sacrificed. Expression of cytokines and their receptors was evaluated by PCR array, and the 6 most expressed inflammatory mediators had their results validated by real time PCR. Hemimaxillas blocks were used for bone volume fraction analysis by micro-computed tomography. The results of the in vitro study demonstrated that hPDL cells are able to produce visfatin and *F. nucleatum* significantly increased this production. After microbial and mechanical co-stimulation, there was a significant reduction in

the expression and synthesis of visfatin and significant increase in the expression and production of COX2 and PGE2. Furthermore, the ratio RANKL/OPG was also increased by reduction of OPG expression. In the in vivo study it was observed that the orthodontic force applied in situations of disease significantly increased the expression of inflammatory mediators in gingival tissues of rats, such as CCL2, CCR2, ITGAM, IL-1β, TNF-α, and IL-6. In addition, the OMP group presented significant alveolar bone loss at 15 days. Therefore, mechanical forces may aggravate periodontal tissue destruction during the progression of periodontal disease through the exacerbation of the inflammatory process characterized by increased production of inflammatory mediators.

Keywords: Periodontal diseases; tooth movement; alveolar bone loss; inflammation.

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1 INTRODUÇÃO

1 INTRODUÇÃO

Com o aumento da expectativa e qualidade de vida nas populações em geral, a procura por tratamento ortodôntico por adultos tem aumentado. Essa busca se deve principalmente devido à demanda estética e funcional dos pacientes. Porém, é cada vez mais frequente nos consultórios ortodônticos a presença de pacientes adultos com história de doença periodontal prévia ou mesmo com doença periodontal ativa, justificados pela alta prevalência das doenças periodontais em populações adultas, que ultrapassa os 50%, mesmo em países desenvolvidos[1, 33]. A periodontite é uma doença crônica inflamatória de origem bacteriana caracterizada pela destruição progressiva e irreversível dos tecidos de suporte dental[12]. Os periodontopatógenos e seus componentes bacterianos, como lipopolissacarídeo, peptidoglicanos, proteases, e as toxinas encontrados no biofilme dental induzem reação inflamatória estimulando as células do hospedeiro e as células residentes do periodonto a produzirem e liberarem mediadores inflamatórios. Estes induzem e mantêm uma resposta imunoinflamatória crônica no periodonto, levando à perda tecidual e, eventualmente, perda dentária[22].

O nível de várias citocinas inflamatórias encontra-se elevado no plasma sanguíneo e em sítios periodontalmente ativos, havendo uma redução após a realização do tratamento periodontal[11, 12, 32, 42]. Acredita-se atualmente que o dano tecidual que ocorre na periodontite está mais associado à essa exacerbada resposta imunoinflamatória do hospedeiro do que ao próprio efeito direto das bactérias[3, 41]. Com o tratamento da doença periodontal é possível restabelecer a saúde periodontal, porém há um comprometimento estético e funcional devido às sequelas periodontais como consequência da perda de tecido de suporte periodontal e da perda dentária. Dentre estas sequelas destaca-se a migração dentária patológica, que acarreta em enorme desconforto estético e funcional para o paciente. Sendo assim, torna-se imprescindível um plano de tratamento multidisciplinar para se alcançar resultados

estético e funcional satisfatórios, além de se recuperar e manter a saúde oral aumentando, dessa forma, o prognóstico em longo prazo da dentição.

A Ortodontia traz vários benefícios para o paciente periodontal, pois ao alinhar os dentes facilita a higienização bucal e, consequentemente, a manutenção da saúde periodontal; permite um melhor posicionamento radicular dentro do processo alveolar e, ao ajustar a oclusão, melhora a distribuição de forças sobre os dentes e restabelece a função do sistema estomatognático, além de resultar em um sorriso harmonioso e estético. O movimento ortodôntico ocorre em resposta à aplicação de força mecânica externa a qual gera um desequilíbrio das forças fisiológicas que mantêm o dente em sua posição normal. Para isso, uma sequência de eventos celulares, moleculares e reações teciduais se inicia. Esse processo é regulado por uma reação inflamatória aguda e asséptica caracterizada pela síntese e liberação local de diversos mediadores inflamatórios, destacando-se as citocinas inflamatórias, que induzem uma remodelação óssea coordenada consistindo na predominância de reabsorção óssea adjacente a áreas de compressão do ligamento periodontal (LP) e na predominância de neoformação óssea adjacente a áreas de tensão do LP, com subsequentes alterações adaptativas do LP, resultando no efeito clínico de movimento dentário[16, 17].

A literatura afirma que é possível realizar movimentação ortodôntica em adultos com periodonto reduzido, desde que saudável, sem que ocorra destruição periodontal adicional[6]. Os mediadores inflamatórios estão presentes durante a movimentação ortodôntica realizada com forças leves em sítios com ausência de doença periodontal, porém não há perda de inserção e/ou altura óssea alveolar adicionais, mesmo em pacientes com periodonto reduzido[2, 6]. Do mesmo modo, estudos experimentais histológicos demonstraram ausência de perdas periodontais adicionais após vários tipos de movimentos ortodônticos realizados em dentes com periodonto reduzido, com defeitos ósseos, porém com saúde tecidual[7, 25]. Além disso, Wennström et al.[38] (1987) demonstraram que as

forças ortodônticas não são capazes de acelerar a destruição periodontal em dentes com lesões periodontais supra-ósseas. No entanto, a destruição periodontal e a perda óssea são agravadas durante a movimentação ortodôntica de dentes afetados por doença periodontal e com lesões infra-ósseas[10, 31]. Em um estudo clínico foi observado que dentes com doença periodontal apresentam aumento na velocidade de perda de inserção quando movimentados[39]. Ademais, nosso grupo de pesquisa verificou, in vivo, que quando o movimento ortodôntico é introduzido em dentes com doença periodontal, maior perda óssea alveolar é detectada comparada aos demais grupos experimentais[5]. Nossos resultados também revelam maior expressão de interleucina-1β (IL-1β) e fator de necrose tumoral-α (TNF-α) nesses grupos amostrais, revelando a participação dessas citocinas na reabsorção óssea. Em geral, os estudos encontrados atualmente na literatura sugerem que, provavelmente, mais células que induzem o aumento na produção de mediadores são estimuladas e liberadas durante a movimentação ortodôntica de dentes comprometidos periodontalmente e que necessitam ser determinados por um método eficiente.

Atualmente, os estudos clínicos e experimentais in vivo proporcionam suporte científico para o planejamento ortodôntico em pacientes previamente acometidos por doença periodontal destrutiva[2, 6, 7, 25]. Entretanto, estudos que correlacionem a perda óssea alveolar com as alterações moleculares nos tecidos periodontais de dentes sob movimentação ortodôntica na presença de doença periodontal são escassos[5]. Da mesma forma, poucos estudos que avaliam as respostas das células periodontais frente a estímulos pró-inflamatórios, associados à ação de força mecânica são encontrados[23]. Mais estudos são necessários para se entender os efeitos regulatórios da doença periodontal e do movimento ortodôntico sobre os tecidos periodontais quando esses processos estão presentes simultaneamente. A confirmação das alterações das respostas celulares e moleculares dos tecidos periodontais pode levar a uma melhor compreensão dos mecanismos biológicos envolvidos na

movimentação dentária e doença periodontal e, diante disso, o desenvolvimento de um planejamento terapêutico seguro e objetivo.

Ainda dentro desse contexto, a presença de outras condições sistêmicas associadas ou não à periodontite deve ser investigada, com o objetivo de se realizar uma ortodontia segura no paciente adulto. Recentemente, a periodontite tem sido associada a certas condições e doenças sistêmicas, como doenças cardiovasculares, diabetes mellitus, artrite reumatóide, obesidade, síndrome metabólica, e outras, que atuam como fatores modificadores do processo imunoinflamatório através de mecanismos patogênicos de interação entre as doenças[30]. A inflamação sistêmica presente na obesidade, por exemplo, pode ser uma ligação patogênica entre a obesidade e outras doenças crônicas, como a periodontite[14]. Os tecidos adiposos produzem várias citocinas conhecidas como adipocinas, por exemplo, visfatina, leptina, resistina e adiponectina. Os níveis séricos dessas adipocinas encontram-se elevados em pacientes obesos e em pacientes apresentando outras condições ou doenças inflamatórias[8, 13]. Além disso, níveis elevados de visfatina tem sido detectado em fluido crevicular gengival e soro de pacientes com doença periodontal, sugerindo que a visfatina seja produzida no periodonto e seja regulada por processo inflamatório[26-28].

Portanto, nós hipotetizamos que a interação de fatores microbianos e biomecânicos em células e tecidos periodontais pode levar à progressão da doença periodontal e ao aumento da reabsorção óssea alveolar.

2 PROPOSIÇÃO

2 PROPOSIÇÃO

Objetivo geral: Avaliar a contribuição das forças biomecânicas na destruição periodontal, por meio da análise do efeito conjunto e isolado de estímulos mecânicos e inflamatórios sobre células e tecidos periodontais.

Objetivo específico 1 (Capítulo 1): O objetivo desse estudo *in vitro* foi avaliar se as células do ligamento periodontal de humanos (hPDL) produziam visfatina e se sim, se essa produção era regulada por estímulo mecânico através de força cíclica tensional e por estímulo inflamatório alcançado com o uso da bactéria inativada *Fusobacterium nucleatum* ATCC 25586 de forma isolada ou concomitante.

Objetivo específico 2 (Capítulo 2): O objetivo desse estudo *in vitro* foi avaliar a regulação de marcadores inflamatórios e de reabsorção óssea em hPDL frente aos estímulos mecânico através de força cíclica tensional e inflamatório através do uso de bactéria inativada *Fusobacterium nucleatum* ATCC 25586, de forma isolada ou concomitante.

Objetivo específico 3 (Capítulo 3): O objetivo do estudo *in vivo* foi avaliar o perfil de expressão de mediadores inflamatórios nos tecidos gengivais e a destruição dos tecidos periodontais de suporte de molares de ratos submetidos à movimentação ortodôntica associada ou não à indução de doença periodontal por meio de ligaduras.

3 MATERIAL E MÉTODO

3 MATERIAL E MÉTODO

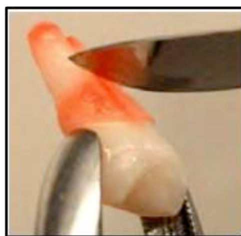
3.1 Estudo in vitro

Este estudo foi realizado durante o estágio de doutorado sanduíche no exterior (PDSE - CAPES) em colaboração com o Prof. Dr. James Deschner, da Universidade de Bonn, Bonn, Alemanha.

3.1.1 Cultura primária de fibroblastos do ligamento periodontal humano (hPDL)

Para o estudo in vitro foram utilizados fibroblastos do ligamento periodontal obtidos de 9 doadores entre 12-14 anos de idade. Previamente ao início dos experimentos foram obtidos o termo de consentimento livre e esclarecido e a aprovação pelo comitê de ética (Anexo 1). As células foram isoladas do terço médio das raízes de pré-molares extraídos por razões ortodônticas e utilizadas entre as passagens 3-5. Após extração os dentes foram lavados com PBS e o ligamento periodontal do terço médio das raízes foi raspado com o auxílio de lâminas de bisturi esterilizadas (Figura 1). As regiões apical e gengival do ligamento periodontal foram descartadas para eliminar contaminação com outros tipos celulares. O material raspado foi cultivado em meio de cultura DMEM suplementado com 10% SFB (soro fetal bovino) e 1% de penicilina/estreptomicina. A cultura celular foi mantida em atmosfera úmida a 37°C e 5% CO_2.

Figura 1 – Remoção de tecido periodontal para isolamento das células hPDL em pré-molar extraído por razões ortodônticas.



3.1.2 Desenho experimental

Células hPDL foram cultivadas em meio de cultura DMEM até atingirem 80% de confluência da área de cultivo. Previamente à colocação do estímulo mecânico e/ou bacteriano (24 horas antes) as células foram desinduzidas em meio suplementado com 1% de SFB, com o intuito de evitar a interferência de componentes do SFB na resposta das células ao tratamento. Para imitar infecção bacteriana *in vitro* as células foram estimuladas com o patógeno oral inativado *Fusobacterium nucleatum* ATCC 25586. A cepa foi pré-cultivada por 48 h em placas de agar Schaedler (Oxoid) em atmosfera anaeróbica. Depois a bactéria foi suspendida em PBS ($OD_{660nm} = 1$, equivalente a $1{,}2 \times 10^9$ bactérias/ml) e exposta duas vezes a ultrasonicação (160 W por 15 min) resultando em morte completa da bactéria através do rompimento da parede celular liberando LPS e outros componentes intracelulares. Para simular o estímulo mecânico, as células foram submetidas a deformações cíclicas tensionais usando um aparelho de tensão (CESTRA) desenvolvido e patenteado pela Universidade de Bonn. As células foram cultivadas em placas de cultura Bioflex revestidas com colágeno (*Flexcell International*, Hillsborough, NC, USA) em meio de cultura apropriado conforme descrito anteriormente. Força cíclica tensional (CTS) de baixa (CTSL, 3%) e alta (CTSH, 20%) magnitudes na frequência de 0.05 Hz foi aplicada às células. As células foram expostas ao *F. nucleatum* ATCC 25586, à CTS, e sua combinação por 1 e 3 dias, respectivamente. Após esses períodos de tratamento as células e o meio de cultura foram coletados. O software específico, *LabVIEW*, foi responsável por conduzir o aparelho e programar diferentes tipos de força (baixa e alta). Em resumo, as células de 9 doadores foram tratadas de acordo com o grupo experimental, conforme descrito abaixo:

- Grupo I: células sem nenhum estímulo;
- Grupo II: células estimuladas com a bactéria inativada *Fusobacterium nucleatum* ATCC 25586;
- Grupos III e IV: células submetidas à força mecânica tensional baixa (III) e alta (IV);

- Grupos V e VI: células estimuladas com a bactéria inativada *Fusobacterium nucleatum* ATCC 25586 e submetidas à força mecânica tensional baixa (V) e alta (VI).

3.1.3 Avaliação da expressão gênica por qPCR

RNA total foi extraído das células hPDL com o kit RNeasy Mini Kit (Qiagen) seguindo o protocolo do fabricante. A quantificação de RNA foi determinada através do espectrofotômetro *NanoDrop* ND-1000 (*Thermo Fisher Scientific*). O kit *iScriptTM Select cDNA Synthesis Kit* (Bio-Rad) foi utilizado para a transcrição reversa de 500 µg de RNA total a 42ºC por 90 min seguido de 85ºC por 5 min, de acordo com as instruções do fabricante. Expressão gênica de visfatina, COX-2, toll like receptor (TLR) 2 e 4, OPG, RANKL e GAPDH foi avaliada por PCR tempo real no aparelho *iCycler iQ* (Bio-Rad), usando *SYBR Green* (Qiagen) e primers específicos (*QuantiTect Primer Assay*, Qiagen). Um microlitro de cDNA foi amplificado em 25 µl de reação contendo 12,5 µl de 2x *QuantiFast SYBR Green PCR Master Mix* (Qiagen), 2,5 µl de primer e água RNase free. A mistura foi inicialmente aquecida a 95ºC por 5 min e seguida de 50 ciclos de desnaturação a 95ºC por 10 s e anelamento/extensão a 60ºC por 30 s. Os resultados foram analisados usando o método de comparação de *cycle threshold* (Ct).

3.1.4 Avaliação protéica por ELISA

Para o ELISA foram utilizados kits comercialmente disponíveis (*R&D Systems* e *HD Hölzel Diagnostika*) de acordo com as instruções dos fabricantes. Foram medidos os níveis das proteínas visfatina, PGE-2, RANKL e OPG nos sobrenadantes das amostras e COX2 no lisado celular. A absorbância foi determinada utilizando uma leitora de ELISA (*POWERWAVE X*; *BioTek Instruments*) a 450 nm com correção do comprimento de onda para 540 nm. Os dados foram normalizados pelo

número de células em cada poço para as amostras de sobrenadante e pela medida de proteína total utilizando o kit *Pierce BCA Protein Assay* (*Thermo Fisher Scientific*) para a amostra do lisado celular.

