UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE ODONTOLOGIA DE ARARAQUARA

VANESSA CAMILA DA SILVA

EFEITOS DA INTRUSÃO ORTODÔNTICA NA REPARAÇÃO DE LESÕES DE FURCA GRAU III EM CÃES, E DA PRESENÇA DE TNFα E/OU IL-1ß NA MECANORESPOSTA DE CÉLULAS ÓSSEAS IN VITRO

ARARAQUARA
2008

UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE ODONTOLOGIA DE ARARAQUARA

VANESSA CAMILA DA SILVA

EFEITOS DA INTRUSÃO ORTODÔNTICA NA REPARAÇÃO DE LESÕES DE FURCA GRAU III EM CÃES, E DA PRESENÇA DE TNFα E/OU IL-1ß NA MECANORESPOSTA DE CÉLULAS ÓSSEAS IN VITRO

Tese apresentada ao Programa de Pós-graduação em Periodontia da Faculdade de Odontologia de Araraquara, Universidade Estadual Paulista "Júlio de Mesquita Filho", para a obtenção do título de Doutor em Periodontia.

Orientador:
Prof. Dr. Joni Augusto Cirelli
Co-orientadora:
Prof. Dra. Rosemary Adriana Chiérici Marcantonio

ARARAQUARA
2008

Silva, Vanessa Camila da
Efeitos da intrusão ortodôntica na reparação de lesões de furca grau III em cães, e da presença de TNFα e/ou IL-1ß na mecanoresposta de células ósseas in vitro. / Vanessa Camila da Silva. – Araraquara : [s.n.], 2008.
130f.; 30 cm.

Tese (Doutorado) – Universidade Estadual Paulista, Faculdade de Odontologia.

Orientador: Prof. Dr. Joni Augusto Cirelli
Co-orientadora: Profa. Dra. Rosemary Adriana Chiérici Marcantonio

1. Defeitos da furca 2. Regeneração tecidual guiada periodontal 3. Movimentação dentária 4. Citocinas 5. Fluxo pulsátil I. Título.

Ficha catalográfica elaborada pela Bibliotecária Marley Cristina Chiusoli Montagnoli CRB 8/5646
Serviço Técnico de Biblioteca e Documentação da Faculdade de Odontologia de Araraquara /U NESP

Vanessa Camila da Silva

EFEITOS DA INTRUSÃO ORTODÔNTICA NA REPARAÇÃO DE LESÕES DE FURCA GRAU III EM CÃES, E DA PRESENÇA DE TNFα E/OU IL-1ß NA MECANORESPOSTA DE CÉLULAS ÓSSEAS IN VITRO

BANCA EXAMINADORA

Presidente e Orientador: Prof. Dr. Joni Augusto Cirelli

2º Examinador: Prof. Dr. Ana Cláudia Moreira de Melo

3º Examinador: Prof. Dr. Enilson Antonio Sallum

4º Examinador: Prof. Dr. Ary dos Santos Pinto

5º Examinador: Prof. Dra. Silvana Regina Perez Orrico

Araraquara, 27 de fevereiro de 2008.

DADOS CURRICULARES

VANESSA CAMILA DA SILVA

NASCIMENTO	08 de Julho de 1978 – São Carlos, SP, Brasil
FILIAÇÃO	Silvio José da Silva Isabel Cristina Gradim da Silva
1996/2000	Curso de Graduação em Odontologia pela Faculdade de Odontologia de Araraquara da Universidade Estadual Paulista "Júlio de Mesquita Filho".
2000/2001	Curso de Especialização em Periodontia pela Associação Paulista dos Cirurgiões Dentistas e Faculdade de Odontologia de Araraquara da Universidade Estadual Paulista "Júlio de Mesquita Filho".
2002/2004	Curso de Pós-Graduação em Odontologia, Área de Periodontia, Nível de Mestrado, na Faculdade de Odontologia de Araraquara da Universidade Estadual Paulista "Júlio de Mesquita Filho".
2004/2008	Curso de Pós-Graduação em Odontologia, Área de Periodontia, Nível de Doutorado, na Faculdade de Odontologia de Araraquara da Universidade Estadual Paulista "Júlio de Mesquita Filho".

DEDICATÓRIA

***Silvio e Cristina*, *meus pais*,** que apoiaram e incentivaram todos os projetos de minha vida. Obrigada pelo amor, carinho e atenção que sempre me dedicaram. Amo vocês.

Guerino (in memoriam), Olinda e Maria, meus avós, sempre interessados em nossos passos vibrando conosco em cada conquista. Obrigada pelo carinho.

Diego, meu irmão, e meus sobrinhos ***Enzo e Júlia,*** agradeço a Deus pela sua existência e por fazer parte desta família.

Aos ***meus tios(as) e primos(as),*** obrigada pelo convívio, amor e paciência.

Aos meus sogros, ***Juarez e Ilzamar,*** cunhados (as), ***Ighor, Saulo, Celso e Rosinha, Fabiana e Priscila*** e meu querido ***Filipe,*** obrigada por todo o carinho e atenção que tem por mim.

Ao meu amado ***Erick,*** toda essa conquista tem sua presença e ajuda em todos os momentos. É muito bom poder viver ao seu lado e ter seu apoio como um porto seguro. Muito obrigada pela sua existência, companheirismo e dedicação ao nosso amor. Amo você.

"Para ser grande: Sê inteiro
Nada teu exagera ou exclui
Sê todo em cada coisa
Põe quanto és, no mínimo que fazes
Assim em cada lago, a Lua toda brilha e alta Vive."

Odes, de Ricardo Reis

AGRADECIMENTO ESPECIAL

A ***Deus*** pela conquista e pela minha vida.

Ao Prof. Dr**.** ***Joni Augusto Cirelli,*** meu orientador e amigo, obrigada pelo exemplo e amizade que sempre me proporcionou ao me orientar em todos os momentos. E pela disponibilidade em discutir, incentivar e me ensinar mesmo quando estávamos em diferentes continentes.

Ao Prof. Dr. ***Luis Carlos Spolidório*** e Prof. Dra. ***Rosemary Adriana Chiérici Marcantonio*** pela atenção e pronta disponibilidade em me receber como sua orientada.

Prof. Dr. ***Jenneke Klein-Nulend,*** thanks for the opportunity you gave to me. It was an enormous pleasure working at the OCB department (Oral Cell Biology) with you and your team. With you I learned that enthusiasm is an important and fundamental step during research.

My Dutch friend ***Astrid***, you are a wonderful researcher and friend. You have the gift of explaining a very complex stuff without complicating. It was very nice to work with you in Amsterdam and I hope we can keep in touch forever. Thanks for the delicious *poffertjes*!!!

My other Dutch friend ***Cor***, a brilliant, gifted technician at OCB. The time I arrived at the department to work with something completely unusual for me, you demonstrated your fortitude and patience by telling me: You can do it!! and I did! You taught me more than culture of cells or fluid flow experiments, you taught me about Netherlands (wonderful book), North Pole, nice food and we spent wonderful moments I got all in my memory!

My Philippine's friend, ***Mel,*** thanks for your friendship and attention. Our chatting at lab after the Dutch time to go home (17h) will be forever in my mind. Thanks for the bike you lent me. It was very nice to live in Amsterdam 'biking' as Dutch people.

Ana, amiga de Portugal que conheci na Holanda, '*pois pois'*, obrigada pela atenção, amizade e pela disponibilidade com que me ajudou desde quando cheguei no departamento. Você tem um lugar especial em meu coração.

Aos amigos ***Fernando Salimon Ribeiro, Daniela Zandim, Fábio Leite, Ana Emília Pontes*** e ***Yeon Jung Kim*** meu muito obrigado pela amizade e carinho. Sei que sempre posso contar com vocês. Sua ajuda foi fundamental na realização deste trabalho e de outros durante o Doutorado.

As amigas de trabalho em São Luís-MA, ***Fátima e Camilla*** que me apoiaram durante esse período e me deram força e incentivo nessa caminhada. Meu muito obrigado por tudo!

AGRADECIMENTOS

À Faculdade de Odontologia de Araraquara, UNESP, na pessoa de sua diretora ***Professora Dra. Rosemary Adriana Chiérici Marcantonio*** e de seu vice-diretor ***Professor Dr. José Cláudio Martins Segalla,*** pela recepção e condições oferecidas para a realização desse trabalho.