3.1.5 Imunofluorescência

As células aderidas ao fundo flexível das placas de cultura Bioflex foram fixadas com paraformaldeído 4% em PBS pH 7.4 (*Sigma-Aldrich*), por 10 min em temperatura ambiente (TA), lavadas com PBS, e tratadas com 0.1% Triton X-100 (*Sigma-Aldrich*) por 5 min em TA. Em seguida, as células foram lavadas novamente com PBS e bloqueadas com um tampão de bloqueio (*non fat dry milk*; Bio-Rad) por 1 h em TA. Após lavagem, as células foram incubadas com o anticorpo primário NFκB p65 (1:400; *Cell Signalling Technology*) por 90 min e com o anticorpo secundário CY3 (1:2000; Abcam) por 45 min. As células foram observadas na objetiva de 20x utilizando o microscópio *Axioplan* 2 (*Carl Zeiss MicroImaging*). As imagens foram capturadas com uma câmera PVCAM com o auxílio de um *software* de captura *VisiView* (*Visitron Systems*).

3.1.6 Imunocitoquímica

Células PDL foram cultivadas sobre lamínulas de vidro redondas (*coverslips*) em placa de 24 poços. As células foram estimuladas ou não com *F. nucleatum* ATCC 25586 e coradas para a presença de visfatina. Após 3 dias, as células foram fixadas com paraformaldeído 4% - pH 7.4 em TA por 15 min e, em seguida, permeabilizadas com 0.1% Triton X-100 por 5 min. Antígenos não específicos foram bloqueados através de incubação com *serum block* (*LSAB System*; *Santa Cruz Biotechnology*, Santa Cruz, CA, USA) por 20 min. Em seguida, as células foram incubadas *overnight* a 4ºC com anticorpo primário policlonal produzido em coelho para visfatina (1:200, *Santa Cruz Biotechnology*). Após,

células foram incubadas com anticorpo secundário anti-coelho, produzido em cabra IgG-HRP (*Cell Signalling Technology*) por 45 min. As células foram expostas ao cromógeno DAB (Dako, Hamburg, Germany) por 3 min em TA para coloração e lavadas 2x com PBS após cada incubação. Hematoxilina de Mayer (Merck Eurolab) foi utilizada como corante de contraste por 2 min. Lamínulas redondas de vidro foram montadas em lâminas de vidro utilizando o meio de montagem DePex (*Serva Electrophoresis*). Fotomicrografias padronizadas foram tiradas usando o microscópio *Axioplan* 2 (*Carl Zeiss MicroImaging*).

3.1.7 Análise Estatística

Análise estatística foi realizada através do *software* SPSS 20.0 (SPSS Inc.). Para análise quantitativa os valores foram expressos em forma de média e erro-padrão. O teste One-way Analysis of Variance foi seguido pelos testes de Dunnet ou de Tukey. Diferenças entre os grupos foram consideradas significantes quando $p<0.05$. Todos os experimentos foram realizados em triplicata.

3.2 Estudo in vivo

Este estudo foi realizado na Faculdade de Odontologia de Araraquara - UNESP, Araraquara, São Paulo.

3.2.1 Desenho experimental

O estudo foi aprovado pelo Comitê de Ética no Uso de Animal da Faculdade de Odontologia de Araraquara - UNESP (Anexo 2). Foram utilizados 144 ratos *Holtzman* adultos, com peso médio de 300 g, que foram mantidos no biotério da Faculdade de Odontologia de Araraquara – UNESP. Os animais foram acomodados em gaiolas de polipropileno e receberam alimentação granulada

(Labina/Purina) e água *ad libitum.* O número de animais utilizados foi determinado a partir dos períodos experimentais propostos, considerando 09 animais em cada grupo, por período experimental. Sendo assim, os 144 animais foram distribuídos em 4 grupos experimentais seguindo os períodos de sacrifício de 1, 3, 7 e 15 dias:

- grupo C (36 animais): grupo controle positivo sem nenhuma intervenção (ortodôntica ou periodontal);
- grupo OM (36 animais): grupo submetido ao movimento ortodôntico e ausência de doença periodontal;
- grupo P (36 animais): grupo submetido à indução de doença periodontal experimental e ausência de movimento ortodôntico;
- grupo OMP (36 animais): grupo submetido à indução de doença periodontal experimental e movimento ortodôntico.

3.2.2 Indução de doença periodontal experimental

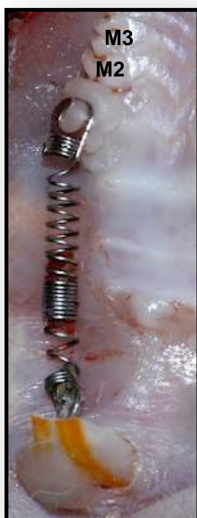
Neste estudo foi utilizado o modelo de indução de doença periodontal por ligadura. Inicialmente os animais foram submetidos à anestesia geral (0,08 mL de 10% ketamina e 0,04 mL de 2% cloridrato de xilasina por 100g de peso corporal) intramuscular na pata posterior e foram posicionados em mesa operatória. O modelo de ligadura foi obtido com a colocação de um fio de algodão ao redor dos primeiros molares superiores bilateralmente.

3.2.3 Instalação de aparelho ortodôntico

Foi utilizado um modelo de movimento dentário em ratos e um tipo de aparelho previamente estabelecidos[5], porém com algumas modificações (Figura 2). Após os animais receberem anestesia

geral, uma mola fechada de níquel-titânio (Sentalloy®, GAC, Dentsply), foi conectada entre o primeiro molar superior e incisivo central superior. Para colocação da mola nos incisivos centrais superiores, sulcos nas superfícies mesial, distal e vestibular desses dentes foram preparados para prevenir que o fio de amarrilho (CrNi, 0.20 mm, código 55.01.208, Morelli) se soltasse devido à curvatura lingual e à erupção contínua dos incisivos. Após os fios serem amarrados, uma fina camada de resina composta foi colocada acima do fio para prevenir seu deslocamento e irritação pulpar devido à exposição dentinária. Já para colocação da mola nos primeiros molares superiores, resina composta foi colocada na superfície oclusal e a mola foi fixada sobre esta. A mola de níquel-titânio foi utilizada para promover uma liberação de força constante de 0.25 N durante o curso do experimento. Além disso, para se evitar interferências oclusais, os primeiros molares inferiores foram extraídos.

Figura 2 - Vista oclusal do aparelho ortodôntico colocado entre o primeiro molar e incisivo central superiores.



3.2.4 Delineamento experimental e sacrifício dos animais

Após 1, 3, 7 e 15 dias do baseline (momento zero) do experimento, 09 animais de cada grupo foram sacrificados por overdose anestésica. A seleção destes períodos considerou a representação dos períodos precoces do desenvolvimento da resposta imune-inflamatória nos tecidos periodontais. Para os animais dos grupos com doença periodontal, a colocação das ligaduras para indução da doença ocorreu 5 dias antes do baseline. A instalação do aparelho ortodôntico e início da movimentação ocorreram no baseline. Após o sacrifício, a maxila de cada animal foi removida e separada em duas hemimaxilas para posterior redução e distribuição aleatória para realização de microtomografia computadorizada e extração de RNA total. Os tecidos coletados das peças destinadas à extração de RNA foram imediatamente congelados em nitrogênio líquido e posteriormente armazenados em freezer -80 °C.

3.2.5 *Array* de citocinas em formato RT-qPCR

Utilizamos os *arrays* focados para a detecção de RNAm de 84 diferentes genes envolvidos na expressão de citocinas inflamatórias e seus receptores (*RT2 ProfilerTM PCR Array Rat Inflammatory Cytokines & Receptors*, *SABiosciences*, Qiagen). O RNA total dos tecidos gengivais (colar gengival removido com o auxílio de lâmina bisturi 15C) coletados foi extraído com o kit *RNeasy Mini* (catálogo no 74106, Qiagen), segundo as instruções do fabricante. Este kit se baseia no método de colunas de purificação e inclui tratamento com DNase (*RNase-Free DNase Set* - catálogo no 79254) de modo a eliminar contaminação com DNA genômico, que pode afetar os resultados da reação de PCR em tempo real. A quantidade e pureza do RNA foram determinadas em espectrofotômetro de luz *NanoVue UV/Visible Spectrophotometer* (*GE Healthcare*, Piscataway, NJ) por meio da avaliação das absorbâncias a 260 nm e da relação entre as absorbâncias a 260/280 nm, respectivamente. A integridade do RNA foi verificada, previamente à realização da reação de transcriptase reversa, por

meio da eletroforese de 1 μg do RNA em gel de formaldeído/agarose e verificação das bandas correspondentes às porções 18S e 28S do RNA ribossômico na proporção 1:2, respectivamente. A reação de transcriptase reversa para conversão de RNA de cada amostra em cDNA, foi realizada utilizando o kit recomendado pelo fabricante do *array* (*RT^2 First Strand cDNA kit*, *SABiosciences*, Qiagen). A reação de PCR em tempo real foi feita utilizando o equipamento *Step One Plus* (*Applied Biosystems*) nas condições de ciclagem otimizadas pelo fabricante (10 min à 95°C para desnaturação inicial seguido de 40 ciclos de 15 segundos à 95°C e 1 min à 60°C). Os valores *cycle threshold* (Ct) de cada poço foram calculados pelo software da termocicladora, exportados para uma planilha de dados fornecida pelo fabricante do *array* (http://sabiosciences.com/pcrarraydataanalysis.php) e analisados pelo método DDCt. Os 6 genes que apresentaram maior expressão tiveram seus resultados validados por meio da técnica do RT-qPCR.

3.2.6 qPCR

O RNA total foi extraído conforme descrito no ítem anterior (3.2.5). DNA complementar foi sintetizado pela transcrição reversa de 500 ng do total de RNA dos tecidos gengivais, seguindo o protocolo do fabricante (*High-Capacity cDNA Reverse Transcription Kit*, *Applied Biosystems*). A reação de PCR foi realizada através do termociclador *Step One Plus Real-Time PCR System* (*Applied Biosystems*). 1 μL do produto da reação de RT foi utilizado em um volume total de reação de PCR de 10 μL. Este volume incluiu, além do produto da reação de RT, água livre de nucleases, *TaqMan Fast Advanced Master Mix* e *TaqMan Gene Expression Assays* (*Applied Biosystems*) para os genes alvo de rato. As condições pré-otimizadas de ciclagem utilizadas foram: 50°C por 2 minutos, 95°C por 20 segundos, 95°C por 1 segundo e 50 ciclos de 60°C por 20 segundos. A expressão gênica tanto dos

genes-alvo quanto do gene constitutivo GAPDH foi quantificada a partir do ΔCT. A expressão do gene alvo foi normalizada para a expressão do gene GAPDH.

3.2.7 Microtomografia computadorizada (micro-CT)

Após o sacrifício dos animais, 6 hemimaxilas de ratos por grupo foram removidas e fixadas em paraformaldeído 4% por 48 horas, e mantidas em álcool 70% em temperatura ambiente até serem escaneadas com o auxílio do microtomógrafo computadorizado (μCT Skyscan 1176, Aartselaar, Kontich, Bélgica). O gerador de raios-x foi operado a 50 kVp, com corrente do feixe em 500 μA, filtro de alumínio de 0.5 mm e com uma resolução de imagem de 12.45 μm. As imagens foram reconstruídas com o auxílio de um software específico (NRecon 1.6.1.5 – Skyscan, Kontich, Bélgica) em todas as três dimensões espaciais, em seguida, todas as imagens das hemimaxilas foram orientadas e salvas em cortes coronais (2000 x 1336) utilizando o *Data Viewer software* (*Data Viewer* 1.4.3.1 - Skyscan, Kontich, Bélgica). Para medir o volume de osso alveolar (BV) e o volume tecidual (TV) a região de interesse (ROI) foi delimitada do ápice das raízes à crista alveolar, sem as raízes e o espaço periodontal, compreendendo assim todo o osso alveolar, usando uma ferramenta específica (*custom processing*) do *software* CTan (CT Analyser 1.10.1.0 - Skyscan, Kontich, Bélgica). O parâmetro arquitetural avaliado foi volume ósseo sobre volume tecidual (BV/TV %) que representa o volume de tecido mineralizado ao redor do defeito.

3.2.8 Análise estatística

A análise estatística foi realizada utilizando o *software GraphPad Prism* 5.0 (*GraphPad Software Inc*., San Diego, CA, USA). Para análise de PCR array, os valores de p foram calculados utilizando os

valores Delta Ct das replicatas para cada gene no grupo controle e nos grupos experimentais e o teste estatístico *Student's t-test*. Para análise de qPCR e reabsorção óssea, a análise estatística foi feita por meio da análise de variância one-way ANOVA, seguido de comparação entre os grupos com o teste de Tukey. O nível de significância adotado foi de 95% ($p < 0.05$).

4 CAPÍTULO 1[*]

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Regulation of Visfatin by Microbial and Biomechanical Signals in PDL Cells

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Abstract

Objectives This in vitro study was established to examine whether visfatin thought to be a link between periodontitis and obesity is produced by periodontal ligament (PDL) cells and, if so, whether its synthesis is modulated by microbial and/or biomechanical signals.

Materials and methods PDL cells seeded on BioFlex® plates were exposed to the oral pathogen Fusobacterium nucleatum ATCC 25586 and/or subjected to biomechanicalstrain for up to 3 days. Gene expression of visfatin and toll- like receptors (TLR) 2 and 4 was analyzed by RT-PCR, visfatin protein synthesis by ELISA and immunocytochemis- try, and NFκB nuclear translocation by immunofluorescence.

Results F. nucleatum upregulated the visfatin expression in a dose- and time-dependent fashion. Preincubation with neutralizing antibodies against TLR2 and TLR4 caused a significant inhibition of the F. nucleatum-upregulated visfa- tin expression at 1 day. F. nucleatum stimulated the NFκB nuclear translocation. Biomechanical loading reduced the stimulatory effects of F. nucleatum on visfatin expression at 1 and 3 days and also abrogated the F. nucleatum-induced NFκB nuclear translocation at 60 min. Biomechanical load- ing inhibited significantly the expression of TLR2 and TLR4 at 3 days. The regulatory effects of F. nucleatum and/or biomechanical loading on visfatin expression were also observed at protein level.

Conclusions PDL cells produce visfatin, and this produc- tion is enhanced by F. nucleatum. Biomechanical loading seems to be protective against the effects of F. nucleatum on visfatin expression.

Clinical relevance Visfatin produced by periodontal tissues could play a major role in the pathogenesis of periodontitis and the interactions with obesity and other systemic diseases.

Keywords Biomechanics . Tension . Forces . Fusobacterium nucleatum . Visfatin . Periodontal ligament.

Introduction

Periodontitis is a chronic inflammatory disease caused by oral pathogenic microorganisms present in dental plaque. These periodontopathogens as well as their components and metabolic products can elicit local immunoinflammatory reactions in periodontal tissues. As a consequence of the exaggerated inflammatory processes and immune responses, periodontal soft and hard tissues are subjected to degradation and resorption, respectively, which can result in periodontal attachment and even tooth loss [1, 2]. Moreover, periodontitis has been shown to be associated with cardiovascular diseases, diabetes mellitus, rheumatoid arthritis, obesity, metabolic syndrome, and other systemic diseases and conditions [3].

The periodontal tissues are periodically exposed to biomechanical loading during mastication, speech, and dental habits. Since the periodontal ligament (PDL) represents a richly vascular and cellular connective tissue, it can absorb and distribute physiological forces, which are at the same time critical for remodeling and maintenance of the periodontium [4, 5]. However, dental overloading due to occlusal discrepancies, dental habits, or strong orthodontic forces can be harmful to periodontal tissues and contribute to progressive periodontal destruction [6–8]. It is well known from in vitro and clinical studies, that biomechanical loading can induce the synthesis of inflammatory mediators and pro- teases, thereby acting as proinflammatory and catabolic signals [9, 10]. For example, excessive mechanical stress due to hyperocclusion has been demonstrated to stimulate osteoclastogenesis and alveolar bone destruction [11]. In summary, both bacterial infection and biomechanical loading can cause immunoinflammatory reactions and, thereby, destructive processes in the periodontium. However, if and how microbial infection and biomechanical loading interact on the level of periodontal cells has yet to be elucidated.

Recently, an association between periodontal diseases and obesity has been reported [12]. Systemic low-grade inflammation present in obesity may be a pathomechanistic link between obesity and other chronic diseases, such as periodontitis [13]. In the adipose tissue, a number of cyto- kines, such as visfatin, leptin, resistin, and adiponectin, are produced and released [14]. These adipokines not only regulate insulin sensitivity and energy expenditure, but also proinflammatory and wound healing processes. Whereas adiponectin exerts anti-inflammatory effects, visfatin, leptin, and resistin

possess proinflammatory characteristics [15, 16]. Interestingly, the serum levels of these adipokines are altered in obesity and overweight [15, 16]. It is thought that adipokines not only contribute to the subclinical inflammatory state in obesity, but that such molecules also represent the pathomechanistic link between type 2 diabetes, obesity, and periodontitis [17].

Visfatin is predominantly secreted by cells from human visceral adipose tissue and was originally denominated as pre-B cell colony enhancing factor. This protein has also an enzymatic nicotinamide phosphoribosyltransferase activity responsible for the synthesis of nicotinamide adenine dinucleotide, which is essential for cell metabolism. Visfatin induces the synthesis of proinflammatory cytokines and acts as a chemotactic factor [18–20]. High serum levels of visfatin have been found in a number of inflammatory diseases and conditions, such as obesity, type 2 diabetes, metabolic syndrome, atherosclerosis, cancer, rheumatoid arthritis, and sepsis [21–27]. Recently, it has been demonstrated that visfatin is present in high levels in gingival crevicular fluid (GCF) and serum from periodontally diseased patients, indicating that visfatin might also be produced in the periodontium and regulated by bacterial infection and/or inflammation [28–30]. However, it is as yet unknown whether PDL cells produce this proinflammatory adipokine, and if so, whether its synthesis is regulated by oral bacteria and biomechanical loading. Hence, the aim of this study was to examine in vitro whether visfatin is produced in PDL cells and, if so, whether its synthesis is modulated by microbial and/or biomechanical signals.

Materials and methods

Culture and treatment of cells

PDL cells from periodontally healthy donors, who under- went tooth extraction for orthodontic reasons, were used. Written informed parental consent and approval of the Ethics Committee of the University of Bonn were obtained. Cells from nine donors were collected from the middle third of the tooth roots and cultured in Dulbecco’s Modified Eagle’s Medium (Invitrogen, Karlsruhe, Germany) supplemented with 10 % fetal bovine serum (FBS, Invitrogen), 100 units of penicillin and 100 µg/ml of streptomycin (both from Biochrom, Berlin, Germany) at 37 °C in a humidified atmosphere of 5 % CO2. Cells of passages 3–5 were seeded (1 × 105 cells/well) on six-well BioFlex® collagen-coated

culture plates (Flexcell International, Hillsborough, NC, USA) and grown to 80 % confluence. One day prior to experiments, the FBS concentration was reduced to 1 %. The medium was changed every other day during the course of the experiment. In order to mimic bacterial infection in vitro, cells were stimulated with the inactivated oral pathogen Fusobacterium nucleatum ATCC 25586 (optical density 0.025, 0.05, and 0.1). The strain was precultivated 48 h on Schaedler agar plates (Oxoid, Basingstoke, UK) in an anaerobic atmosphere. Thereafter, bacteria were suspended in phosphate-buffered saline (PBS) (OD660nm = 1, equivalent to 1.2 × 109 bacterial cells/ml) and exposed two times to ultra- sonication (160 W for 15 min) resulting in a complete killing. Moreover, biomechanical loading conditions were simulated in vitro by application of cyclic tensile strain (CTS) of low (CTSL, 3 %) and high (CTSH, 20 %) magnitudes at a rate of 0.05 Hz to cells. Like in previous experiments, a strain device (CESTRA) developed at the University of Bonn was used to apply biomechanical loading [31–34]. In the present study, cells were exposed to F. nucleatum ATCC 25586, CTS, and their combination for 1 and 3 days, respectively.

Real-time RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qia- gen, Hilden, Germany) following manufacturer's protocol. By using the iScriptTM Select cDNA Synthesis Kit (Bio-Rad, Munich, Germany), 500 μg of total RNA was reverse tran- scribed at 42 °C for 90 min followed by 85 °C for 5 min, following manufacturer's instructions. Gene expression of visfatin, toll-like receptor (TLR) 2 and 4, and glyceraldehyde-3-phosphate dehydrogenase as housekeeping gene was analyzed by real-time RT-PCR using the iCycler iQ detection system (Bio-Rad), SYBR Green (Qiagen), and specific primers (QuantiTect Primer Assay, Qiagen). One microliter of cDNA was amplified as a tem- plate in a 25-μl reaction mixture containing 12.5 μl of 2x QuantiFast SYBR Green PCR Master Mix (Qiagen), 2.5 μl of primers and RNase free water. The mixture was heated initially at 95 °C for 5 min and then followed by 50 cycles of denaturation at 95 °C for 10 s and combined annealing/ex- tension at 60 °C for 30 s. Data were analyzed using the comparative threshold cycle method.

ELISA

Protein levels of visfatin in cell supernatants were measured using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The absorbance was determined using a micro- titer plate reader (POWERWAVE X; BioTek Instruments,

Winooski, VT, USA) at 450 nm. Data were normalized by the cell number.

Immunofluorescence

Cells attached to the flexible bottom of the culture plates were fixed with 4 % paraformaldehyde in PBS pH7.4 (Sig- ma-Aldrich, Munich, Germany) for 10 min at room temperature (RT), washed with PBS, and treated with 0.1 % Triton X-100 (Sigma-Aldrich) for 5 min at RT. Then cells were washed again with PBS and blocked with a blocking buffer (nonfat dry milk; Bio-Rad) for 1 h at RT. After washing, the cells were incubated with primary rabbit antibody NFκB p65 (1:400; Cell Signaling Technology, Danvers, MA, USA) for 90 min and with secondary antibody CY3 (1:2,000; Abcam, Cambridge, MA, USA) for 45 min. Cells were observed under a × 20 objective using an Axioplan 2 imaging microscope (Carl Zeiss MicroImaging, Jena, Germany). The images were captured with a PVCAM cam- era and the VisiView capturing software (Visitron Systems, Puchheim, Germany).

Immunocytochemistry

Cells were cultured on glass coverslips in a 24-well plate. Cells were stimulated or not with F. nucleatum ATCC 25586 and stained for the presence of visfatin. After 3 days, cells were fixed in 4 % paraformaldehyde at pH7.4 and RT for 15 min and then permeabilized in 0.1 % Triton X-100 for 5 min. Nonspecific antigens were blocked by incubation with serum block (LSAB System; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 20 min. Subsequently, cells were incubated overnight at 4 °C with rabbit polyclonal antibody to visfatin (Santa Cruz Biotechnology). Afterwards, cells were labeled with goat anti-rabbit IgG-HRP secondary antibody (Cell Signaling Technology) for 45 min. For staining, cells were exposed to DAB chromogen (Dako, Hamburg, Germany) for 3 min at RT. After each incubation step, cells were washed twice with PBS. Counterstaining was performed with Mayer's Hematoxylin (Merck Eurolab, Dietikon, Switzerland) for 2 min. Cover- slips were mounted in DePex mounting medium (Serva Electrophoresis, Heidelberg, Germany). Standardized photomicrographs were taken using an Axioplan 2 imaging microscope (Carl Zeiss MicroImaging).

Statistical analysis

Statistical analysis of the data was performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). For quantitative analysis, values were expressed as mean and standard errors of the mean. One-way analysis of variance followed by the post hoc Dunnett's or Tukey's tests was applied.

Differences between groups were considered significant at $p < 0.05$. All experiments were performed in triplicate and repeated at least twice.

Results

Time- and dose-dependent stimulation of visfatin production by F. nucleatum ATCC 25586

First, we sought to examine whether PDL cells produce visfatin and, if so, whether this production is regulated by bacteria. Our experiments revealed that visfatin was constitutively expressed by PDL cells and upregulated by F. nucleatum ATCC 25586 (Fig. 1a,b). As shown in Fig. 1a, F. nucleatum ATCC 25586 had no significant effect on visfatin mRNA expression up to 12 h, but it stimulated significantly the visfatin expression at 24 h (3.8-fold; $p<0.05$) and 72 h (3.9-fold; $p<0.05$). Furthermore, the F. nucleatum-induced increase in visfatin expression was dose-dependent. At 24 h, low concentration of F. nucleatum ATCC 25586 enhanced the visfatin expression by 3.8-fold ($p < 0.05$), whereas higher concentrations caused a more than 6-fold increase in its expression ($p<0.05$) (Fig. 1b). The stimulatory effect of F. nucleatum ATCC 25586 on visfatin was also observed at protein level by immunocytochemistry and ELISA (Fig. 1c and Table 1).

Regulation of F. nucleatum-induced effects on visfatin by biomechanical loading

Next, we studied whether the response of PDL cells to F. nucleatum ATCC 25586 is modulated by biomechanical loading, because the periodontium is often subjected simultaneously to microbial infection and biomechanical, i.e., occlusal, loading. Biomechanical loading alone slightly decreased the constitutive visfatin mRNA expression in PDL cells at 1 and 3 days, but these effects were not significant (Fig. 1d and e). However, biomechanical loading modulated the actions of F. nucleatum ATCC 25586 on visfatin in PDL cells. CTSL abrogated the stimulatory effect of F. nucleatum ATCC 25586 on visfatin by 17 % ($p>0.05$) and by 70 % ($p < 0.05$) at 1 and 3 days, respectively. CTSH reduced the F. nucleatum-induced stimulation of visfatin expression by 86 % ($p < 0.05$) at 1 day and completely blocked ($p < 0.05$) the visfatin upregulation at 3 days (Fig. 1d,e). The counter- regulatory effects of CTSL and CTSH were also observed at protein level, as analyzed by ELISA (Table 1).

Involvement of TLRs in the actions of F. nucleatum ATCC 25586 on visfatin

We then sought to unravel the mechanisms underlying the stimulatory effect of F. nucleatum ATCC

25586 on visfatin expression. PDL cells were preincubated with neutralizing antibodies against TLR2 and TLR4 and, subsequently, stimulated with F. nucleatum ATCC 25586. Our experiments revealed that preincubation with antibodies against TLR2 and TLR4 caused a significant ($p<0.05$) inhibition of the F. nucleatum-induced visfatin stimulation by 20 and by 24 %, respectively, at 1 day (Fig. 2a).

Exploitation of the NFkB pathway by F. nucleatum ATCC 25586

Upon ligand binding, TLRs can trigger the NFκB signaling pathway. We therefore examined whether F. nucleatum ATCC 25586 activates this pathway in PDL cells. As evidenced by immunofluorescence microscopy, F. nucleatum ATCC 25586 stimulated the nuclear translocation of NFκB and caused a maximal NFκB accumulation within the nucleus at 60 and 90 min (Fig. 2b). Then, we examined whether this F. nucleatum-stimulated NFκB transactivation is regulated by biomechanical loading. As demonstrated in Fig. 2c, CTSL and CTSH remarkably inhibited the F. nucleatum-induced NFκB nuclear translocation at 60 min.

Regulation of TLRs by biomechanical loading

We then examined whether the inhibitory effects of biomechanical loading on F. nucleatum ATCC 25586 actions might be caused by downregulation of TLRs. Although CTSL had no regulatory effect on TLR2 at 1 day, CTSH reduced significantly the constitutive TLR2 mRNA expression by 35 % ($p<0.05$) at this time point (Fig. 2d). At 3 days, the TLR2 expression was significantly inhibited by 12 % ($p<0.05$) by CTSL and by 69 % ($p<0.05$) by CTSH (Fig. 2d). The TLR4 expression was not significantly affected by biomechanical loading at 1 day (Fig. 2e). At 3 days, the expression of TLR4 was inhibited by 12 % ($p < 0.05$) by CTSL and by 52 % ($p<0.05$) by CTSH (Fig. 2e).

Discussion

This study shows for the first time that the proinflammatory adipokine visfatin is produced by PDL cells. In addition, visfatin was upregulated by the oral pathogen F. nucleatum ATCC 25586, and this increase was significantly counterregulated by biomechanical loading. These findings suggest that visfatin may be produced at sites of periodontal infection with F. nucleatum ATCC 25586 and, thereby, contributes to periodontal inflammation and destruction. By contrast, biomechanical loading of the PDL might be at least partially protective against the stimulatory effects of F. nucleatum ATCC 25586 on visfatin.

Recently, the role of visfatin in inflammatory diseases and conditions has been widely investigated. Plasma visfatin levels have been found to be increased in patients with obesity, metabolic syndrome, diabetes mellitus, cardiovascular diseases, and insulin resistance [21–27]. Furthermore, it has also been demonstrated that body-weight reduction by physical exercise can lead to decreased visfatin levels [35]. In recent years, evidence has accumulated that obese individuals have an increased risk of periodontitis, and it is thought that adipokines might represent the pathomechanistic link between both diseases [12, 17]. Visfatin has been shown to stimulate a variety of cells to produce inflammatory mediators and proteases by using different intracellular pathways [36]. Moreover, this adipokine can inhibit apopto- sis of inflammatory cells [37, 38]. However, if and how visfatin affects periodontal cells is as yet unclear. Interestingly, Pradeep and coworkers have recently reported in- creased visfatin levels in GCF from gingivitis and periodontitis patients, as compared to periodontally healthy individuals [28–30]. Furthermore, periodontal treatment caused a decrease in visfatin levels [28-30]. These reports suggest that visfatin might also be produced in the periodontium and that its synthesis is enhanced by periodontal infection. Our study demonstrates for the first time that PDL cells produce visfatin and, thereby, suggests that PDL cells may contribute to the increased visfatin levels in GCF in periodontitis. However, if and to what extent other immunoinflammatory or structural cells of the periodontium pro- duce visfatin has yet to be elucidated.

In the present study, F. nucleatum ATCC 25586, which was used to mimic a bacterial infection, increased the visfatin production in a dose- and time-dependent manner. F. nucleatum is a gram-negative, anaerobic microorganism, acts as a bridge bacterium between early and late colonizers during plaque development, and is associated with both gingivitis and periodontitis [39, 40]. In vitro studies have shown that F. nucleatum ATCC 25586 can invade epithelial cells, fibroblasts, and PDL cells [41–43]. In addition, F. nucleatum ATCC 25586 supports other periodontal pathogens to invade host cells [44]. Nevertheless, periodontitis is caused by a complex bacterial biofilm. Further studies should clarify whether other microorganisms associated with periodontitis are also capable of stimulating the synthesis of visfatin.

Although biomechanical loading alone had only minor effects on the visfatin production in PDL cells, they inhibited the F. nucleatum-induced upregulation of visfatin in a magnitude-dependent manner. These findings indicate that biomechanical loading, for example, caused by occlusal loading

or orthodontic treatment may not necessarily augment the stimulatory action of F. nucleatum ATCC 25586 on visfatin. At least for the strain regimens used in our experiments, biomechanical loading had a protective role against the actions of F. nucleatum ATCC 25586 on this proinflammatory adipokine. Our results concur with previous reports, which have also demonstrated that biomechanical loading can exert anti-inflammatory effects [31, 45, 46].

Our study revealed that one of the pathways by which F. nucleatum ATCC 25586 upregulates visfatin are TLR2 and TLR4. Interestingly, biomechanical loading decreased time- and magnitude-dependently the expression of hese TLRs, suggesting that the counterregulatory effects of CTS on the action of F. nucleatum ATCC 25586 might be mediated at least in part by downregulation of these receptors, especially at 3 days. TLRs are strong activators of the NFκB pathway [47]. F. nucleatum LPS was shown to be a strong ligand for TLR2 and a weak ligand for TLR4 for activation of NFκB [48]. Interestingly, we could observe that CTS inhibited the NFκB nuclear translocation stimulated by F. nucleatum TCC 25586, indicating that both F. nucleatum ATCC 25586 and CTS exploit this pathway for their opposite effects.

In our experiments, a suspension of F. nucleatum ATCC 25586 was used. Since this suspension was exposed to intensive ultrasonication, it can be assumed that the suspension contained disrupted cell wall particles with a high amount of LPS. However, other bacterial components also present in the suspension may interact with the receptors. The F. nucleatum ATCC 25586 concentration used in this study was determined by dose–response experiments. A low F. nucleatum concentration, as can be found in subgingival plaque, was chosen for the subsequent experiments, because this concentration exerted a pronounced stimulatory effect on visfatin, while also allowing to study the modulatory effects of biomechanical loading on the F. nucleatum-stimulated visfatin synthesis [49].

Like in previous experiments, tensile forces were applied to the cells [31–34]. However, during mastication, dental habits, and orthodontic treatment, the tooth-supporting periodontal tissues are subjected to complex forces. Whether compressive, hydrostatic, and shear forces as well as their combinations exert similar effects, as observed for tensile strain in the present study, needs to be examined. As in our previous investigations, cells were exposed to biomechanical loading of low and high magnitudes, which have been shown to occur in the periodontium [50, 51].

In summary, the present study demonstrates for the first time that visfatin is produced by PDL cells. Moreover, the oral pathogen F. nucleatum ATCC 25586 caused an upregulation of visfatin, whereas biomechanical loading counterregulated the F. nucleatum-induced stimulation of this adipokine. Within the limits of this study, we conclude that visfatin may be produced by the PDL in the presence of periodontal infection with F. nucleatum ATCC 25586. In addition, biomechanical loading of the PDL might be at least partially protective against the F. nucleatum-induced stimulation of visfatin synthesis.