Aos Professores da Disciplina de Periodontia da Faculdade de Odontologia de Araraquara: ***Benedicto Egbert Corrêa de Toledo, Ricardo Samih Georges Abi Rached, Elcio Marcantonio Junior, José Eduardo Cézar Sampaio, Rosemary Adriana Chiérici Marcantonio, Silvana Regina Perez Orrico e Carlos Rossa Junior,*** pelo ensino, incentivo, amizade e apoio durante toda minha formação. Vocês me ensinaram muito mais que somente Odontologia.

Aos demais professores desta Faculdade e àqueles que lecionaram durante o meu curso de Pós-Graduação.

À Coordenadora do Curso de Pós-Graduação em Periodontia, Prof. Dra. ***Silvana Regina Perez Orrico***, pelo nível do curso oferecido e pelo carinho que tem por todos nós.

À ***Regina Lúcia, Maria do Rosário, Terê, Zezé, Vera, Sueli, Thelma e Toninho***, funcionários da Periodontia, sempre dispostos a nos ajudar. Obrigada pela amizade.

À ***Ana Cláudia G. C. Miranda,*** pelo trabalho laboratorial na confecção das lâminas e principalmente pela amizade e carinho com que sempre me tratou.

A ***Mara, Rosangêla*** e demais funcionários da Seção de Pós-Graduação e Graduação pela organização, cooperação e competência.

Aos funcionários da Biblioteca pelo apoio constante, em especial à ***Marley, Ceres e Maria Helena,*** pela ficha catalográfica, correção e revisão da redação técnica e das referências bibliográficas.

Ao protético ***Diego***, pela ajuda na confecção dos componentes protéticos e ao ***Marcelo***, da Anatomia, pela ajuda na redução das peças.

À ***Carolina Chan Cirelli*** pela amizade e ajuda na realização da parte ortodôntica desse trabalho.

Ao Dr. ***Alessandro Galhardo,*** veterinário, pela atenção dedicada, ajuda com os animais e pela amizade.

Ao ***Fernando***, do Biotério, e ***Wellington Leão Favero*** pela ajuda na escovação dentária e demais cuidados dos cães.

Ao Professor Dr. ***Carlos Benatti Neto (Tato)*** pela amizade e ajuda fundamental na análise histológica.

Ao Professor Dr. ***José Vanderlei Menani,*** do Departamento de Fisiologia da FOAr-UNESP, pelo apoio na obtenção das fotografias histológicas.

Ao Professor Dr. ***Romeu Magnani*** pela colaboração na estatística desse trabalho.

Aos amigos de turma ***Fernando, Eduardo Ishi, Andréa, Beatriz, Dani Gonçalves, Elisângela, Fernanda, Juliana Moraes, Juliana Rico, Ivy, Patrícia*** e ***'Maurício'*** pela amizade, cooperação, festas, aprendizado e pelo apoio. "Nenhum caminho é longo demais quando amigos nos acompanham."

A todos os amigos da Pós-Graduação em Periodontia e a todos os amigos dos demais cursos pela convivência, colaboração, festas e sonhos compartilhados.

Às minhas amigas (os) e colegas de trabalho do Fórum em São Luís-MA, ***Gracielle, Madalena, Terezinha, Costa, Edson, Ana, Onilde, Jô, Renata, Kátia, Valéria, Rui, Sheila, Marta e Gildete*** meu muito obrigado pela amizade e cooperação durante todo esse período.

Às minhas amigas e companheiras de casa, ***Cristiane e Daniela*** foi muito bom conviver com vocês. Vocês vão estar sempre em meu coração.

Aos meus amigos em Amsterdam, ***Euler, Tati, Max, Lia, Valesk, Miguel e Vanessa*** e todos aqueles que conheci e que me ajudaram a me sentir em casa mesmo a muitas milhas de distância dela.

Jolanda, I will never forget your contagious laugh, wonderful cakes, dutch barbecue and your way to say '*Vanessa flow'*. In Brazilians' defenses we do not have an official paranymph, however be sure that if I could choose so, it would be you!

Marion, thanks for your special attention in teaching me Dutch words and tell me about offers and promotions written in Dutch language in supermarkets and so ever. Thanks for helping me with everything I needed.

Chun Ling, Zufu, Agnes, Marlene, Aviral, Veerla, Dennis, Marjolein, Ton Schoenmaker, Djien Tan, Ton Bronckers, Vincent, Jack, Marco, Ruud, Dirk-Jan, Clara, Marco, Nina, Elena, Lucienne, Nina, Don, Behrouz and Ineke**,** friends from Amsterdam, thanks for your attention and friendship.

Aos ***animais*** que permitiram a realização deste trabalho.

Ao ***CNPq*** (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Bolsa de Doutorado, Processo 142080/2004-7), à ***Capes*** (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Bolsa de Doutorado Sanduíche, Processo BEX0481/06-8) agradeço pelo apoio financeiro; e a ***Neodent® Implante Osteointegrável***, Curitiba, PR, por fornecer os mini-implantes e kit de instalação usado neste estudo.

A todos os amigos que silenciosamente e cada um a seu modo, tão bem souberam me ajudar. Mais uma vez ***Obrigada!!!***

SUMÁRIO

PREFÁCIO

Essa tese é constituída de dois trabalhos:

Um artigo científico desenvolvido durante o curso de Doutorado nessa instituição:

• Capítulo 1, "Intrusion of teeth with class III furcation. Clinical, histologic, and histometric study in dogs", submetido para publicação no periódico Journal of Clinical Periodontology.

Um artigo científico desenvolvido durante o estágio do Programa de Doutorado sanduíche, na Academic Center of Dentistry Amsterdam (ACTA), Holanda:

• Capítulo 2, "TNFα and IL-1ß inhibit fluid shear stress-induced nitric oxide production by osteocytes and osteoblasts", submetido para publicação no periódico Journal of Bone and Mineral Research.

LISTA DE ABREVIATURAS

ATP: adenosina trifosfato

BA: enxerto ósseo autógeno (bone autograft)

EGTA: etileno glicol-bis (2-aminoetiléter)-N,N,N',N'-ácido tetraacético

GdCl3: cloreto de gadolínio

GTR: regeneração tecidual guiada (guided tissue regeneration)

I: intrusão ortodôntica

IL-1ß: interleucina-1 beta

LP: ligamento periodontal

NO: óxido nítrico (nitric oxide)

NOS: enzima óxido nítrico sintetase (nitric oxide synthase enzyme)

OFD: raspagem com acesso via retalho periodontal (open flap debridment)

OPG: osteoprotegerina

PFF: fluxo de fluido pulsátil (pulsatile fluid flow)

RANK-L: receptor ativador do fator nuclear-κB ligante

TMB-8: 8-(NN-dietilamino)octil-3,4,5-trimetoxibenzoato HCl

TNFα: fator de necrose tumoral alfa

da Silva VC. Efeitos da intrusão ortodôntica na reparação de lesões de furca grau III em cães, e da presença de TNFα e/ou IL-1ß na mecanoresposta de células ósseas in vitro [tese doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2008.

Resumo

O objetivo dessa tese foi avaliar os efeitos da intrusão ortodôntica no processo de reparação de lesão de furca grau III em cães, e da presença de fator de necrose tumoral alfa (TNFα) e/ou interleucina-1 beta (IL-1ß) na mecanoresposta de células com característica de osteócitos e osteoblastos in vitro. No estudo in vivo, lesões de furca grau III foram criadas em pré-molares inferiores de sete cães. Após 75 dias, as lesões foram aleatoriamente tratadas com cirurgia a retalho (OFD) associada ou não à regeneração tecidual guiada (GTR) e enxerto ósseo autógeno (BA). Após um mês, iniciou-se a intrusão ortodôntica (I) em parte dos dentes tratados pelas duas diferentes abordagens (grupos teste), por meio de ancoragem em miniimplantes. Os cães foram sacrificados após três meses de movimentação e um mês de contenção. Todas as lesões de furca grau III foram reduzidas para grau II ou I nos grupos teste. O mesmo foi observado em 50% das lesões nos grupos controle (sem movimentação). O nível de inserção clínico foi reduzido nos grupos teste, no final da contenção ($p<0.01$). O grupo OFD+I apresentou maior preenchimento ósseo que os demais grupos ($p<0.05$), demonstrando superioridade desta associação, no tratamento de lesões de furca grau III, em cães. Esses resultados levantaram a hipótese de que o processo de degradação da membrana e/ou enxerto ósseo interagiria negativamente sobre o processo de reparo quando associado a forças ortodônticas, pois a presença de mediadores inflamatórios estaria intensificada. É conhecido que a alteração tecidual proveniente da movimentação ortodôntica é resultado da ação de mediadores químicos sobre as células dos tecidos periodontais. Portanto, buscamos avaliar in vitro a ação de duas citocinas pró-inflamatórias, TNFα e IL-1ß, sobre células ósseas em presença e ausência de carga mecânica. Assim, no estudo in vitro, MLO-Y4 osteócitos e

MC3T3-E1 osteoblastos foram incubados com TNFα (0.5-30 ng/ml), IL-1β (0.1-10 ng/ml) ou inibidores de cálcio TMB-8, $GdCl_3$, ou EGTA por 30 min seguidos por 30 min de fluxo de fluido pulsátil (PFF; 0.6±0.3 Pa, 5 Hz). Apoptose, quantificada por ensaio de Caspase-Glo 3/7, não foi afetada por TNFα e IL-1β. O PFF estimulou a produção de óxido nítrico (NO) e de $[Ca]_i^{2+}$, avaliados pelo reagente de Griess e Fluo-4 AM respectivamente. Ambos TNFα e IL-1ß reduziram a produção de NO em todos os períodos e suprimiram o aumento nos níveis de cálcio induzidos pelo PFF em ambos os tipos celulares. Em osteócitos, a produção de NO aos 5 min foi reduzida por todos os inibidores de cálcio ($p<0.05$). Em osteoblastos, NO foi reduzido por TMB-8 e $GdCl_3$, mas não EGTA. Concluindo, TNFα e IL-1β reduziram a produção de NO induzido pelo PFF em células MLO-Y4 e MC3T3-E1, provavelmente devido à inibição do aumento dos níveis de cálcio associados ao PFF.

Palavras-chave: Defeitos de furca; regeneração tecidual guiada periodontal; movimentação dentária; citocinas; fluxo pulsátil.

da Silva VC. Effects of orthodontic intrusion on the healing of class III furcation lesions in dogs, and of the presence of TNFα and/or IL-1ß on the mechanoresponse of bone cells in vitro [tese doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2008.

Abstract

The aim was to assess the effects of orthodontic intrusion on the healing of class III furcation lesions in dogs, and of the presence of tumor necrosis factor alpha (TNFα) and/or interleukin-1 beta (IL-1ß) on the mechanoresponse of osteocyte and osteoblast-like cells in vitro. In the in vivo study, class III furcation lesions were created in lower pre-molars of seven mongrel dogs. After 75 days, teeth were randomly treated with open flap debridment (OFD) associated or not to guided tissue regeneration (GTR) and bone autograft (BA). After one month, teeth were randomly assigned to orthodontic intrusion using mini-implants anchorage or no movement. Dogs were sacrificed after three months of movement and one month contention. All class III furcations were closed or reduced to class II or I lesions in the intrusion groups while 50% of the class III lesions in non-moved teeth remained unchanged. Clinical attachment level was reduced in the intrusion groups by the end of contention ($p<0.01$). OFD + I presented smaller soft tissues area and larger bone tissue area than other groups ($p<0.05$). Orthodontic intrusion with mini-implants anchorage improved healing of class III furcation defects after OFD in dogs. Based on these results we hypothesized that degradation of membrane and/or bone autograft can negatively interfere on repair when associated to orthodontic movement because inflammatory mediators are intensified. It‘s known that cytokines are present during orthodontic movement which are acting on periodontal cells. Therefore we evaluated in vitro the effects of two pro-inflammatory cytokines, TNFα and IL-1ß, on bone cells in presence or absence of mechanical loading. In the in vitro study, MLO-Y4 osteocytes and MC3T3-E1 osteoblasts were incubated with TNFα (0.5-30 ng/ml), IL-1β (0.1-10 ng/ml) or the calcium inhibitors TMB-8, $GdCl_3$, or EGTA for 30 min, followed by

30 min of pulsating fluid flow (PFF; 0.6±0.3 Pa, 5 Hz) with or without cytokines or calcium inhibitors. Apoptosis, quantified using the Caspase-Glo 3/7 assay, was not affected by TNFα and IL-1ß. PFF stimulated nitric oxide (NO) production and $[Ca]_i^{2+}$ which was measured using Griess reagent and Fluo-4 AM respectively. Both TNFα and IL-1ß decreased PFF-induced NO production at all time points and abolished the PFF-induced rise in $[Ca^{2+}]_i$ in both cell types. In MLO-Y4 cells, PFF-induced NO production at 5 min was inhibited by all calcium inhibitors, but in MC3T3-E1 cells this effect was inhibited by TMB-8 and $GdCl_3$, but not EGTA. In conclusion, TNFα and IL-1β inhibit fluid shear stress-induced NO production by MLO-Y4 and MC3T3-E1 cells, likely via prevention of the rapid rise in $[Ca^{2+}]_i$ associated with the onset of PFF.

Keywords: Furcation defects; guided tissue regeneration periodontal; tooth movement; cytokines; pulsatile flow.

1 INTRODUÇÃO

O tratamento ortodôntico tem sido cada vez mais procurado por pacientes adultos em busca por função e estética dentária[124,127]. Em muitos casos, a movimentação dentária ortodôntica em adultos se faz necessária pela ocorrência de perdas dentárias e migrações patológicas conseqüentes da doença periodontal[24,28]. Nessas situações, o tratamento periodontal regenerativo prévio a ortodontia tem sido sugerido com o objetivo de restabelecer o periodonto perdido facilitando o controle de placa e a distribuição da carga mecânica durante a ortodontia[1,63,97,115,133]. A terapia ortodôntica também tem sido utilizada previamente ao tratamento periodontal regenerativo com o objetivo de alinhamento dentário e redução do tamanho do defeito periodontal facilitando o prognóstico do tratamento periodontal regenerativo, se ainda necessário[70,133].

A influência da movimentação dentária ortodôntica como um provável fator que estimule a regeneração periodontal vem sendo recentemente estudada, no entanto não foi ainda totalmente elucidada[23,51,97,98,111,112,150]. É conhecido que o ligamento periodontal (LP) contém células indiferenciadas com a habilidade de se diferenciar em cementoblastos e osteoblastos[82,100,158] e que a aplicação de carga mecânica in vitro é capaz de promover a diferenciação de células mesenquimais indiferenciadas em osteoblastos[74]. Assim, durante o movimento ortodôntico, o LP em contato direto com o defeito ósseo pode estimular a aposição óssea[95]. Vários autores observaram maior formação óssea e periodontal quando a ortodontia foi realizada em direção a defeitos ósseos[32,95,96,145]. Ganho de inserção conjuntiva e óssea também foi observado após movimento intrusivo de dentes com periodonto reduzido e saudáveis[37,88]. Sendo assim, o provável potencial regenerativo da ortodontia poderia ser utilizado em defeitos periodontais críticos com pobre prognóstico ao tratamento periodontal regenerativo. No capítulo 1, nós testamos a hipótese de que o movimento de intrusão ortodôntica poderia favorecer o ganho de inserção e preenchimento ósseo em lesões de furca grau III, usando mini-implantes como ancoragem ortodôntica.

No entanto, sabe-se que as alterações observadas em tecidos submetidos à carga mecânica ortodôntica são resultantes de eventos biológicos complexos[78] visto que inúmeras moléculas são liberadas durante a ortodontia tanto para estimulação quanto para inibição da formação e/ou reabsorção óssea[34,49,78]. Essas moléculas devem exercer efeito na reparação de defeitos periodontais quando presentes (como as lesões de furca grau III, no capítulo 1).