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

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TABLES

Table 1 Visfatin protein levels in supernatants of PDL cells treated with *F. nucleatum* ATCC 25586 either alone or in combination with low (CTSL) and high (CTSH) biomechanical loading

Groups	**Visfatin Protein Synthesis (ng/10^5 cells)**	
	Day 1	**Day 3**
Control	213.44 ± 1.10	143.34 ± 1.68
F. nucleatum	1030.22 ± 9.40*	354.83 ± 3.22*
***F. nucleatum* + CTSL**	725.28 ± 6.50**	253.05 ± 2.34**
***F. nucleatum* + CTSH**	613.35 ± 5.30**	132.18 ± 1.39**

* p<0.05 (significantly different from control); **p<0.05 (significantly different from control and *F. nucleatum*-stimulated cells)

FIGURES

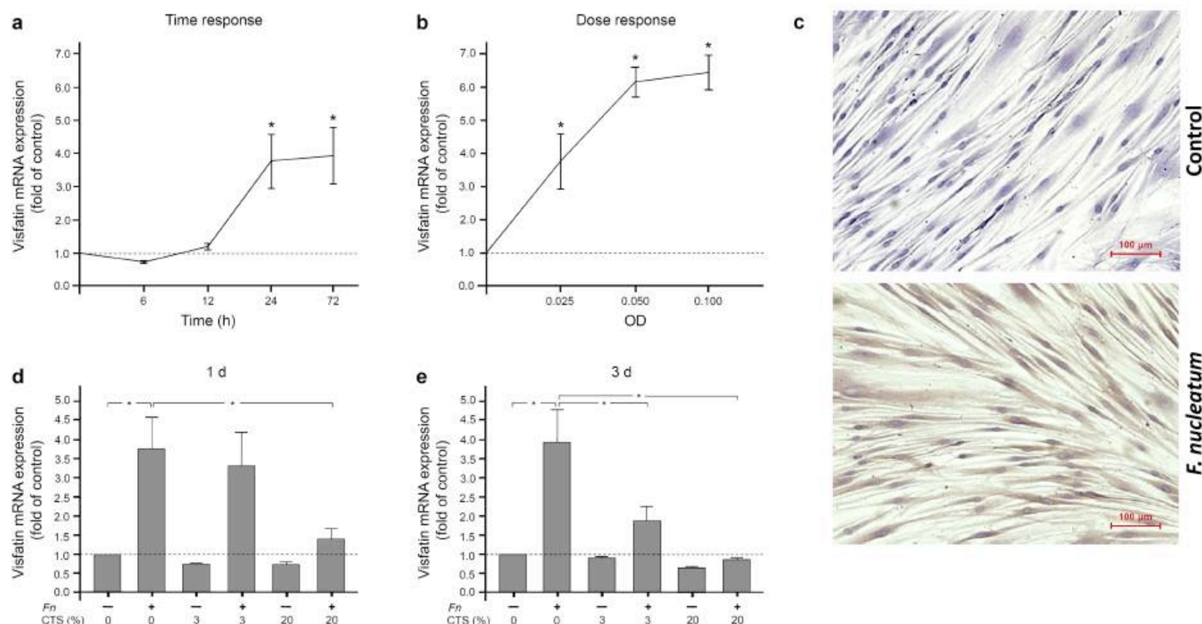

Fig. 1 **a** Stimulation of visfatin expression by *F. nucleatum* ATCC 25586 (OD: 0.025) in PDL cells over time. *: significantly ($p<0.05$) different from unstimulated cells (n=9); **b** Stimulation of visfatin expression by various concentrations of *F. nucleatum* ATCC 25586 in PDL cells at 24 h. *: significantly ($p<0.05$) different from unstimulated cells (n=9); **c** Visfatin protein synthesis in PDL cells in the presence and absence of *F. nucleatum* ATCC 25586 (OD: 0.025) at 3 days, as analyzed by immunocytochemistry. Images from one representative experiment are shown; **d** and **e** Regulation of visfatin expression in the presence of *F. nucleatum* ATCC 25586 (*Fn*; OD: 0.025) and/or biomechanical loading (CTS) of low (3%) and high (20%) magnitudes in PDL cells at 1 (**d**) and 3 days (**e**). *: significant ($p<0.05$) difference between groups (n=9).

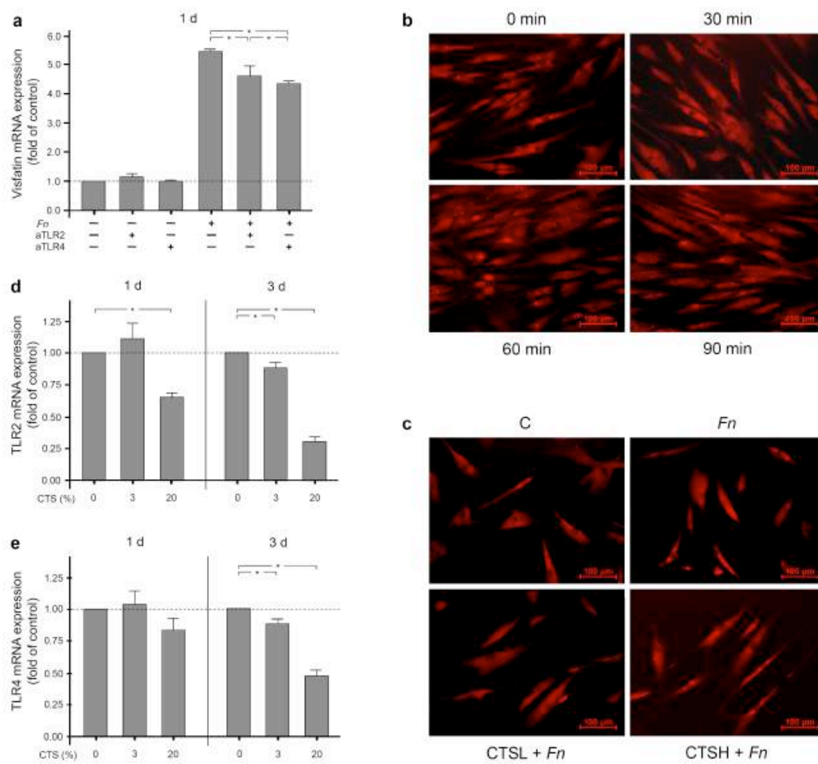

Fig. 2 a Stimulation of visfatin expression by *F. nucleatum* ATCC 25586 (OD: 0.025) in the presence and absence of blocking TLR2 or TRL4 antibodies in PDL cells at 1 day. Unstimulated PDL cells in the presence and absence of blocking antibodies served as controls. *: significant ($p<0.05$) difference between groups (n=9); **b** Stimulation of NFκB nuclear translocation by *F. nucleatum* ATCC 25586 (OD: 0.025) over time, as analyzed by immunofluorescence. Images from one representative experiment are shown; **c** Inhibition of *F. nucleatum* (*Fn*; OD: 0.025)-stimulated p65 nuclear translocation by biomechanical loading of low (CTSL; 3%) and high (CTSH; 20%) magnitudes in PDL cells at 60 min. Images from one representative experiment are shown; **d** and **e** Expression of TLR2 (**d**) and TLR4 (**e**) in PDL cells subjected to biomechanical loading of low (3%) and high (20%) magnitudes at 1 and 3 days. *: significant ($p<0.05$) difference between groups (n=9).

5 CAPÍTULO 2*

*Artigo publicado na revista *Mediators of Inflammation*

Biomechanical loading modulates proinflammatory and bone resorptive mediators in bacterial-stimulated PDL cells

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ABSTRACT

The present study aimed to evaluate *in vitro* whether biomechanical loading modulates proinflammatory and bone remodeling mediators production by periodontal ligament (PDL) cells in the presence of bacterial challenge. Cells were seeded on BioFlex® culture plates and exposed to *Fusobacterium nucleatum* ATCC 25586 and/or cyclic tensile strain (CTS) of low (CTSL) and high (CTSH) magnitudes for 1 and 3 days. Synthesis of cyclooxygenase-2 (COX2) and prostaglandin E2 (PGE2) was evaluated by ELISA. Gene expression and protein secretion of osteoprotegerin (OPG) and receptor activator of nuclear factor kappa-B ligand (RANKL) were evaluated by quantitative RT-PCR and ELISA, respectively. *F. nucleatum* increased the production of COX2 and PGE2, which was further increased by CTS. *F. nucleatum*-induced increase of PGE2 synthesis was significantly ($p<0.05$) increased when CTSH was applied at 1 and 3 days. In addition, CTSH inhibited the *F. nucleatum*-induced upregulation of OPG at 1 and 3 days, thereby increasing the RANKL/OPG ratio. OPG and RANKL mRNA results correlated with the protein results. In summary, our findings provide original evidence that CTS can enhance bacterial-induced syntheses of molecules associated with inflammation and bone resorption by PDL cells. Therefore, biomechanical, such as orthodontic or occlusal, loading may enhance the bacterial-induced inflammation and destruction in periodontitis.

Keywords: COX2; OPG; RANKL; periodontal diseases; biomechanics; *Fusobacterium nucleatum*; periodontal ligament.

1. Introduction

Periodontitis is characterized by a pathological process triggered by the host response against pathogenic bacteria present in the dental biofilm. It is a chronic inflammatory disease that affects the periodontium resulting in tissue destruction and even in the loss of the dental organ [1]. Host immune response against this infection leads to the production of inflammatory mediators. Cytokines such as interleukin-1β and tumor necrosis factor-α are the primary mediators responsible for stimulating the production of secondary mediators like chemokines and cyclooxygenases (COX) [2]. These inflammatory molecules activate osteoclasts and induce bone resorption as a result of an exacerbated host response. Thus, understanding the regulation of proinflammatory mediators and their effects in periodontal tissues have been the objective of many studies [2-6].

Besides pathological conditions, orthodontic tooth movement also stimulates those biological mediators in response to therapeutic mechanical forces. Tooth displacement occurs as a result of periodontal tissues remodeling process, which is predominantly characterized by bone resorption and bone formation on pressure and tension sides, respectively. PDL cells are constantly subjected to several types of mechanical forces, such as compression, tension, shear stress. In addition, they are considered as mechanoresponsive cells that mediate the response of the connective tissue to mechanical loading [7]. During orthodontic movement, several proinflammatory mediators are synthesized and released, especially cytokines and prostaglandins, playing an important role in bone remodeling. However, these molecules may also interfere with the underlying disease bacterial-induced inflammatory process and exacerbate periodontitis [8]. Although some studies [8-10] have been conducted to understand the effects of concomitant periodontal disease and orthodontic movement on periodontal tissues, the interactions between periodontitis and biomechanical loading are as yet not well established.

Among the many molecules involved in the inflammatory process, COX is highly expressed during both periodontal disease and orthodontic tooth movement. After several types of stimuli the membrane phospholipids of some cells release arachidonic acid, which is catalyzed by COX into prostanoids like prostaglandins and thromboxane. Two isoforms of COX are described, COX1 and COX2. COX1 is constitutively expressed in many tissues and is required to maintain organ and tissue homeostasis. In contrast, COX2 expression is induced by proinflammatory cytokines and lipopolysaccharide. COX2 is responsible for prostaglandin E2 (PGE2) production [11], which has an important role in the pathogenesis of periodontal diseases. High levels of PGE2 are detected in the gingiva and gingival crevicular fluid of patients with periodontal disease [12-15], acting as inflammatory mediator. Also, PGE2 has been associated with bone resorption during the progression of periodontal diseases [14, 16] by stimulating and activating osteoclast production [17], and by upregulating receptor activator of nuclear factor kappa-B (RANK) ligand (RANKL) expression [18]. Moreover, blocking endogenous PGE2 production with indomethacin has shown to significantly inhibit the increase of osteoclasts by LPS-induced COX2 [19]. In addition to the role in the disease process, some studies have demonstrated high expression of COX2 and/or PGE2 in periodontal ligament (PDL) cells after *in vitro* mechanical loading [20-25], and high PGE2 level in gingival crevicular fluid during orthodontic movement at both compression and tension sides [26], suggesting its participation in bone remodeling process [7]. This role of PGE2 in bone remodeling can be confirmed due to the increase in orthodontic tooth movement achieved after PGE2 administration [27-30]. Furthermore, COX2/PGE2 were demonstrated to be responsible for RANKL upregulation in PDL cells under mechanical stress *in vitro* [21].

RANKL is a key molecule in osteoclast differentiation and activation. Increased level of this molecule is detected in periodontal disease and orthodontic tooth movement [21, 31]. RANKL effects

are counteracted by osteoprotegerin (OPG) and the balance between them regulates bone resorption [32]. The level of OPG in periodontitis seems to be lower when compared to that in healthy patients [31], but OPG regulation during mechanical stress is still uncertain [33, 34]. Some reports suggest that OPG expression remains unchanged while other reports suggest that its expression is upregulated in PDL cells subjected to biomechanical loading [21, 34, 35]. The fact is that RANKL and OPG are involved in PDL and bone remodeling.

However, no previous study has evaluated the expression of those important molecules COX2/PGE2 and RANKL/OPG when both conditions, bacterial challenge and mechanical force, are concomitantly present. Thus, the aim of the present study was to evaluate *in vitro* whether biomechanical loading modulates bacterial regulation of proinflammatory and bone remodeling mediators in PDL cells.

2. Materials and Methods

2.1 Cell culture

The experiment was approved by the Ethics Committee of the University of Bonn and informed parental consent was obtained. Human periodontal ligament (hPDL) fibroblasts were used. Cells were obtained from six periodontally healthy donors, who underwent tooth extraction for orthodontic reasons. Cells were derived from the middle third of the tooth roots and maintained in DMEM (Dulbecco´s Modified Eagle´s Medium, Invitrogen, Karlsruhe, Germany) supplemented with 10% FBS (fetal bovine serum, Invitrogen), 100 units of penicillin and 100 µg/mL of streptomycin (Biochrom, Berlin, Germany) at 37ºC in a humidified atmosphere of 5% CO_2. Cells were seeded between passages 3-5 (1 x 10^5 cells/well) on 6-well BioFlex® collagen-coated culture plates (Flexcell International, Hillsborough, NC, USA) and grown to 80% confluence. FBS concentration was

reduced to 1% 24 hours prior to experiments in order to avoid interference from its components and to synchronize the cell cycle.

2.2 Cell stimulation

Cells were stimulated with the inactivated oral pathogen *Fusobacterium nucleatum* ATCC 25586 (OD_{660nm}: 0.025, 0.05, and 0.1) in order to mimic cell-microbial interactions *in vitro*. In an anaerobic atmosphere, bacteria strain was pre-cultivated for 48 hours on Schaedler agar plates (Oxoid, Basingstoke, United Kingdom). Subsequently, bacteria were suspended in PBS (OD_{660nm} = 1, equivalent to 1.2 x 10^9 bacterial cells/ml) and subjected twice to ultrasonication (160 W for 15 min). Different OD concentrations were used in the first experiment to evaluate the dose response of PDL cells stimulated with *F. nucleatum* ATCC 25586. Afterwards, the lowest concentration capable of upregulating COX2 was chosen (OD 0.025) and used in the subsequent experiments. As in previous experiments, a strain device (CESTRA) developed at the University of Bonn was used to apply biomechanical forces to cells [36-38]. In addition to bacterial challenge, biomechanical forces were simulated by the application of cyclic tensile strain (CTS) of low (CTSL, 3%) and high (CTSH, 20%) magnitudes at a rate of 0.05 Hz. PDL fibroblasts were exposed to *F. nucleatum* ATCC 25586, CTS, and to their combinations for 1 and 3 days. Moreover, cells were stimulated with *F. nucleatum* ATCC 25586 in the presence and absence of 10 µg/mL blocking antibodies against TLR2 (mouse anti-human TLR2 mAb TL2.1, 16-9917-82, eBioscience, San Diego, CA, USA) and TLR4 (mouse anti-human TLR4 HTA125, 16-9917-82, eBioscience) for 1 day. Cell viability of treated and control cells was > 95%.