O principal mecanismo a nível celular que regula a remodelação do tecido ósseo submetido à carga mecânica é conhecido como mecanotransdução, onde células ósseas convertem sinais mecânicos em químicos e/ou elétricos[19]. Esse processo envolve uma cascata de eventos que incluem a presença de ATP, cálcio, prostaglandinas e óxido nítrico (NO) que levam a remodelação óssea[117]. Quando a carga mecânica é aplicada sobre o tecido ósseo, ocorre o movimento do fluido intersticial na rede lacuno-canalicular ao redor dos osteócitos[75,76,109,149]. Subsequentemente, essas células produzem moléculas como NO que sinalizam osteoblastos e osteoclastos para induzir remodelação óssea[19,72,131]. Por outro lado, a remodelação tecidual é modulada também por citocinas inflamatórias[25,64,77,81]. Dentre elas, TNFα e IL-1ß estão presente em altos níveis no lado de pressão do LP nos primeiros três dias após ativação do movimento ortodôntico[13,56,84,141]. Em virtude dos níveis elevados encontrados, é sugerido que elas apresentam um papel importante na reabsorção óssea durante o movimento ortodôntico[84,141]. Estudos em animais demonstram que a ausência de TNFα resulta em reduzido movimento dentário[66,155], no entanto os mecanismos envolvidos na sua função permanecem desconhecidos. Considerando-se que o entendimento de diferentes eventos celulares podem ajudar a explicar as respostas teciduais de estudos in vivo, foi objetivo do capítulo 2 avaliar se a presença de TNFα e/ou IL-1ß poderia interferir na resposta de células ósseas submetidas à carga mecânica afetando o mecanismo de formação óssea. Para isso utilizamos a técnica de fluxo de fluido pulsátil (PFF), que é um estimulo mecânico experimental que simula a carga mecânica in vivo[19,73].

2 PROPOSIÇÃO

O objetivo geral foi avaliar se a intrusão ortodôntica influencia no processo de reparação de lesão de furca grau III em cães, e se a presença de TNFα e/ou IL-1ß afeta a mecanoresposta de células com característica de osteócitos e osteoblastos in vitro.

Os objetivos específicos foram:

1- avaliar parâmetros clínicos periodontais em pré-molares de cães com lesões de furca grau III, aplicada em fase recente de cicatrização após tratamento periodontal com cirurgia a retalho associada ou não a regeneração tecidual guiada e enxerto ósseo autógeno;

2- avaliar histológica e histometricamente os efeitos da intrusão nos tecidos periodontais de pré-molares de cães com lesões de furca grau III, aplicada em fase recente de cicatrização após tratamento periodontal com cirurgia a retalho associada ou não a regeneração tecidual guiada e enxerto ósseo autógeno;

2 - avaliar, in vitro, se a presença de TNFα e/ou IL-1β afeta a produção de NO e o nível de cálcio intracelular em células com característica de osteócitos e osteoblastos submetidas à carga mecânica.

3 CAPÍTULO 1

Este capítulo é constituído por uma introdução específica ao assunto "Intrusão ortodôntica de dentes com lesões de furca grau III em cães." e pelo seguinte artigo:

da Silva VC, Cirelli CC, Ribeiro FS, Leite FRM, Benatti Neto C, Marcantonio RAC, Cirelli JA. Intrusion of teeth with class III furcation. Clinical, histologic and histometric study in dogs. Submetido para publicação no periódico Journal of Clinical Periodontology.

No Anexo 1 estão incluídas as estatísticas descritivas com média, desvio padrão, mediana, valores máximo e mínimo e os valores de probabilidade p correspondente aos testes estatísticos utilizados no artigo a seguir. As tabelas A1-A6 apresentam respectivamente os dados clínicos, em mm, de profundidade de sondagem, nível gengival, nível de inserção, e número de sítios por grupo com placa visível, sangramento gengival marginal e sangramento a sondagem. As tabelas A7-A8 apresentam respectivamente os dados histométricos em porcentagem, referentes à área da lesão e à extensão linear da raiz. As medidas originais da análise histométrica foram transformadas em valores percentuais para minimizar a interferência do tamanho dos dentes no resultado da análise.

No Anexo 2 consta o certificado de aprovação final do Comitê de Ética em Experimentação Animal da Faculdade de Odontologia de Araraquara – UNESP.

Intrusão ortodôntica de dentes com lesões de furca grau III em cães.

Dentre os defeitos ósseos, as lesões de furca grau III se constituem em defeito crítico com pobre prognóstico[26,50] devido a dificuldade de acesso e controle de placa[146] e pela presença de células osteogênicas apenas na parede inferior do defeito[87]. Tentativas de restabelecimento do periodonto perdido têm sido propostas com o emprego de técnicas e/ou biomateriais regenerativos como a regeneração tecidual guiada (GTR), enxertos ósseos, proteínas derivadas da matriz do esmalte (Emdogain®), peptídeos sintéticos (Pepgen P-15®) e fatores de crescimento[8,40,45,118,119]. Dentre esses, a GTR tem sido a mais empregada usando membranas para atuar como barreiras mecânicas visando excluir o tecido epitelial e conjuntivo gengival da superfície radicular, para permitir um repovoamento desta por células mesenquimais indiferenciadas provenientes do ligamento periodontal[52,102]. Enxertos ósseos podem ser associados à GTR com o objetivo de conferir suporte à proliferação vascular e celular (osteocondução) e promover osteogênese e/ou osteoindução (no caso do enxerto autógeno) estimulando a formação óssea[15,116]. O fechamento das lesões de furca grau III após GTR, associada ou não a enxerto ósseo, é um evento ocasional e, portanto, com pobre prognóstico[11,45,105,114,118]. É freqüente ainda, a observação de extrusão dentária ou mal posicionamento na cavidade bucal em dentes acometidos por essas lesões, principalmente em função da ausência de dente antagonista[24,28]. Nessas situações o uso associado da intrusão ortodôntica poderia ser vantajoso para o reposicionamento dentário e eliminação ou redução da lesão de furca.

O tratamento ortodôntico em dentes que apresentam problemas periodontais é possível na ausência de processos inflamatórios e infecciosos e quando há adequado controle de placa bacteriana[42,150]. Nestas condições, os dentes podem ser movimentados sem que haja perda adicional de inserção, com possibilidade de ganho de inserção periodontal[23,88]. Visto que as lesões de furca grau III possuem prognóstico desfavorável ao tratamento periodontal[45,105,118], a combinação ortodontia-periodontia pode ser uma alternativa viável[86] pois o

movimento ortodôntico é capaz de promover a ativação de células locais indiferenciadas que beneficia o tratamento periodontal regenerativo[95,96,145].

Melsen et al.[88] estudaram a reação tecidual relacionada com a intrusão ortodôntica em dentes com periodonto reduzido e concluíram que a combinação do tratamento periodontal com intrusão ortodôntica resulta em ganho de inserção periodontal quando forças leves são utilizadas com adequado controle de placa bacteriana. Por outro lado, o efeito benéfico da intrusão tem sido questionado pelo maior risco de reabsorção radicular e movimento dos dentes de ancoragem[14,134]. Com o objetivo de evitar efeitos adversos, forças leves e miniimplantes para ancoragem são indicados[35,103,107,152].

INTRUSION OF TEETH WITH CLASS III FURCATION. CLINICAL, HISTOLOGIC AND HISTOMETRIC STUDY IN DOGS.

Vanessa C. da Silva[1], Carolina C. Cirelli[1], Fernando S. Ribeiro[1], Fábio R. M. Leite[1], Carlos Benatti Neto[2], Rosemary A. C. Marcantonio[1], Joni A. Cirelli[1].

[1] UNESP - São Paulo State University, Department of Periodontology, School of Dentistry at Araraquara - SP, Brazil

[2] UNESP - São Paulo State University, Department of Physiology and Pathology, School of Dentistry at Araraquara - SP, Brazil

Running title: Intrusion of class III furcation lesions.

Keywords: Furcation defects; surgical periodontal treatment; periodontal regeneration; tooth intrusion.

Correspondence address : Joni Augusto Cirelli, PhD, Department of Diagnosis and Surgery, School of Dentistry at Araraquara, São Paulo State University – UNESP, Rua Humaitá, 1680, Araraquara, SP, 14801-903, Brazil. Tel: +55 16 33016375, fax: +55 16 33016369, email: cirelli@foar.unesp.br

Conflict of Interest and Sources of Funding Statement

All authors have no conflict of interest.