2.3 Quantitative RT-PCR

Total RNA extraction was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer´s protocol. RNA concentration was determined by NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA) spectrophotometer. 500 ng of total RNA was reverse transcribed using the iScript^{TM} Select cDNA Synthesis Kit (Bio-Rad, Munich, Germany) at 42ºC for 90 min followed by 85ºC for 5 min, following manufacturer´s instructions. Using the iCycler iQ detection system (Bio-Rad), SYBR Green (Qiagen) and specific primers (QuantiTect Primer Assay, Qiagen), gene expression of glyceraldehyde- 3-phosphate dehydrogenase (GAPDH), COX2, OPG, and RANKL was evaluated by quantitative RT-PCR. One microliter of cDNA was amplified as a template in a 25 µl reaction mixture containing 12.5 µl of 2x QuantiFast SYBR Green PCR Master Mix (Qiagen), 2.5 µl of primers and RNase free water. The PCR mixture was heated initially at 95ºC for 5 min and then followed by 50 cycles of denaturation at 95ºC for 10 s and combined annealing/extension at 60ºC for 30 s. This analysis was performed in triplicate. Data were analyzed using the comparative threshold cycle method.

2.4 ELISA

Commercially available ELISA kits (DYC4198-2, DY805, DY626, R&D Systems Europe, Abingdon, United Kingdom, and HZ-5203, Hölzel Diagnostika, Cologne, Germany) were used for ELISA analyses according to the manufacturer´s instructions to measure the levels of COX2 in cell lysates and of OPG, soluble RANKL, and PGE2 in cell supernatants. Using a microtiter plate reader (POWERWAVE X; BioTek Instruments, Winooski, VT, USA) the absorbance was determined at 450 nm with wavelength correction at 540 nm. COX2 data were normalized by total protein measured

using Pierce BCA Protein Assay Kit (23227, Thermo Scientific, Pierce Biotechnology, Rockford, USA), while OPG, RANKL, and PGE2 data were normalized by the numbers of cells in the wells.

2.5 Statistical analysis

Statistical analysis of the data was performed with GraphPad Prism 5 software (GraphPad Software Inc., San Diego, USA) using mean ± standard deviation. One-way analysis of variance test (ANOVA) followed by the Dunnett´s or Tukey´s post-hoc tests were used to determine the presence of significant differences among experimental groups. Significant differences were considered when $p < 0.05$. All experiments were performed in triplicate and repeated at least twice.

3. Results

3.1 Stimulation of COX2 and OPG expressions by *F. nucleatum* ATCC 25586

COX2 and OPG were constitutively produced by PDL cells. To mimic an inflammatory environment, cells were stimulated with *F. nucleatum* ATCC 25586 leading to a significant ($p<0.05$) upregulation of the COX2 and OPG mRNA expression in a time-dependent manner. As shown in Figure 1A and C, *F. nucleatum* ATCC 25586 had no significant effect on COX2 and OPG mRNA expression up to 12 hours, but at 24 hours it significantly ($p<0.05$) increased the COX2 and OPG expressions. *F. nucleatum* ATCC 25586 enhanced significantly ($p<0.05$) the mRNA expression of COX2 in a dose-dependent manner, whereas OPG was not influenced by the varying concentrations of bacteria at 24 hours (Figures 1B and D).

3.2 Stimulation of COX2 expression by *F. nucleatum* ATCC 25586 via TLRs

In order to analyze whether *F. nucleatum* ATCC 25586 uses TLRs to upregulate COX2, cells were stimulated with *F. nucleatum* ATCC 25586 in the presence and absence of blocking antibodies against TLR2 and TLR4 for 24 hours. The results demonstrated that COX2 mRNA expression was decreased after treating PDL cells with anti-TLRs (Figure 1E). The inhibition of the *F. nucleatum*-induced COX2 expression occurred for both anti-TLRs being more evident and significant ($p<0.05$) for anti-TLR4.

3.3 Regulation of *F. nucleatum*-stimulated COX2 and PGE2 syntheses by CTS

In order to study whether biomechanical loading modulates the *F. nucleatum*-induced effects on COX2 and PGE2, CTS was applied to PDL fibroblasts. As evidenced by ELISA, CTSL and CTSH alone had only small effects on COX2 and PGE2 syntheses. However, biomechanical loading modulated the effects of *F. nucleatum* ATCC 25586 on COX2 and PGE2 in PDL cells. When PDL cells were concomitantly stimulated with CTS and *F. nucleatum* ATCC 25586, CTS tended to increase the *F. nucleatum*-induced effects on COX2 production (Figure 2A). Furthermore, CTS aggravated the *F. nucleatum*-induced effects on the PGE2 production (Figures 2B and C). As compared to CTSL, CTSH caused a more pronounced and significant increase in the *F. nucleatum*-induced PGE production ($p<0.05$) at 1 and 3 days (Figures 2B and C).

3.4 Regulation of *F. nucleatum*-induced RANKL and OPG expressions by CTS

Next, we investigated the expression of RANKL and OPG in PDL cells challenged with *F. nucleatum* ATCC 25586 in the presence and absence of biomechanical loading. *F. nucleatum* ATCC 25586 tended to increase the RANKL expression at 1 day but not at 3 days and a significant ($p<0.05$), albeit

discrete upregulation of the OPG expression after both 1 and 3 days (Figures 3A-D). Whereas CTSL had no effects on RANKL and OPG expressions (data not shown), CTSH caused a significant ($p<0.05$) upregulation of the *F. nucleatum*-induced RANKL expression at 3 days and a significant ($p<0.05$) downregulation of the *F. nucleatum*-induced OPG expression at 1 and 3 days (Figures 3A-D). Furthermore, CTSH increased significantly the RANKL/OPG ratio in the presence and absence of *F. nucleatum* ATCC 25586 at both time points (Figures 3E and F). As shown in Figures 4A-F, the effects of biomechanical strain were also observed at protein level, as analyzed by ELISA.

4. Discussion

Many attempts have been made to study the effects of inflammatory and/or mechanical stimulation on PDL cells [37-40]. Occasionally the results are ambiguous especially due to differences in methodology such as culture conditions, type of inflammatory induction, type of mechanical apparatus, and type of strain regime. Our study aimed to investigate *in vitro* whether biomechanical loading would modulate bacterial regulation of important proinflammatory and bone remodeling mediators in PDL cells. The main finding of this study was that a CTS stimulus enhanced the *F. nucleatum*-induced increase of COX2 and PGE2. The association of CTSH and *F. nucleatum* ATCC 25586 resulted in an RANKL/OPG ratio significantly higher compared to *F. nucleatum* ATCC 25586 alone.

F. nucleatum ATCC 25586 upregulates COX2 via TLRs signaling in PDL cells, especially by TLR4 due to the fact that preincubation with TLR4 antibody resulted in significant inhibition of the *F. nucleatum*-induced COX2 stimulation. Also, *F. nucleatum* is probably using other pathways for signaling in PDL cells as our data from the TLR inhibition experiment showed only partial inhibition of COX2 expression after blocking the TLRs 2 and 4. In addition, *F. nucleatum* was previously

shown to stimulate TLR2 and 4 expressions in PDL cells [41]. Mechanical force driven by CTS to PDL cells increased COX2 production as previously demonstrated [11, 37]. When PDL cells were subjected to both stimuli, bacterial and mechanical force, COX2 production showed a tendency to be higher than the effect of each stimulus alone, and PGE2 expression and production were significantly increased in this situation. Thus, mechanical force increased the effect of *F. nucleatum* ATCC 25586 on COX2-PGE2 production exhibiting a proinflammatory effect.

Studies have reported that PGE2 mediates bone resorption through the activation of osteoclasts and RANKL in response to mechanical stress *in vitro* [11] and *in vivo* [25]. Due to this fact, we have decided to investigate whether RANKL and OPG expression and production would be modulated by *F. nucleatum* ATCC 25586 and CTS in PDL cells. *F. nucleatum* ATCC 25586 induced the expression and synthesis of RANKL and OPG in PDL cells at both time points, 1 and 3 days. CTSH in the presence of *F. nucleatum* ATCC 25586 inhibited the expression and synthesis of OPG while it stimulated the increase in RANKL. As a result, the RANKL/OPG ratio was low for the groups stimulated by *F. nucleatum* ATCC 25586 alone and high for the other two groups, CTSH alone and CTSH associated with *F.nucleatum* ATCC 25586.

In our study, CTS aggravated *F. nucleatum*-induced increase in the production of COX2 and PGE2, and reduced the expression and production of OPG, leading to an increase in RANKL/OPG ratio in this group compared to *F. nucleatum* ATCC 25586 alone. These results suggest an exacerbated proinflammatory and bone resorptive effect of CTS in the presence of bacteria. Our results corroborate in part with other previous studies in that biomechanical loading can exert proinflammatory effects under inflammatory conditions in PDL cells [37, 40, 42]. All of these studies applied CTS of low and high strains in cells treated with IL-1β. Some of them reported that high strain was proinflammatory and enhanced the IL-1β-induced production of inflammatory mediators

[40, 42], while other study reported a proinflammatory effect for both strains only at 1 day [37]. Long et al. [40] have detected an anti-inflammatory effect of CTSL and Nokhbehsaim et al. [37] found this same effect when cells were subjected to long-term application of CTS. In addition to the proinflammatory effect of biomechanical loading, mechanical stress driven by hydrostatic pressure has shown to intensify the production of proinflammatory cytokines in PDL cells stimulated with periodontopathogenic bacteria [39]. The contradiction in the results exists especially due to differences in experimental features, for example, the type of inflammatory mediator that is being evaluated, and the type of force that is being used.

Some studies reported that PDL cells express RANKL in response to mechanical stress [21, 35] while other studies reported PDL cells do not express RANKL [33] or express low levels of RANKL [34]. Regarding to the expression of OPG, downregulation was detected in response to CTS in PDL cells [33]. Corroborating with this study, our results also revealed downregulation of OPG in response to CTS and, in addition, an inhibitory effect of CTS on *F. nucleatum*-induced increase in OPG expression and protein synthesis. On the other hand, in another study, PDL cells subjected to CTS have expressed high levels of OPG and this effect was inhibited after concomitant stimulation with LPS [34]. Although contradictions can be observed in the literature regarding OPG expression after CTS stimulus in all studies, RANKL/OPG ratio increased when both CTS and inflammatory challenge were associated, suggesting that those conditions together have a bone resorptive effect.

In addition, when osteoblasts are cultured with inflammatory conditioned medium and subjected to CTS, an upregulation of c-fos and reduction of osteogenicity was observed [43]. C-fos is a transcription factor important for the activation of genes involved in osteoclastogenesis [44]. This study shows that CTS in combination with inflammation is impairing the osteogenic capacity of osteoblasts. Although we have not evaluated c-fos, our *in vitro* study demonstrates a proinflammatory

and bone resorptive (increase in the RANKL/OPG ratio) effect of CTS when associated with *F. nucleatum* ATCC 25586.

Periodontitis has a polymicrobial nature as it is originated from a complex bacterial biofilm. In order to mimic an inflammatory infection *in vitro*, *F. nucleatum* ATCC 25586, which is a gram-negative and anaerobic microorganism associated with both gingivitis and periodontitis, was used. In our study, *F. nucleatum* ATCC 25586 upregulated COX2, PGE2, and OPG syntheses in PDL cells. *F. nucleatum* ATCC 25586 is considered as a bridge bacterium because it is located in the middle of the subgingival biofilm inducing the adhesion of late colonizers during plaque development by coaggregation [45, 46]. *F. nucleatum* ATCC 25586 can invade some cells and help other periodontopathogens to invade host cells [47, 48]. However, more studies are necessary to evaluate whether other microorganisms associated with periodontal diseases are also able to activate the production of the mediators we have evaluated in the present study. Another limitation of this study is that it did not examine the involvement of COX2-PGE2 on the RANKL/OPG ratio when cells were concomitantly stimulated by biomechanical loading and bacteria. This could be shown by blocking COX2 with indomethacin, for example. However, a recent study demonstrated that COX2 inhibition with celecoxib resulted in RANKL downregulation and osteoclastogenesis reduction in PDL cells stimulated with heat-inactivated bacteria [49].

The sRANKL ELISA kit used in the present study can be interfered by OPG, according to the manufacterer's datasheet. In our samples, the OPG concentration was higher than 156 pg/ml, so that interference cannot be completely excluded in our experiments. However, this ELISA kit is frequently used by other investigators and it is difficult to avoid OPG in the samples.

The periodontium is constantly subjected to complex biomechanical forces such as mastication, orthodontic tooth movement, and functional occlusal habits. In the present study, we

investigated the interactions of biomechanical forces and inflammatory signals in PDL cells. Our results revealed that biomechanical loading enhanced the *F. nucleatum*-induced upregulation of COX2 and PGE2 production and the RANKL/OPG ratio in PDL cells. These findings indicate that biomechanical loading can aggravate the destructive effects of inflammation on periodontal tissues during periodontitis. The strain regimens used in our experiments showed that biomechanical loading has a proinflammatory effect favoring the actions of *F. nucleatum* ATCC 25586. Previous studies corroborate with our results [37, 40, 42].

In summary, our findings provide original evidence that CTS can enhance the synthesis of molecules associated with inflammation and bone resorption. Therefore, biomechanical, such as orthodontic or occlusal, loading, may enhance the bacterial-induced inflammation and destruction in periodontitis.

Conflict of Interest

The authors declare that there are no conflicts of interest in this study.

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Figure Legends

Figure 1 – A - COX2 expression in PDL cells stimulated by *F. nucleatum* ATCC 25586 over time. B - COX2 expression in PDL cells stimulated by various concentrations of *F. nucleatum* ATCC 25586 at 1 day. C - OPG expression in PDL cells stimulated by *F. nucleatum* ATCC 25586 over time. D - OPG expression in PDL cells stimulated by various concentrations of *F. nucleatum* ATCC 25586 at 1 day. E - COX2 expression in PDL cells stimulated by *F. nucleatum* ATCC 25586 (OD 0.025) in the presence or not of anti-TLR2 or anti-TLR4 antibodies at 1 day. *Significant difference between groups ($p < 0.05$).

Figure 2 – A - Synthesis of COX2 in lysates of PDL cells treated with *F. nucleatum* ATCC 25586 and/or cyclic tensile strain (CTS) of low (CTSL, 3%) and high (CTSH, 20%) magnitudes at 36 hours. B and C - Production of PGE2 in supernatants of PDL cells treated with *F. nucleatum* ATCC 25586 and/or CTSL and CTSH at 1 (B) and 3 (C) days. *Significant difference compared to other groups ($p < 0.05$).

Figure 3 – A and B - RANKL expression in PDL cells stimulated by *F. nucleatum* ATCC 25586 and/or CTSH at 1 (A) and 3 (B) days. C and D - OPG expression in PDL cells stimulated by *F. nucleatum* ATCC 25586 and/or CTSH at 1 (C) and 3 (D) days. E and F - RANKL/OPG mRNA ratio in PDL cells stimulated by *F. nucleatum* ATCC 25586 and/or CTSH at 1 (E) and 3 (F) days. *Significant difference compared to other groups ($p < 0.05$), ■Significant difference compared to control ($p < 0.05$), # Significant difference ($p < 0.05$).

Figure 4 - A and B - Synthesis of RANKL protein in supernatants of PDL cells stimulated by *F. nucleatum* ATCC 25586 and/or CTSH at 1 (A) and 3 (B) days. C and D - Synthesis of OPG protein in supernatants of PDL cells stimulated by *F. nucleatum* ATCC 25586 and/or CTSH at 1 (C) and 3 (D) days. E and F - RANKL/OPG protein ratio in supernatants of PDL cells stimulated by F. nucleatum ATCC 25586 and/or CTSH at 1 (E) and 3 (F) days. *Significant difference compared to other groups ($p < 0.05$), ■Significant difference compared to control ($p < 0.05$), # Significant difference ($p < 0.05$).

Figures

Figure 1

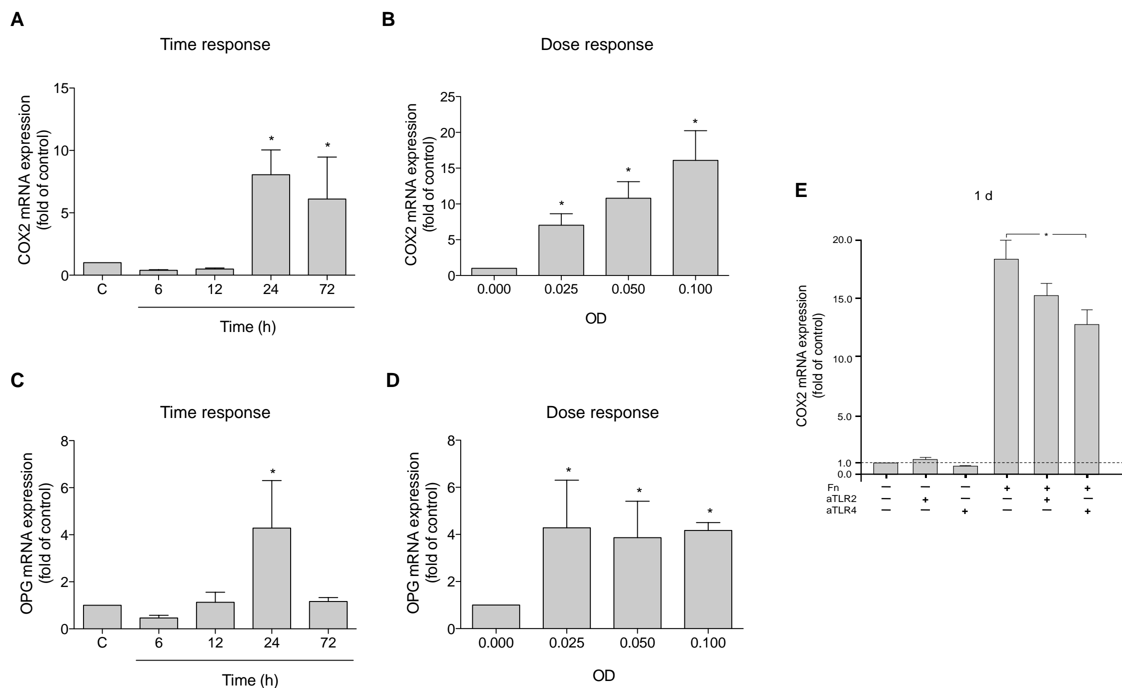


Figure 2

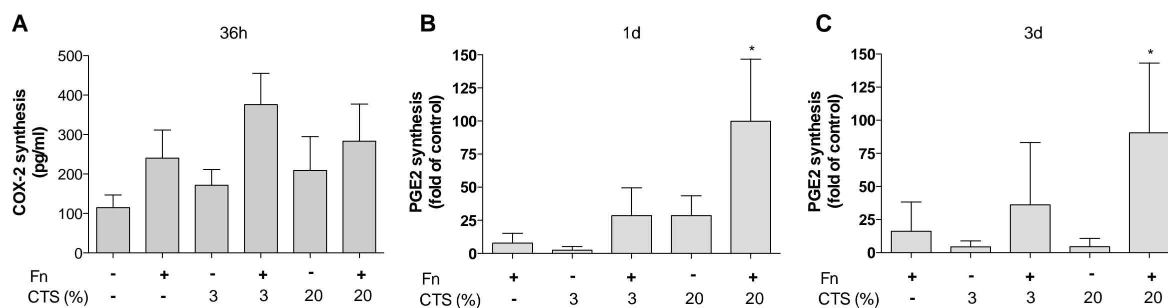


Figure 3

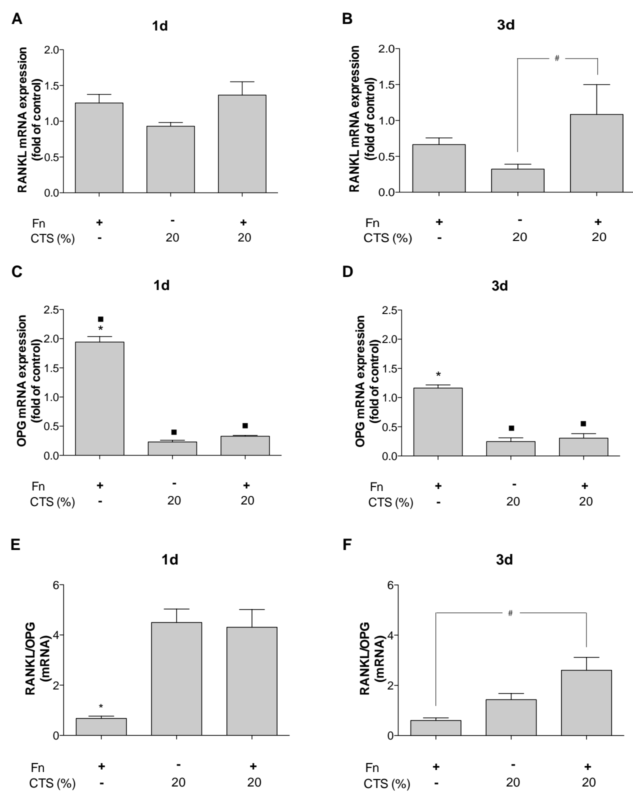
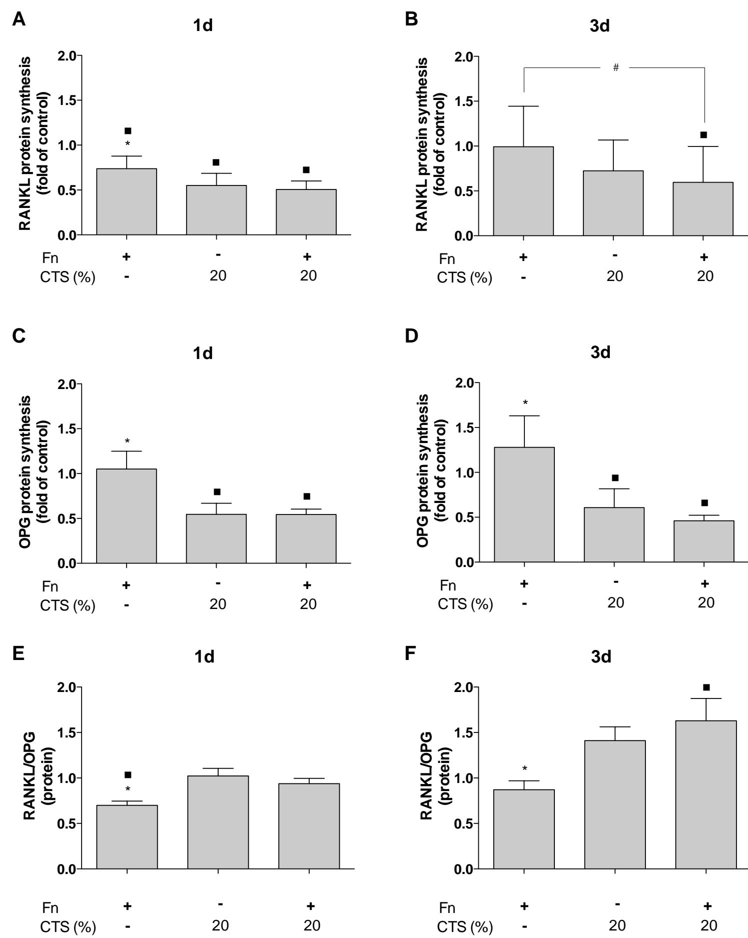


Figure 4



6 CAPÍTULO 3[*]

[*]De acordo com o manual da FOAr/UNESP, adaptadas das normas Vancouver. Disponível no site: http://www.foar.unesp.br/#!/biblioteca/manual

Co-regulation of inflammation and bone resorption by orthodontic forces and periodontal disease

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Keywords: tooth movement, periodontal diseases, proinflammatory mediators, alveolar bone loss, cytokines, chemokines

Abstract

The aim of the present study was to evaluate the inflammatory profile of the periodontal tissues in response to biomechanical forces and periodontal inflammation, correlating it with the alveolar bone resorption levels. A total of 144 male adult *Holtzman* rats were randomly assigned into four experimental groups: negative control (C), ligature-induced periodontal disease (P), orthodontic movement (OM), P followed by OM (OMP). After 1, 3, 7, and 15 days of the orthodontic movement start, 9 animals from each experimental group were sacrificed. Bone volume fraction (BVF) was measured using micro-computed tomography. Gene expression profile of cytokines and receptors of inflammation was evaluated by PCR array assay and the 6 most expressed genes had their expression validated by real time PCR. The OMP group exhibited continuous and significant ($p < 0.05$) alveolar bone loss compared to C and OM since day 1, but it was only at 15 days that the OMP group presented a relevant effect on bone loss compared to all the other groups as demonstrated by a significant ($p<0.05$) decrease in BVF. PCR array analysis revealed the induction of several inflammatory mediators and receptors. The 6 most upregulated genes found in the OMP group compared to the C group were: CCL2, CCR2, IL-1β, IL-6R, Itgam, and TNF-α. In summary, mechanical loading modulates the response of periodontal tissues to periodontal disease through the increased expression of proinflammatory mediators, which might be involved in the alveolar bone resorption. Thus, this study suggests that orthodontic forces may aggravate the periodontal breakdown in periodontitis by the enhancement of periodontal inflammation.

Introduction

Lately, the aging population is expanding worldwide and so is the prevalence of periodontal diseases[1, 35]. Furthermore, the search for orthodontic treatment has increased among adult people especially due to esthetic and functional reasons, leading the orthodontists to often deal with an increased number of patients affected by moderate to advanced periodontal disease[18]. In such cases, a multidisciplinary approach involving periodontics and orthodontics has shown to be the best option of treatment in order to restore and maintain health, esthetic, and function of the dentition. Additionally, orthodontic therapy has demonstrated to benefit oral health-related quality of life by reducing psychological discomfort and disability in response to the esthetic improvement after severe malocclusion treatment[34]. However, some adverse effects on the periodontium of those patients have been associated to the orthodontic treatment, such as: increased periodontal support destruction induced by an association of periodontal disease and hyperocclusal forces[14, 40], and changes in the subgingival microbiological environment favored by orthodontic devices, resulting in an exacerbation of periodontal disease activity and pocket depth[20, 33]. On the other hand, the mechanisms of how biomechanical loading affects periodontal disease progression are still unclear.

Periodontitis is a chronic inflammatory disease that affects the periodontal tissues, resulting in their destruction and, eventually, tooth loss. Pathogens present in the subgingival dental biofilm are essential for the initiation and progression of periodontitis. Periodontopathogens, their components and products can trigger local inflammatory reactions and host immune responses involving the production of proinflammatory mediators[12, 13]. As a result of the exaggerated immunoinflammatory reaction, the periodontal tissues and the alveolar bone undergo degradation and resorption, respectively[25].

Orthodontic tooth movement occurs in response to mechanical force by remodeling changes of the periodontal ligament and alveolar bone[18]. Tissue remodeling is regulated and maintained by an aseptic acute inflammatory response characterized by the release of proinflammatory mediators[6, 17, 18, 31]. Clinical studies have shown high levels of cytokines and chemokines in gingival crevicular fluid during orthodontic therapy[6, 32, 38]. Although some in vitro studies[9, 23, 27] have found that biomechanical forces can also exert anti-inflammatory effect, according to our previous in vivo study, orthodontic forces could aggravate periodontal destruction by intensifying periodontal inflammation[7].

Interestingly, inflammatory mediators are produced and released during the progression of both, periodontal diseases and orthodontic tooth movement. The expression of cytokines, such as IL-1β, TNF-α, IL-6, as well as chemokines, such as CC chemokine ligand 2 (CCL2) and its receptor CCR2 is increased during those two distinct conditions[6, 32, 36-38]. However, it is still unclear how orthodontic loading and inflammation co-regulate these mediators. Hence, the present study aimed to evaluate the inflammatory profile of the periodontal tissues in response to biomechanical forces and periodontal inflammation, correlating it with the alveolar bone resorption levels.

Materials and Methods

Experimental Protocol

The experimental protocol was approved by the local Ethical Committee on Animal Experimentation, and performed in accordance with the guidelines from the National Council for the Control of Animal Experimentation (CONCEA). The study protocol also followed all recommendations of the ARRIVE guidelines. A total of 144 male adult Holtzman rats, average weigh 300g, were maintained in the animal facilities of the School of Dentistry at Araraquara under controlled temperature (22-25oC) with a 12h light/dark cycle. Animals were housed in plastic cages receiving standard laboratory diet

and water ad libitum. After 1 week of acclimatization, general anesthesia was induced with intramuscular injections of ketamine chlorhydrate 10% (0.08 mL/100g body weight) and xylasine chlorhydrate 2% (0.04 mL/100g body weight). The animals were randomly assigned into four experimental groups: 1- negative control (sham operated), 2 - ligature-induced periodontal disease (P), 3 - orthodontic movement (OM), 4 - ligature-induced periodontal disease, followed by orthodontic movement (OMP). After 1, 3, 7, and 15 days of the orthodontic movement start, 9 animals from each experimental group were sacrificed in each period by anesthetic overdose. The maxillary jaws were hemisected, and half of the block samples including molars with their surrounding tissues were fixed in 4% paraformaldehyde for 48 hours and stored in 70% ethanol for alveolar bone resorption analysis by micro-computed tomography (micro-CT). The other half of the blocks had the gingival tissue around the maxillary first molars carefully dissected for extraction of total RNA for PCR array and RT-qPCR.

Ligature-induced periodontal disease

At baseline, animals were anaesthetized and cotton ligatures were placed around the cervical area of the upper first molars bilaterally and knotted mesially, aiming to induce experimental periodontal disease. For P and OMP groups, the ligature-induced periodontal disease was initiated five days before baseline to guarantee disease development and bone loss[7] before the placement of the orthodontic appliance.

Orthodontic tooth movement

To induce orthodontic tooth movement, a previously established rat tooth movement model and orthodontic appliance design were adopted with some changes[7]. After anesthesia, a closed coil nickel-

titanium spring (Sentalloy®, GAC, Dentsply) was connected between the maxillary first molar and maxillary central incisor teeth. For spring placement around the maxillary central incisor teeth, grooves were prepared on their surfaces to prevent displacement of the 0.20 mm steel wire (CrNi, 55.01.208, Morelli) and a thin layer of composite resin was placed covering it. For spring placement on the maxillary first molars, composite resin was placed at the occlusal surface, and then the spring was positioned over it. A nickel-titanium spring was used to provide a relatively constant force of 50 g over the course of the experiment. To avoid oclusal interference, lower first molars were extracted.

PCR array

PCR array was used to screen mRNA of 84 different genes involved in the expression of inflammatory cytokines and receptors (RT2 Profiler™ PCR Array Rat Inflammatory Cytokines & Receptors, SA Biosciences, Qiagen, Hilden, Germany) in gingival tissues of 1-day samples from all experimental groups. Total RNA was extracted from gingival tissue samples using RNeasy Mini Kit (Qiagen), according to the manufacturer's protocol. DNase treatment was performed to eliminate DNA contamination using DNA- free kit (Ambion, Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. The quantity and purity of total RNA were determined using NanoVue UV/Visible Spectrophotometer (GE Healthcare, Piscataway, NJ) by evaluating the absorbance at 260 nm and the 260/280 nm ratio, respectively. RNA integrity was confirmed by electrophoresis in formaldehyde agarose gel. Complementary DNA was synthesized by reverse transcription of 1 μg total RNA using RT2 First Strand cDNA kit (SA Biosciences, Qiagen) following the manufacturer's protocol. Data analysis was performed by a Web-Based PCR Array Data Analysis accessed by the online link: http://www.sabiosciences.com/pcrarraydataanalysis.php, which converts values from Ct

to fold change. The 6 most upregulated genes expressed in the OMP group in comparison to the C group were selected and validated them by qPCR.

Quantitative Real-Time PCR

To validate the 6 most upregulated genes, 500 ng of total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's protocol. Quantitative PCR was performed using a Step One thermocycler (Applied Biosystems). PCR reaction included 1 μL of the RT reaction product in a 10 μL total volume PCR reaction mix that contained TaqMan Fast Advance Master Mix (Applied Biosystems), nuclease free water, and TaqMan Gene Expression Assays (Applied Biosystems) for rat genes (Table 1). The cycling conditions used for all primers were pre-optimized: 50oC for 2 min and 40 cycles of: 95oC for 15 seconds and 600C for 1 min. The determination of the relative levels of gene expression was performed using the cycle threshold method and normalized to the housekeeping gene GAPDH, which was not altered by the experimental conditions. Results are represented as the mean mRNA expression from duplicate measurements of 4 samples from different animals at 3 days, normalized by the internal control GAPDH and expressed as fold change over the levels of expression of the normalized target gene determined in cDNA samples prepared from healthy control gingival tissues.

Micro-computed tomography analysis (micro-CT)

After the sacrifice of the animals, dissected maxillae of 6 rats per group were carefully harvested, fixed in 4% paraformaldehyde for 48 hours, and stored in 70% ethanol in room temperature until they were imaged by μCT scanning (μCT Skyscan 1176, Aartselaar, Kontich, Belgium). The x-ray generator was operated at 50 kVp, beam current at 500μA, 0.5mm aluminum filtration at an image

resolution of 12.45µm. The images were reconstructed with specific software (NRecon 1.6.1.5 – Skyscan, Kontich, Belgium) in all three spatial dimensions and then, all the maxillae images were orientated and saved in coronal slices (2000 x 1336) utilizing the Data Viewer software (Data Viewer 1.4.3.1 - Skyscan, Kontich, Belgium). To measure alveolar bone volume (BV) and tissue volume (TV) a region of interest (ROI) was delineated from the root apices to the alveolar crest, minus the roots and PDL space, comprising the entire alveolar bone, using the despeckle tool (custom processing) in CtAn software (CT Analyser 1.10.1.0 - Skyscan, Kontich, Belgium). The architectural parameter evaluated was bone volume under tissue volume (BV/TV %) that represents the volume of the mineralized tissue around the defect.