Vanessa C. da Silva received funding from CNPq (Brazilian Counsel for Scientific and Technological Development, scholarship n^0. 142080/2004-7).The mini-implants and related supplies used in the present study were supplied by Neodent® Implante Osteointegrável, Curitiba, PR, Brazil.

Abstract

Aim: To assess orthodontic intrusion effects on periodontal tissues of dogs pre-molars with class III furcations previously treated with open flap debridement (OFD) associated or not to bone autograft (BA) and guided tissue regeneration (GTR).

Materials and Methods: Class III furcations were created in lower pre-molars of seven mongrel dogs. After 75 days, teeth were randomly treated with OFD or GTR /BA. After 1 month of daily plaque control, metallic crowns were assembled on pre-molars and connected apically to mini-implants by nickel-titanium springs. Teeth were randomly assigned to orthodontic intrusion or no movement. Dogs were sacrificed after three months of movement and one month contention.

Results: All class III furcations were closed or reduced to class II or I lesions in the intrusion groups while 50% of the class III lesions in non-moved teeth remained unchanged. Intruded teeth presented higher probing depth and lower gingival marginal level than non-moved teeth ($p<0.01$). Clinical attachment level was reduced in the intrusion groups by the end of contention ($p<0.01$). OFD + I presented smaller soft tissues area and larger bone tissue area than other groups ($p<0.05$).

Conclusion: Orthodontic intrusion with mini-implants anchorage improved healing of class III furcation defects after OFD in dogs.

Clinical Relevance

Scientific rationale for the study: Orthodontic treatment has been associated to periodontal regenerative techniques to improve prognosis for patients affected by advanced periodontal disease. In this study, dogs' teeth with class III furcations were intruded after open flap debridement associated or not to bone autograft and guided tissue regeneration in dogs. **Principal findings:** Clinical elimination or reduction of furcations was obtained after periodontal treatment and intrusion. Larger bone area was obtained after open flap debridement and intrusion in the furcation region. **Practical implications:** Orthodontic intrusion can be associated to surgical periodontal treatment to improve the prognosis of class III furcations.

Introduction

Furcation lesions have uncertain prognosis and frequently lead to tooth loss (Carnevale et al. 1995; Karring & Cortellini 1999). Regenerative approaches improve results for furcation treatment; however unpredictable periodontal regeneration is still obtained for Class III furcation defects (Becker 1999; Rossa et al. 2000; Fernandes et al. 2005).

Combined orthodontic and periodontal therapies has demonstrated to be a viable alternative for advanced periodontal bone defects, increasing clinical attachment gain and bone defect filling (Melsen et al. 1988; Melsen et al. 1989; Corrente et al. 2003). In the absence of periodontal disease combined with an adequate plaque control, reduced periodontium and periodontal defects are not a limitation for orthodontic movement (Ericsson & Thilander 1978; Polson et al. 1984; Boyd et al. 1989; Re et al. 2000). Recently, the use of periodontal regenerative techniques before orthodontic treatment are proposed to improve prognosis of tooth movement, facilitating the control of subgingival inflammation and stress distribution (Hossain et al. 1996; Nemcovsky et al. 1996; Stelzel & Flores-de-Jacoby 1998; Aguirre-Zorzano et al. 1999; Araújo et al. 2001; Re et al. 2002). On the other hand, orthodontic movement performed before regenerative treatment facilitate to reduce periodontal defects and increase of clinical attachment levels, because of teeth repositioning in alveolar bone (Stelzel & Flores-de-Jacoby 1998; Cirelli et al. 2003) and increased cellular activity (Roberts & Chase 1981; Melsen 2001). After orthodontic treatment, regenerative treatment can still be performed in reduced periodontal defects improving prognosis (Stelzel & Flores-de-Jacoby 1998).

Teeth movement toward alveolar bone defects demonstrates to be a stimulating factor for bone apposition, indicating a beneficial effect of orthodontic movement on regenerative therapy (Vardimon et al. 2001; Nemcovsky et al. 2004). Knippenberg et al. (2007) demonstrated the diferentiation of mesenchymal stem cells into bone cells after mechanical loading *in vitro*. The combination of body movement after GTR and bovine-derived xenograft in treatment of class II furcation lesions in dogs has shown a tendency to larger new formed bone area

compared to no-moved teeth (da Silva et al. 2006). Thus, orthodontic movement performed after regenerative treatment could stimulate periodontal and bone healing, improving the prognosis of periodontal lesions.

Favorable results have also been demonstrated with orthodontic intrusion. New connective tissue attachment and bone defect filling were observed during intrusion of periodontally involved teeth in absence of gingival inflammation (Melsen et al. 1988; Melsen et al. 1989; Cardaropoli et al. 2001; Corrente et al. 2003; Re et al. 2004). Nevertheless, intrusion is considered a critical movement as it requires fixed orthodontic appliances in several teeth to anchorage reinforcement (Alessandri Bonetti & Giunta 1996). The results are unpredictable and may lead to root resorption and extrusion and inclination of the anchorage teeth (Alessandri Bonetti & Giunta 1996). Besides, patients previously affected by periodontal diseases have reduced periodontium that also difficult conventional orthodontics. Recently, to avoid undesirable effects, anchorage using mini-implants has been used to obtain pure intrusion of molars (Ohmae et al. 2001; Chang et al. 2004; Yao et al. 2004).

So far, it is unknown whether an association of regenerative periodontal treatment with intrusion can improve healing of class III furcation lesions. The aim of this study was to assess the effects of orthodontic intrusion, obtained with mini-implants, in periodontal tissues of teeth with class III furcation lesions previously treated with open flap debridement associated or not to autogenous bone and guided tissue regeneration with absorbable membrane in dogs.

Materials and Methods

This study was approved by the Ethics Committee on Animal Experimentation of the School of Dentistry of Araraquara - UNESP. A total of 28 third and fourth lower pre-molars of seven mongrel dogs (weighing between 15 and 20kg) were used. For all clinical procedures, dogs were pre anesthetized using levopremazin chloritate (Neozine®, Aventis Pharma Ltd., SP, Brazil), administered intramuscularly 0.2 ml/kg of body weight and further anaesthetized intravenously with 0.5 ml/kg of body weight of sodium thiobarbiturate 25 mg/ml (sodium Tiopental®, Abbott Laboratories, SP, Brazil) and kept on intravenous hydration with a 0.9% physiological solution during surgery. Immediately after each surgery, dogs were given 10 ml of an hepatic protector (Frutoplex LN, Marjan e Comércio, São Paulo, Brazil) intravenously and 2 ml of dipirone intramuscularly.

Teeth were randomly divided into four groups as follow:

Open flap debridement (OFD): teeth with class III furcation defect treated with open flap debridement.

Open flap debridement plus intrusion (OFD+I): teeth with class III furcation defect treated with open flap debridement followed by intrusion movement.

Guided tissue regeneration and bone autograft (GTR/BA): teeth with class III furcation defect treated with open flap debridement associated with guided tissue regeneration and bone autograft.

Guided tissue regeneration and bone autograft plus intrusion (GTR/BA+I): teeth with class III furcation defect treated with open flap debridement associated with guided tissue regeneration and bone autograft followed by intrusion movement.

Seven pre-molars were assigned per group in such a way that each animal had one tooth in each group. Randomization was therefore performed, for each dog, by drawing two identified cards: one regarding the group and another regarding the tooth.

Clinical measurements were performed by a calibrated examiner at the designated time-points: before creation and cronification of furcation lesions (C),

immediately before the periodontal regenerative treatment (R), at the beginning (BI-before intrusion) and the end of orthodontic movement (AI-after intrusion) as well as at the end of contention (F-final). The following clinical data were obtained from the lower third and fourth pre-molars: presence of visible plaque (Pl), presence of marginal gingival bleeding (GB), probing depth (PD), bleeding on probing (BP), marginal gingival level (MGL) and clinical attachment level (CAL). Horizontal classification of furcation lesions (Hamp et al. 1975) was measured using a Nabers probe (HF, Hu-Friedy, Chicago, IL, USA) at R and F periods.

Creation and cronification of class III furcation lesions

Scaling and tooth prophylaxis using rubber cup and prophylactic toothpaste were initially performed in the entire mouth. After a week, a class III furcation lesion, 4 mm high, was created in the third and fourth lower pre-molars. The cavities were filled with gutta-percha to prevent spontaneous regeneration of the lesions (Cirelli et al. 1997) and allow lesion cronification (Wikesjo et al. 1991). Flaps were sutured in a coronal position. Sutures were removed after one week. During a 60-day-cronification period, dogs were fed with water-softened feed in order to accumulate plaque and to promote radicular contamination and development of chronic inflammation.