Data Analysis

Statistical analyses were performed with the software GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). For the PCR array analysis, the p values were calculated based on Student's t-test of the replicates 2^ (- Delta Ct) values for each gene in the control and treatment groups. For the qPCR and BVF, data were synthesized using mean ± standard deviation. One-way analysis of variance test (ANOVA) followed by the Tukey´s post hoc test was used to determine the presence of any significant difference between groups for inflammatory mediators mRNA expression and alveolar bone volume. Significant differences were considered when $p < 0.05$.

Results

Gene expression regulation

First, we sought to screen the expression of 84 rat inflammatory cytokines and receptors transcripts in gingival tissues of teeth subjected or not to experimental periodontal disease and/or orthodontic tooth

movement by performing a PCR array-based approach. In the OMP group, 75 genes were upregulated out of the 84 genes evaluated, but only 12 genes presented significant difference compared to C group. For the P group 69 genes were upregulated and for the OM 57 (Figure 1). As shown in Table 2 and Figure 2, we selected 6 genes significantly upregulated ($p < 0.05$) in the OMP group compared to the C group: CCL2, CCR2, IL-1β, IL-6R, Itgam, and TNF-α. Then, the results from the PCR array analysis for those 6 genes were validated by qPCR (Figure 3), showing that both conditions, disease and mechanical loading, induced a significant upregulation of the mRNA expression of the previously cited genes. At day 3, the OMP group demonstrated a significant ($p < 0.05$) increase in the mRNA expression levels of CCL2 and IL-6 genes compared to the C group, CCR2 gene compared to C and OM groups, IL-1β, Itgam, and TNF-α genes compared to all other groups. Expression levels of Itgam gene paralleled the expression of the inflammatory cytokines IL-1β and TNF-α. The chemokine CCL2 and its receptor CCR2 also showed a tendency to parallel their expression with the cytokines.

Alveolar bone loss

The three-dimensional sagittal micro-computed tomography views of the maxillary molars from each group at 15 days are shown in Figure 4-a. Graphs of all experimental periods with the results of bone volume are shown in Figure 4-b. Alveolar bone loss was obvious in the OMP and P groups during all time points. The OMP group exhibited continuous and significant ($p < 0.05$) alveolar bone loss compared to C and OM since day 1, but it was only at 15 days that the OMP group presented a relevant effect on bone loss ($p<0.05$) compared to all the other groups as demonstrated by a significant ($p<0.05$) decrease in bone volume fraction.

Discussion

The present study evaluates for the first time the expression of 84 genes implicated in the inflammatory response during orthodontic movement, associated or not to periodontal disease, using PCR array. The results demonstrated increased expression of cytokines, chemokines and their receptors in diseased periodontal tissues submitted to biomechanical forces. Furthermore, in this situation, the loss of alveolar bone was significantly ($p < 0.05$) higher compared to other experimental groups. These results suggest that orthodontic loading may contribute to periodontal tissues inflammation and breakdown through co-regulating inflammatory mediators and bone resorption with the periodontal disease process.

Alveolar bone volume measured in all experimental groups during all time points showed significant bone loss for the diseased groups P and OMP, justified by the experimental periodontal disease induction. In addition, this study revealed a tendency of the OMP group to have more bone loss than all the other groups from the third day after the beginning of the orthodontic movement, reaching significant difference at 15 days. These findings indicate that mechanical loading, which leads to an aseptic inflammatory response, performed in the presence of periodontal disease, results in greater effect on periodontal tissues involving more inflammation and bone uncoupling than both isolated conditions.

After screening 84 inflammatory cytokines and receptors genes, our data demonstrated that 75 genes were upregulated in the OMP group, but only 12 were significantly different in comparison to C group. Six out of these 12 genes were validated by qPCR and the results confirmed the PCR array results. Among the mediators evaluated by the PCR array, the chemokine CC chemokine ligand 2 (CCL2), and its receptor CC chemokine receptor 2 (CCR2) were significantly upregulated in the OMP group. Chemokines are a family of small chemoattractant cytokines. Their main function is to

control and direct the trafficking cells in homeostatic and inflammatory processes. The CCL2 also known as monocyte chemotactic protein-1 (MCP-1), is produced by several cell types and regulates the migration and infiltration of monocytes, memory T lymphocytes, and natural killer cells[10], the maturation of T-cell, and the immune response by T-helper-cell polarization. The cellular effects of CCL2 are mediated by its receptor CCR2. CCR2 is expressed in osteoclast precursors and after binding to CCL2, osteoclast differentiation occurs. Also, osteoblasts secrete CCL2 in response to orthodontic force application, and express CCL2 in response to inflammatory processes in vivo[30]. This bone remodeling role of CCL2 has been extensively studied, and many reports have demonstrated increased expression of CCL2 during orthodontic tooth movement[2-4, 11] as well as in other inflammatory conditions where bone remodeling occurs, like in rheumatoid arthritis[22], tumors[24], and periodontal disease[19, 29]. Increased levels of CCL2 have been detected in gingival crevicular fluid and gingiva from sites with periodontal disease[19, 29]. In addition, CCL2 has shown to be a potential target for host response modulation and for therapeutic intervention of many diseases, such as multiple sclerosis[5, 26], rheumatoid arthritis[5], atherosclerosis[16]. Corroborating with other studies, the expression of CCL2 and CCR2 was increased in P and OM groups. The relevant finding of our study was that the OMP group presented even higher expression of these molecules, suggesting that they are involved in the mechanism inducing exacerbated bone loss observed in this group.

Several pro-inflammatory cytokines were evaluated in the PCR array. Among them, TNF-α, IL-1 β and IL-6 were highly upregulated. Cytokines, small proteins important for cell signaling, are produced by all nucleated cells and are usually involved in tissue reactions through the regulation of host responses to infection, immune responses, inflammation, and trauma. Pro- and anti-inflammatory cytokines play a role in periodontal tissues and bone remodeling. IL-1β and TNF-α are the most important proinflammatory cytokines, and high levels of these cytokines have been detected in

gingival crevicular fluid during tooth movement and periodontal disease progression. Our data concur with these previous studies in relation to the increased expression of these cytokines in P and OM groups. Interestingly, the present results for the OMP group are in agreement with our previous study showing that both cytokines are highly upregulated in gingival tissues subjected to periodontal disease and mechanical forces. In addition to those cytokines, IL-6 is an important inflammatory mediator that triggers inflammatory and immune response. IL-6 activates bone resorption in conjunction or not with other bone resorptive mediators. It binds to the receptor IL-6R and uncontrolled production of both, IL-6 and IL-6R, is associated with the pathogenesis of many diseases. High levels of IL-6 have been detected in gingival tissues and gingival crevicular fluid from subjects presenting periodontitis. Orthodontic loading has been demonstrated to increase the expression of IL-6 in gingival crevicular fluid[38]. In our study, IL-6 and IL-6R were upregulated in all experimental groups, although their expression was again more evident for the OMP group.

The last group of mediators evaluated in the PCR array was the integrins. Integrins are proteins expressed on cells surface. They mediate cell adhesion to other cells and to extracellular matrix proteins, acting as important transducers of vital signals to cell function. Additionally, integrins are important to polymorphonuclear cells responses to infection and injury, during cell adhesion, migration, phagocytosis, and oxidant production. Integrin alpha M (ITGAM), also known as macrophage-1 antigen (Mac-1) and cluster of differentiation molecule 11B (CD11B), is an innate immune receptor expressed on leukocytes involved in the immune response. ITGAM acts in inflammatory processes by promoting leukocyte adhesion and migration, by stimulating cytokine production, cell activation. Also, ITGAM is implicated with the complement system. Increase in the number of ITGAM-positive cells has been detected in rat PDL subjected to experimental orthodontic tooth movement[39]. Furthermore, ITGAM, as well as other innate immune receptors, has been found

in higher levels in the gingivae of old mice compared to young ones, and this expression was correlated to the increased bone loss and increased expression of proinflammatory mediators[21]. Our data showed high levels of ITGAM in all experimental groups, but only the OMP group presented significant difference compared to the other groups.

Periodontal tissues are exposed to complex forces during orthodontic tooth movement, as well as during mastication, dental habits, and dental trauma. Many experimental tooth movement models have been used to study the cellular and molecular reactions of bone remodeling in response to mechanical force application. Recently, diverse animal models have been described hindering the comparison of different studies[15, 28]. The chosen orthodontic appliance in the present study was the NiTi closed-coil spring, which provides a constant force of 0.25 N during the whole time of the experiment due to material properties. In addition, our model demonstrated to induce reliable tooth movement of the maxillary first molars and stimulated the expression of proinflammatory mediators. In this study, we did not use ligature wire around the first molars in order to prevent any interference of the wire in the periodontal tissues, once this device could act like the cotton ligature and induce gingival inflammation. Thus, instead of using ligature wire, the coil spring was bonded on the occlusal surface of the first molar.

Most of the researchers from the periodontics and orthodontics fields agree and affirm that orthodontic treatment should be performed only in healthy periodontal tissues. This study was conducted in an attempt to better understand the molecular mechanisms involved in diseased periodontal tissues submitted to orthodontic forces, in order to elucidate that statement. Relevant mediators, including chemokines, cytokines and integrins involved in that destructive process were highlighted. Even with modifications in the orthodontic appliance, the results were similar to our previous study in relation to the significant increase in inflammatory mediators and in bone loss when

periodontally compromised teeth were subjected to orthodontic force. In other words, the results suggest the need of eliminating the periodontal infection/inflammation before starting orthodontic treatment, as well as maintaining the periodontal health throughout the duration of the therapy, since it has been demonstrated a significant increase in inflammation and destruction of periodontal tissues.

In summary, the present study demonstrates that mechanical loading modulates the response of periodontal tissues to periodontal disease through the increased expression of proinflammatory mediators, which might be involved in the alveolar bone resorption. Therefore, it can be concluded that orthodontic forces may aggravate the periodontal breakdown in periodontitis by the enhancement of periodontal inflammation.

Acknowledgments

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Table and figure legends

Table 2

Gene regulation and p value in rat gingival tissues derived from the experimental groups OM, P, and OMP compared to C at 1 day.

Figure 1 Heat map analysis showing the gene expression profile in gingival tissues in response to biomechanical forces and periodontal inflammation driven by orthodontic movement and experimental periodontal disease, respectively. Green color means upregulation while red means downregulation.

Figure 2 Regulation of gene expression in rat gingival tissues by inflammatory and/or mechanical stimulus at 1 day, as analyzed by PCR array. Six genes are shown as they were significantly ($p < 0.05$) increased in the OMP group compared to the C group.

Figure 3 Regulation of gene expression in rat gingival tissues by inflammatory and/or mechanical stimulus at 3 days, as analyzed by qPCR. Graphs with the mRNA expression of CCL2 (a), CCR2 (b), IL-1β (c), IL-6 (d), Itgam (e), and TNF-α (f) are shown. Results are expressed as mean and ± SD. *Significant difference compared to all other groups ($p < 0.05$), ☐Significant difference compared to C and OM groups ($p < 0.05$), ■Significant difference compared to C group ($p < 0.05$).

Figure 4 a) Three-dimensional sagittal micro-CT views of maxillary molars of different animals from each experimental group (C, OM, P, and OMP) at 15 days. Tooth movement is shown in the OM and OMP groups. b) Comparison of BVF percentage measured in a selected ROI of the maxillary first molars using micro-CT. Results are expressed as mean ± SD. *Significant difference compared to all other groups ($p < 0.05$), ☐Significant difference compared to C and OM groups ($p < 0.05$), ■Significant difference compared to C group ($p < 0.05$).

Tables and figures

Table 1

Table 1 - Inventoried TaqMan primers and probes (TaqMan Gene Expression Assays, Applied Biosystems).

Target gene	Assay ID	Acession #	Amplicon (bp)
GAPDH	Rn 99999916-s1	NM_017008.3	87
CCL2	Rn 00580555-m1	NM_031530.1	95
CCR2	Rn 01637698-s1	NM_021866.1	103
IL-1β	Rn 00580432-m1	NM_031512.2	74
IL-6	Rn 99999011-m1	NM_012589.1	90
ITGAM	Rn 00709342-m1	NM_012711.1	76
TNF-α	Rn 01525859-g1	NM_012675.3	92

Table 2

Table 2 - Regulation of gene expression evaluated by PCR array.

Gene	Fold regulation			*P* value		
	OM	P	OMP	OM	P	OMP
CCL2	2.67	24.19	32.35	0.50	0.05	0.03
CCR2	1.17	4.52	6.85	0.58	0.12	0.001
IL1β	1.53	11.32	57.01	0.35	0.11	0.04
IL6R	2.46	9.29	21.84	0.45	0.10	0.03
ITGAM	1.61	5.42	8.12	0.42	0.06	0.002
TNF-α	0.95	2.21	4.30	0.65	0.03	0.02