Periodontal treatment

After furcation cronification, gutta-percha was removed and teeth were scaled and root planed. The crowns of the third and fourth lower pre-molars were prepared using a high-speed bur to achieve parallel proximal surfaces. Impressions were taken with a silicone-based material (Xantopren® & Optosil®, Heraeus Kulzer South America, São Paulo, SP, Brazil). Silver metallic crowns were made with orthodontic hooks at the buccal and lingual surfaces.

After 15 days, a mucoperiosteal flap was raised and the roots scaled with hand instruments (Fig. 1a). The dimensions of the furcation lesions were measured and found to be, in average, 4.05 ± 0.95 mm high, 4.46 ± 0.94 mm deep and found to be, in average, 4.05 ± 0.95 mm high, 4.46 ± 0.94 mm deep and 2.96

± 0.72 mm width, with no statistical differences among them (p = 0.451, 1 way-ANOVA). Reference notches using low-speed bur nº ½ (KG Sorensen, São Paulo, SP, Brazil) were made on the mesial and distal roots at the bone crest level in the furcation region to orientate the histologic and histometric analyses.

Regenerative treatment of furcation lesions using bone autograft and absorbable membrane of copolymer of glycolide and lactide (Resolut ® Adapt W.L. Gore & Associates Inc., Flagstaff, AZ, USA) was performed at the buccal and lingual side (Fig. 1b, c). The bone autograft was obtained by scraping the buccal apical surface of the lower first molar. Membranes were sutured with a 4-0 poliglactin 910 absorbable suture (Vycril, Ethicon S.A., SP, Brazil). The flap was repositioned coronarilly with a suspending and inter-proximally interrupted 4-0 silk suture (Ethicon, Atraloc, Johnson & Johnson S.A., SP, Brazil) in order to cover the whole membrane and improve the region stability. Sutures were removed after 10 days. Benzyl penicillin and streptomycin (Pentabiótico, Fort Dodge®, Campinas, SP, Brazil) were administered IM (0.1 ml/kg of body weight) immediately after surgery and 5 days later.

Daily plaque control was performed by applying a 0.2% chlorhexidine gel with a soft brush until the end of the study. During and after this period, the dogs continued to be fed with water-softened feed to minimize mechanical trauma.

Installation of mini-implants and orthodontic movement.

After 30 days, tooth prophylaxis was performed and the metallic crowns were cemented with an adhesive material (Panavia 21X Dental Adhesive, Kuraray CO. LTDA, Osaka 530, Japan). For orthodontic anchorage, 7 mm length and 1.3 mm diameter titanium mini-implants (Neodent® Implante Osteointegrável, Curitiba, PR, Brazil) were installed 3 mm apically to a line joining the root apexes, at the middle distance between the roots of each treated pre-molar (verified by periapical radiographs) in both buccal and lingual cortical plates. A mucoperiosteal flap was opened 2 mm above muco-gingival junction, to avoid contact with the furcation region, and the alveolar bone was denuded. The cortical bone was drilled with a 1.1 mm round bur (Neodent®) under saline irrigation. The mini-implants were

installed in apical direction with 45º angulation to the bone surface, using a miniature screwdriver up to 10 N, verified with a torque-meter (Neodent®). This angulation was applied to avoid contact between mini-implants and root surfaces. Then, two nickel-titanium springs (GAC International, Inc., NY, USA), 7 mm long, providing a light and continuous tension of 25g, were installed connecting the mini-implants to the hook on the metallic crown of each tooth, followed by periapical radiograph (Fig. 2). The final intrusive force in the test teeth was 50g. Inactive identical appliances were placed on control pre-molars, to provide identical conditions for bacterial plaque accumulation and hygiene as in the test groups. The mini-implant was covered with the sutured flap. Sutures were removed after seven days and tooth prophylaxis was performed every 20 days.

After 90 days, teeth stabilization was carried out for one month. Contention was prepared using a stainless steel wire 0.25 mm (MORELLI, Sorocaba, SP, Brazil) passing through the hole of the mini-implant's head and over the hook of the metallic crown without causing any force to the tooth. A new periapical radiograph exam was taken.

After contention period, the appliances were removed and the dogs were sacrificed with an overdose of Thiopental. The third and fourth lower pre-molars were removed in block.

Histologic and histometric analyses

The biopsies were fixed in 10% formalin for 48h and decalcified in Morse solution during four months for routine histological processing and paraffin embedding. The mini-implants were removed carefully before paraffin embedding.

Histological semi-serial sections, 5 µm thick, were cut in the sagital plane through the entire bucco-lingual extension of the tooth (Jung Supercut 2065 Leica, Chicago, IL, USA). Five sections were selected for each tooth: the two most external sections showing the reference notches on both root surfaces (buccal and lingual sections) and three other sections which were equally spaced between the external sections, representing the central and internal portions of the defects. Haematoxylin and eosin (HE) and Masson trichrome staining were used.

Histometric analysis was performed with image analysis software (Sigma Scan Pro, Jandel Scientific, San Rafael, CA, USA) to obtain the following measurements (Fig. 3):

Cementum formation (Ce): linear radicular extension of the defect, between the notches on the mesial (M) and distal (D) roots of pre-molars, covered with new cementum.

Connective tissue (CT): linear radicular extension of the defect in direct contact with connective tissue.

Epithelial migration (Ep): linear radicular extension of the lesion covered with epithelial tissue.

Linear periodontal regeneration (LPR): linear radicular extension of the lesion with new cementum, bone and periodontal ligament regeneration.

Bone filling area (Bo): lesion area filled with newly formed bone, apically defined by a straight line joining the two radicular notches.

Soft tissue area (ST): lesion area filled with epithelium, connective tissue and periodontal ligament.

Bone autograft filling area (BA): lesion area filled with bone autograft.

Histometric linear and volumetric data were presented in % of total root linear extension and lesion area. Percentages were used intending to minimize interference of tooth size in the results.

Statistical analysis of the clinical and histometric data

Analysis of variance followed by Tukey's post hoc test was used to measure PD, MGL and CAL. Friedman test was applied to analyze Pl, GB and BP followed by non parametrical multiples comparisons test. T test compared the amount of intrusion between intrusion groups and Wilcoxon test compared horizontal classification of the furcation lesion between R and F periods. Statistical analysis of the effect of the treatments on the histometric linear measurements, in millimeters, and upon the area measurements, in square millimeters were made using Friedman test followed by non parametrical multiples comparisons test. The level of 5% was considered a decision rule for a significance difference.

Results

Clinical analysis

The average amount of tooth intrusion observed was 4.4 ± 1.9 mm in the OFD+I group and 4.7 ± 2.5 mm in the GTR/BA+I group, with no significant difference between groups (p>0.05). Also, there were not significant differences among groups for Pl, GB and BP, demonstrating that similar plaque control was obtained in all groups (data not shown).

The furcation lesions were re-evaluated and clinically re-classified in the end of contention period (Table 1). Class III furcations were still present in four teeth in the OFD group and in three teeth in the GTR/BA. Both intrusion groups did not present any class III furcation lesion. Instead, the initial lesions were closed or reduced to class II or I furcations ($p < 0.05$).

PD significantly increased in the intrusion groups in the end of intrusion and contention periods, compared to previous time-points and to non-intrusion groups at the same time-points (Fig. 4a; $p < 0.01$).

Intrusion groups presented significant variation of MGL which was coronally positioned at the end of intrusion and end of contention when compared to previous time-points and to non intrusion groups (Fig. 4b; $p < 0.01$).

CAL statistically reduced in the OFD+I group at the beginning of intrusion and stabilized after this time-point (Fig. 4c, p<0.05). Although in OFD+I group lower CAL values after intrusion and contention period were observed, these values were not significantly different from OFD group. GTR/BA+I group tended to present lower CAL after intrusion and contention time-points. This tendency was also observed when GTR/BA+I group was compared to GTR/BA group at the end of contention (Fig. 4c, p=0.07).

Histologic and histometric analysis

The majority of the samples presented an epithelial migration at the roof of the furcation region, frequently on the external cuts. A light lymphoplasmocytary inflammatory infiltration with sub-epithelial exacerbations was frequently observed. Teeth presented new cementum formation in the furcation region from

radicular notches up to the epithelial tissue. There was apposition of new cellular cementum filling radicular resorptions in the furcation and in the lateral radicular surfaces of teeth tending to surface smoothing. A fibrous connective tissue with collagenous fibers in a regular disposition and a primary trabecular newly formed bone were filling the furcation defects (Figs. 5 to 8). There was no evidence of any significant difference among groups on the linear extension of cementum formation, epithelial migration and periodontal regeneration (Fig. 9).

A significant effect of the orthodontic treatment on the furcation defect filling was observed in the OFD+I group, which presented smaller soft tissues area and larger bone area in the furcation region compared to OFD and GTR/BA groups (Fig. 6, 10, $p<0.05$).

A complete regeneration of the furcation lesion with newly formed cementum, periodontal ligament and bone tissues was observed in one dog of the OFD group, two dogs of the GTR/BA group, and one dog of the GTR/BA+I group. Bone autograft evolved by newly formed bone or connective tissue was observed in both GTR/BA (Fig. 7b) and GTR/BA+I groups.

There were areas of active resorption with osteoclasts in the Howship lacunas at the apical and furcation region in one dog of the OFD+I (Fig. 6b) and three dogs of the GTR/BA+I. Mini-implants were evolved by bone tissue in all groups as shown in OFD group (5b).

Discussion

This study evaluated the effects of intrusion orthodontic movement on periodontal tissues healing in teeth with class III furcation lesions previously treated with OFD or regenerative techniques (GTR/BA) in dogs.

To obtain the intrusion movement, mini-implants were inserted apically in both buccal and lingual bone plates of each tooth in the same level to prevent tooth inclination and lead to pure intrusion (Yao et al. 2005). Both intrusion groups obtained more than 4 mm of total apical movement without significant differences between them. These values were also observed by other authors using mini-implants (Ohmae et al. 2001) and were higher than in studies using anchorage provided by teeth (Diedrich et al. 1992; Ng et al. 2005; Ng et al. 2006). The primary stability obtained was sufficient to apply orthodontic force in the same day of mini-implant installation without failures during intrusion period (Favero et al. 2002; Miyawaki et al. 2003; Kyung et al. 2003; Liou et al. 2004). Histological, loading and unloading mini-implants were involved by bone as observed previously (Ohmae et al. 2001; Salina et al. 2006).

Orthodontic force was initiated at the early healing phase (30 days after surgery) (Matsuura et al. 1995; Araújo et al. 1997) to increase the mitotic activity of periodontal cells (Roberts & Chase 1981; Bumann et al. 1997; Melsen 2001), as used in previous study (Diedrich et al. 1992; Diedrich et al. 2003). Besides cells activation in the periodontal ligament, mechanical loading in bone tissues results in flow of interstitial fluid in the canalicular non-mineralized matrix resulting in bone apposition (Klein-Nulend et al. 1995a; Klein-Nulend et al. 1995b). Currently, adult mesenchymal stem cells with multilineage potential (Pittenger et al. 1999) and bone marrow stromal cells have been investigated for their usage in generating bone tissue after *in vitro* loading (Thomas & el Haj 1996; Meinel et al. 2004).

Clinically, a significant reduction of furcation lesion was observed in both intrusion groups ($p<0.05$). At the final of contention, class III furcations were present only in non intrusion groups (Table 1). Although results of periodontal treatment of class III furcation lesions tended to be inconsistent and unpredictable (Palioto et al. 2003; Fernandes et al. 2005; Roriz et al. 2006), the interaction of

periodontal and orthodontic treatments leaded to significant clinical closure of class III furcation lesions in this study. The intrusion groups presented an increased probing depth and decreased marginal gingival level as observed in other studies (Bondevik 1980; Murakami et al. 1989). No sample from any group presented clinical attachment loss. OFD+I and GTR/BA+I groups presented a gain in clinical attachment level of 34.73% and 31.18%, respectively, at the end of contention (Fig. 4c). These results are in line with other studies (Amiri-Jezeh et al. 2004) and demonstrate that periodontal attachment was maintained or improved during intrusion. It is hypothesized that the increased mitotic activity induced by the orthodontic stimulus results in an improved prognosis for a new attachment (Roberts & Jee 1974; Melsen et al. 1988; Melsen & Agerbaek 1991).

The dimensions of furcation lesions measured after the cronification period were 4.05 ± 0.95 mm high without any significant difference among groups. An incomplete regeneration of class III furcation lesion was observed in more than 50% of the non intrusion groups, which as demonstrated by other studies (Anderegg et al. 1991; Park et al. 1995; Mehlbauer et al. 2000; Rossa et al. 2000; Rupprecht et al. 2001; Palioto et al. 2003; Fernandes et al. 2005). Class III furcation lesions are critical defects especially when the vertical opening is larger than 3 mm (Pontoriero et al. 1989). In such defects, osteogenic cells are supplied only from the bottom of the furcation region which may explain the incomplete regeneration observed (Polson et al. 1984; Mehlbauer et al. 2000).

In intrusion groups, at the end of tooth movement the whole furcation defect was moved apically to bone crest level. It would be expected a complete newly formed bone at all furcation regions after intrusion, but it was not observed in all cases. In the OFD+I group, higher bone filling area and lower soft tissue area were present when compared to non-intrusion groups ($p<0.05$), but these results were not observed in the GTR/BA+I group. In addition, OFD+I group demonstrated a tendency to a higher linear periodontal regeneration (53.45%), i.e., presence of new cementum, periodontal ligament and bone. However, GTR/BA treatment was expected to favour the migration of periodontal and bone cells into the furcation region, once GTR promotes exclusion of epithelial and gingival cells

from the periodontal defects, favouring periodontal regeneration (Nyman et al. 1982). Besides, GTR plus bone autograft can improve the prognostic of the periodontal regeneration in furcation lesions (Reynolds et al. 2003). Also, bone autograft is considered to be the gold standard material for any regeneration procedure because of its osteogenetic, osteoinductive and osteoconductive properties (Simion & Fontana 2004). An explanation for obtained results in the GTR/BA+I group is that the application of orthodontic movement 30 days after the regenerative treatment may raised the inflammatory process expected due to membrane and bone autograft absorption, accelerating this process and interfering with tissue regeneration in the GTR/BA+I group. An increasing in macrophages and leukocytes migration (Gianelly 1969; Krishnan & Davidovitch 2006) and osteoclasts activation (Noxon et al. 2001) is usually observed during orthodontic movement. Diedrich et al. (2003) also observed an unfavorable interaction of periodontal regeneration, orthodontic remodeling and membrane degradation in the treatment of three-wall bony defects in dogs.

Intrusion is a critical orthodontic movement regarding external root resorption since pressure is highly concentrated at the conical apex and furcation region (Parker & Harris 1998; Jeon et al. 1999; Han et al. 2005; Harris et al. 2006). However, several reports indicate that 50 g, which was used in our study, is an adequate load to promote pre-molar intrusion avoiding severe root resorption (Dellinger 1967; Cooke & Bedi 1985; Diedrich et al. 1992; Southard et al. 1995; Faltin et al. 2001). In this study, new apposition of cellular cementum was regularizing areas of radicular resorption when present. However, some areas with active root resorption were observed in both intrusion groups either at the apex or furcation region which might be due to failure of dentin-protecting cementoblastic layer formation on the radicular area as observed in other study (Katzhendler & Steigman 1999).

Within the limits of this study, it was possible to conclude that mechanical loading applied in an early healing phase after surgical periodontal treatment on teeth with class III furcation defect promoted a clinical elimination or reduction of the class III defects and improved bone filling in the defect area. Intrusion after

one month of the regenerative periodontal treatment with membrane and bone autograft did not improve bone formation in the defect area, suggesting that the interaction between orthodontic movement and biomaterial degradation must be analysed in future research.