Figure 1

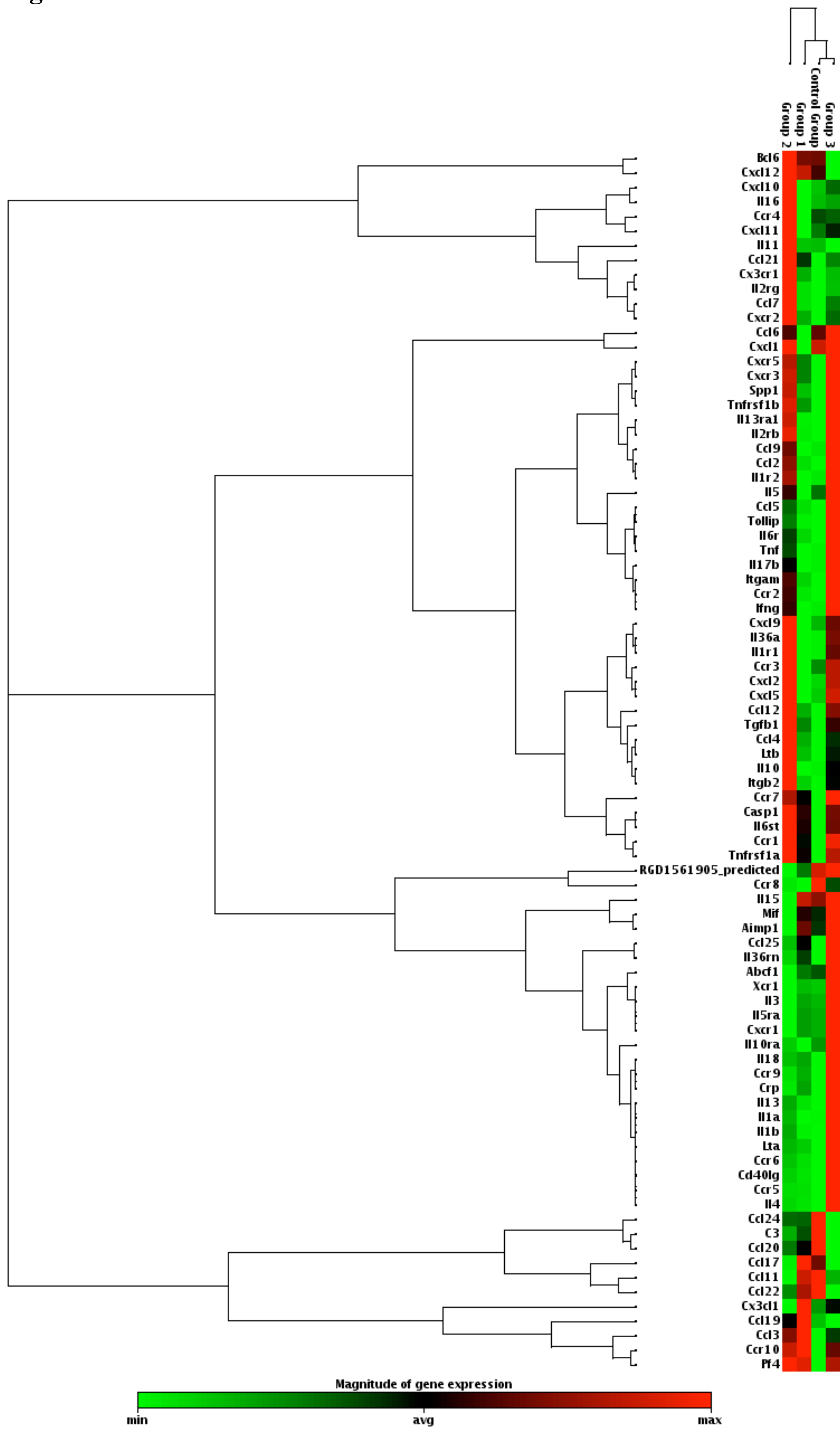


Figura 2

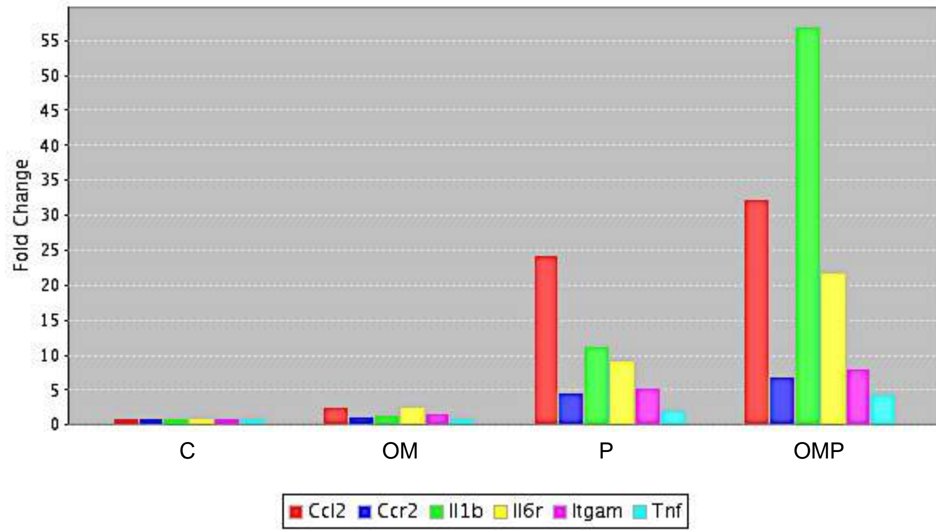


Figure 3

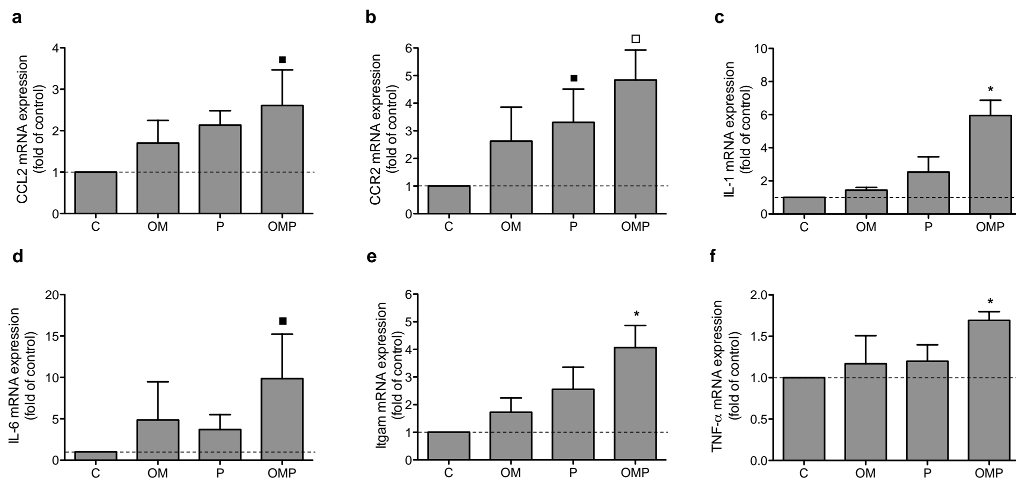
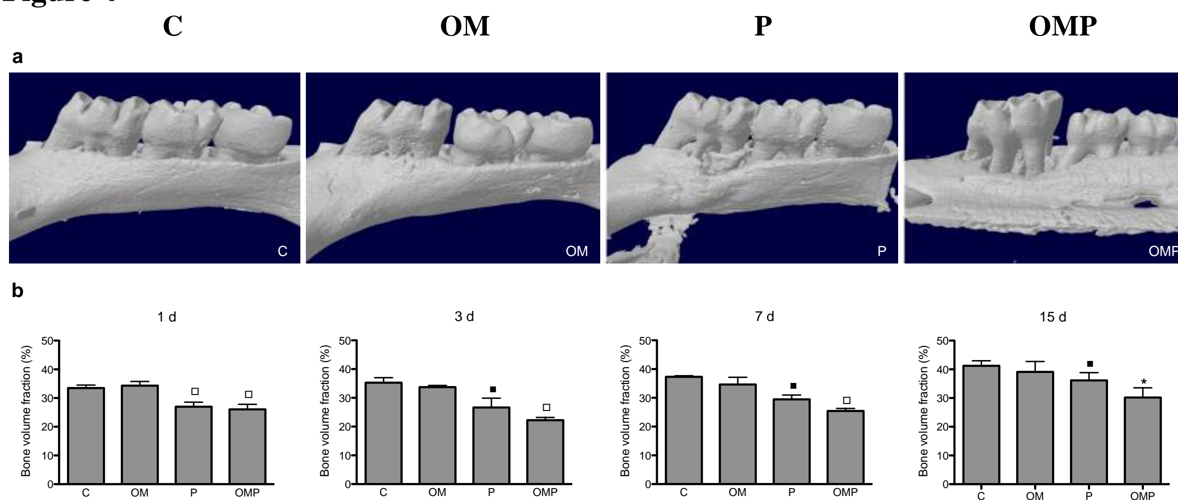


Figure 4



7 CONSIDERAÇÕES FINAIS

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O capítulo 1 teve como objetivo avaliar a regulação de visfatina em células hPDL e se a interação dos sinais microbiano e biomecânico é capaz de modular essa regulação. Os resultados mostraram que a visfatina é produzida pelas células hPDL e que sua produção está aumentada em resposta ao estímulo bacteriano por *F. nucleatum*. Por outro lado, a força biomecânica resultou em um efeito protetor, reduzindo a ação de *F. nucleatum* sobre a expressão e produção de visfatina. A importância de se estudar a regulação da visfatina se deve principalmente pelo fato de ser um marcador inflamatório presente em várias condições e doenças sistêmicas como: obesidade, síndrome metabólica, diabetes mellitus e doenças cardiovasculares. Além disso, um aumento nos níveis de visfatina já foi detectado no soro, no fluido crevicular gengival e na saliva de pacientes com doença periodontal crônica em comparação a pacientes periodontalmente saudáveis, sugerindo um papel da visfatina como um marcador inflamatório também para a doença periodontal[26, 34]. Sabe-se que após terapia periodontal, os níveis de visfatina são reduzidos no soro e no fluido crevicular gengival[28]. Da mesma forma, outro estudo clínico demonstrou aumento de visfatina no soro e fluido crevicular gengival de pacientes com doença periodontal e diabetes mellitus, sugerindo um potencial papel da visfatina como mediador de ligação patogênica entre a doença periodontal e outras alterações sistêmicas como diabetes mellitus[27]. Sugere-se que as células hPDL estão contribuindo para o aumento de visfatina no fluido crevicular gengival pelo fato dessas células produzirem visfatina. Além disso, recentemente foi demonstrado que a visfatina também é produzida por fibroblastos gengivais de humanos (HGF)[9]. Um aumento significante na produção de visfatina é encontrado nas células HGF após estímulos inflamatório e bacteriano[9]. Ademais, tecidos gengivais humanos referentes a pacientes apresentando gengivite e periodontite crônica também resultaram em aumento significante na produção de visfatina. A

produção local de visfatina pelas células hPDL e HGF representa um possível mecanismo pelo qual a doença periodontal tenha impacto sobre algumas condições e doenças sistêmicas.

O objetivo do capítulo 2 foi avaliar a regulação de marcadores da inflamação e reabsorção óssea por estímulos bacteriano e mecânico em células hPDL. Os resultados demonstraram que a força teve ação pro-inflamatória sobre o efeito de *F. nucleatum*, aumentando ainda mais a expressão e síntese de COX2 e PGE2. Além disso, a associação entre força e *F. nucleatum* resultou em maior aumento na razão RANKL/OPG comparado ao *F. nucleatum* sozinho, devido à inibição na expressão de OPG pela força biomecânica. Esses dados sugerem que a interação entre bactéria e força gera um efeito pró-inflamatório e de reabsorção óssea. Nossos resultados estão de acordo com outros estudos prévios em relação ao efeito pró-inflamatório da força em condições de inflamação, porém alguns estudos encontram esse efeito pró-inflamatório somente para força de alta magnitude[20, 21], enquanto outro somente para o período de 1 dia para forças de baixa e alta magnitudes[23]. Outro tipo de força mecânica realizado por meio de pressão hidrostática também mostrou intensificar a produção de citocinas inflamatórias em células hPDL estimuladas com bactéria periodontopatógena. Essas de RANKL e OPG após estímulo mecânico é contraditória na literatura. Alguns trabalhos relatam que as células hPDL expressam RANKL em resposta à força mecânica[15, 40], enquanto para outros essas células não expressam[24] ou expressam muito pouco RANKL [35]. Assim como o nosso estudo, um outro trabalho detectou redução na expressão de OPG após aplicação de força mecânica em células hPDL[24], porém outro encontrou aumento com posterior redução quando as células foram estimuladas por força e LPS[35]. Apesar das contradições, sugere-se um efeito de reabsorção óssea após a interação entre força e bactéria devido ao aumento da razão RANKL/OPG que ocorre por redução na expressão e produção de OPG.

Já, o capítulo 3 teve como objetivo avaliar a regulação de mediadores e receptores inflamatórios nos tecidos periodontais de ratos submetidos à força ortodôntica associada ou não à doença periodontal experimental. Foi observado que após essa associação entre força e doença, ocorreu um aumento relevante na expressão de citocinas, quimiocinas e receptores inflamatórios. Além disso, houve um aumento na reabsorção óssea alveolar a partir do dia 3, com diferença estatística em 15 dias. Esses resultados sugerem que a força mecânica na presença da doença periodontal modula a expressão de marcadores inflamatórios responsáveis por exacerbar a destruição periodontal. Houve um aumento na expressão de várias quimiocinas cuja principal função é a quimiotaxia de células imunes em processos de homeostase e de inflamação. Estudos demonstraram um aumento na expressão de CCL2 e CCR2 durante o movimento ortodôntico, assim como em outras condições inflamatórias em que ocorre remodelação óssea, como doença periodontal[18, 27] e artrite[19]. A relevância dos nossos resultados consiste no aumento significante na expressão de CCL2 e CCR2 no grupo OMP, sugerindo o envolvimento dessas moléculas no mecanismo de indução de maior perda óssea nesse grupo experimental. Além das quimiocinas, houve um aumento na expressão de várias citocinas pró-inflamatórias, dentre elas as mais conhecidas e que tiveram seus resultados validados foram IL-1β, IL-6 e TNF-α. Níveis aumentados dessas citocinas estão presentes no fluido crevicular gengival de pacientes com periodontite e durante o movimento ortodôntico[4, 11, 29, 32, 36] No presente estudo, a expressão dessas citocinas foi novamente maior no grupo OMP. As integrinas, proteínas de adesão celular, são moléculas responsáveis por intermediar a adesão de uma célula à outra e às proteínas da matriz extracelular, atuando como transdutores de sinais vitais importantes para o funcionamento das células. Aumento no número de células positivas ao ITGAM foi demonstrado no ligamento periodontal de ratos submetidos ao movimento ortodôntico[37]. Ademais, em nosso estudo a expressão de ITGAM nos grupos experimentais foi similar à da IL-1β, sendo que o grupo OMP

apresentou um aumento significante. Os dados desse capítulo sugerem que a interação entre força biomecânica por meio da força ortodôntica e a inflamação periodontal resulta em um aumento na expressão de vários mediadores inflamatórios responsáveis por intensificar a inflamação periodontal e a destruição dos tecidos de suporte dental.

8 CONCLUSÃO

8 CONCLUSÃO

Os resultados obtidos permitiram concluir que:

Conclusão geral: A interação da força biomecânica com a inflamação resulta em um aumento na expressão de mediadores inflamatórios responsáveis pela progressão da doença periodontal.

Conclusão específica 1 (Capítulo 1): As células hPDL produzem visfatina e sua produção está aumentada após estímulo bacteriano com *F. nucleatum*, sugerindo que a doença periodontal tenha uma ligação patogênica com alterações sistêmicas através desse mediador inflamatório. A força biomecânica apresentou ação inibitória em relação aos efeitos de *F. nucleatum,* reduzindo a expressão e produção de visfatina.

Conclusão específica 2 (Capítulo 2): A força biomecânica apresentou ação pró-inflamatória em relação aos efeitos de *F. nucleatum* nas células hPDL, aumentando ainda mais a expressão e produção de COX2 e PGE2. Além disso, houve um aumento na razão RANKL/OPG devido à redução na expressão de OPG.

Conclusão específica 3 (Capítulo 3): In vivo, a força ortodôntica foi capaz de modular a resposta dos tecidos periodontais à doença periodontal por meio do aumento na expressão de mediadores pró-inflamatórios, os quais podem estar envolvidos com a reabsorção de osso alveolar. Portanto, as forças ortodônticas podem agravar a destruição periodontal na periodontite por meio do aumento da inflamação periodontal.

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ANEXOS

Anexo 1 - Comitê de Ética - células humanas

Rheinische Friedrich-Wilhelms-Universität

Medizinische Fakultät

Ethik–Kommission

53105 Bonn, 23.02.2011
Sigmund-Freud-Str. 25
Biomedizinisches Zentrum
Zimmer 2G 029

Durchwahl: 287 – 51 931
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Ethik-Kommission - Medizinische Fakultät Bonn
Biomedizinisches Zentrum, Sigmund-Freud-Str. 25, 53105 Bonn

persönlich / vertraulich
Herr
Prof. Dr. J. Deschner
Poliklinik für Parodontologie / Zahnheilkunde
Welschnonnenstr. 17
53111 Bonn / durch Boten

Lfd. Nr. 043/11
Bitte stets angeben!

Betr.: **Ihr Antrag an die Ethik-Kommission**
Antragsteller: **Prof. Dr. J. Deschner**
Studientitel: **KFO 208 „Ursachen und Folgen von Parodontophatien TP 4 Einfluss von Adipokinen und Adipositas auf die parodontale Destruktion und Regeneration (Fortsetzungsantrag zu Lfd.-Nr. 073/08)**
Sponsor: **DFG**

- Antrag - Projektbeschreibung vom 22.02.08
- Aktualisierte Patientenaufklärung / Einwilligungserklärung

Sehr geehrter Herr Kollege Deschner,

die Ethik-Kommission für klinische Versuche am Menschen und epidemiologische Forschung mit personenbezogenen Daten der Medizinischen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn hat o.g. Unterlagen geprüft und erhebt keine berufsethischen oder berufsrechtlichen Bedenken gegen die Weiterführung das o.g. Forschungsvorhabens.

Mit freundlichen Grüßen

Prof. Dr. K. Racké
Vorsitzender der Ethik-Kommission

Anexo 2 - Comitê de Ética no Uso de Animal

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

Proc. CEUA nº 23/2012

Araraquara, 01 de março de 2013.

Senhores Pesquisadores:

A Comissão de Ética no Uso de Animal - CEUA desta Faculdade, após a avaliação do projeto de sua responsabilidade intitulado *"CONTRIBUIÇÃO DAS FORÇAS BIOMECÂNICAS NA DESTRUIÇÃO PERIODONTAL EM RATOS"* (Proc. CEUA nº 23/2012) AUTORIZA a realização da pesquisa, ficando a apresentação do RELATÓRIO FINAL para SETEMBRO/2014.

Atenciosamente.

Prof. Dr. PAULO SÉRGIO CERRI
Coordenador da CEUA

Ao
Prof. Dr. JONI AUGUSTO CIRELLI
DD. Pesquisador Responsável
a/c Andressa Vilas Boas Nogueira
Departamento de Diagnóstico e Cirurgia

Comissão de Ética no Uso de Animal - CEUA

Não autorizo a publicação deste trabalho até 23 de março de 2017.
(Direitos de publicação reservados ao autor)

Araraquara, 27 de março de 2014.

ANDRESSA VILAS BOAS NOGUEIRA