Acknowledgments

The authors would like to thank the CNPq (Brazilian Counsel for Scientific and Technological Development, scholarship n^0. 142080/2004-7) for financial support, Mrs. Ana Claúdia G. C. Miranda for technical support in the histologic procedures, Dr. Ana Emília Pontes and Dr. Daniela Leal Zandim for collaboration in the experiments and Mr. Fernando Luis Capelli and Wellington Leão Favero for dealing with dogs. In addition, we would like to express our gratitude to Neodent® Implante Osteointegrável, Curitiba, PR, Brazil for providing the mini-implants and related supplies used in the present study.

References

Aguirre-Zorzano, L. A., Bayona, J. M., Remolina, A., Castanos, J., Diez, R. & Estefania, E. (1999) Postorthodontic stability of the new attachment achieved by guided tissue regeneration following orthodontic movement: report of 2 cases. *Quintessence International,* **30,** 769-774.

Alessandri Bonetti G. & Giunta, D. (1996) Molar intrusion with a removable appliance. *Journal of Clinical Orthodontics,* **30,** 434-437.

Amiri-Jezeh, M., Marinello, C. P., Weiger, R. & Wichelhaus, A. (2004) [Effect of orthodontic tooth intrusion on the periodontium. Clinical study of changes in attachment level and probing depth at intruded incisors]. *Schweizer Monatsschrift für Zahnmedizin,* **114,** 804-816.

Anderegg, C. R., Martin, S. J., Gray, J. L., Mellonig, J. T. & Gher, M. E. (1991) Clinical evaluation of the use of decalcified freeze-dried bone allograft with guided tissue regeneration in the treatment of molar furcation invasions. *Journal of Periodontology,* **62,** 264-268.

Araújo, M. G., Berglundh, T. & Lindhe, J. (1997) On the dynamics of periodontal tissue formation in degree III furcation defects. An experimental study in dogs. *Journal of Clinical Periodontology,* **24,** 738-746.

Araújo, M. G., Carmagnola, D., Berglundh, T., Thilander, B. & Lindhe, J. (2001) Orthodontic movement in bone defects augmented with Bio-Oss. An experimental study in dogs. *Journal of Clinical Periodontology,* **28,** 73-80.

Becker, W. (1999) Periodontal regeneration: myth or reality? *Journal of the California Dental Association,* **27,** 118-124.

Bondevik, O. (1980) Tissue changes in the rat molar periodontium following application of intrusive forces. *European Journal of Orthodontics,* **2,** 41-49.

Boyd, R. L., Leggott, P. J., Quinn, R. S., Eakle, W. S. & Chambers, D. (1989) Periodontal implications of orthodontic treatment in adults with reduced or normal periodontal tissues versus those of adolescents. *American Journal of Orthodontics and Dentofacial Orthopedics,* **96,** 191-198.

Bumann, A., Carvalho, R. S., Schwarzer, C. L. & Yen, E. H. (1997) Collagen synthesis from human PDL cells following orthodontic tooth movement. *European Journal of Orthodontics,* **19,** 29-37.

Cardaropoli, D., Re, S., Corrente, G. & Abundo, R. (2001) Intrusion of migrated incisors with infrabony defects in adult periodontal patients. *American Journal of Orthodontics and Dentofacial Orthopedics,* **120,** 671-675.

Carnevale, G., Pontoriero, R. & Hurzeler, M. B. (1995) Management of furcation involvement. *Periodontology 2000,* **9,** 69-89.

Chang, Y. J., Lee, H. S. & Chun, Y. S. (2004) Microscrew anchorage for molar intrusion. *Journal of Clinical Orthodontics,* **38,** 325-330.

Cirelli, C. C., Cirelli, J. A., Rosa Martins, J. C., Lia, R. C., Rossa, C., Jr. & Marcantonio, E. Jr (2003) Orthodontic movement of teeth with intraosseous defects: Histologic and histometric study in dogs. *American Journal of Orthodontics and Dentofacial Orthopedics,* **123,** 666-673.

Cirelli, J. A., Marcantonio, E. Jr, Marcantonio, R. A. C., Lia, R. C., Goissis, G. & Rossa, C. Jr. (1997) Evaluation of anionic collagen membranes in the treatment of class II furcation lesions: an histometric analysis in dogs. *Biomaterials,* **18,** 1227-1234.

Cooke, M. S. & Bedi, R. (1985) Intrusion of an extrusion: case report of an innovative treatment alternative. *British Dental Journal,* **159,** 84-87.

Corrente, G., Abundo, R., Re, S., Cardaropoli, D. & Cardaropoli, G. (2003) Orthodontic movement into infrabony defects in patients with advanced periodontal disease: a clinical and radiological study. *Journal of Periodontology,* **74,** 1104-1109.

da Silva, V. C., Cirelli, C. C., Ribeiro, F. S., Costa, M. R., Lia, R. C. & Cirelli, J. A. (2006) Orthodontic movement after periodontal regeneration of class II furcation: a pilot study in dogs. *Journal of Clinical Periodontology,* **33,** 440-448.

Dellinger, E. L. (1967) A histologic and cephalometric investigation of premolar intrusion in the Macaca speciosa monkey. *American Journal of Orthodontics,* **53,** 325-355.

Diedrich, P., Wehrbein, H. & Schneider, B. (1992) Zur problematik der orthodontischen intrusion parodontal erkrankter zähne. *Parodontologie,* **2,** 87-102.

Diedrich, P., Fritz, U., Kinzinger, G. & Angelakis, J. (2003) Movement of periodontally affected teeth after guided tissue regeneration (GTR)--an experimental pilot study in animals. *Journal of Orofacial Orthopedics,* **64,** 214-227.

Ericsson, I. & Thilander, B. (1978) Orthodontic forces and recurrence of periodontal disease. An experimental study in the dog. *American Journal of Orthodontics,* **74,** 41-50.

Faltin, R. M., Faltin, K., Sander, F. G. & Arana-Chavez, V. E. (2001) Ultrastructure of cementum and periodontal ligament after continuous intrusion in humans: a transmission electron microscopy study. *European Journal of Orthodontics,* **23,** 35-49.

Favero, L., Brollo, P. & Bressan, E. (2002) Orthodontic anchorage with specific fixtures: related study analysis. *American Journal of Orthodontics and Dentofacial Orthopedics,* **122,** 84-94.

Fernandes, J. M., Rego, R. O., Spolidorio, L. C., Marcantonio, R. A., Marcantonio, J. E. & Cirelli, J. A. (2005) Enamel matrix proteins associated with GTR and bioactive glass in the treatment of class III furcation in dogs. *Brazilian Oral Research,* **19,** 169-175.

Gianelly, A. A. (1969) Force-induced changes in the vascularity of the periodontal ligament. *American Journal of Orthodontics,* **55,** 5-11.

Hamp, S. E., Nyman, S. & Lindhe, J. (1975) Periodontal treatment of multirooted teeth. Results after 5 years. *Journal of Clinical Periodontology,* **2,** 126-135.

Han, G., Huang, S., Von den Hoff, J. W., Zeng, X. & Kuijpers-Jagtman, A. M. (2005) Root resorption after orthodontic intrusion and extrusion: an intraindividual study. *Angle Orthodontist,* **75,** 912-918.

Harris, D. A., Jones, A. S. & Darendeliler, M. A. (2006) Physical properties of root cementum: part 8. Volumetric analysis of root resorption craters after application of controlled intrusive light and heavy orthodontic forces: a microcomputed tomography scan study. *American Journal of Orthodontics and Dentofacial Orthopedics,* **130,** 639-647.

Hossain, M. Z., Kyomen, S. & Tanne, K. (1996) Biologic responses of autogenous bone and beta-tricalcium phosphate ceramics transplanted into bone defects to orthodontic forces. *The Cleft Palate-Craniofacial Journal,* **33,** 277-283.

Jeon, P. D., Turley, P. K., Moon, H. B. & Ting, K. (1999) Analysis of stress in the periodontium of the maxillary first molar with a three-dimensional finite element model. *American Journal of Orthodontics and Dentofacial Orthopedics,* **115,** 267-274.

Karring, T. & Cortellini, P. (1999) Regenerative therapy: furcation defects. *Periodontolology 2000,* **19,** 115-137.

Katzhendler, E. & Steigman, S. (1999) Effect of repeated orthodontic treatment on the dental and periodontal tissues of the rat incisor. *American Journal of Orthodontics and Dentofacial Orthopedics,* **116**, 642-650.

Klein-Nulend, J., Semeins, C. M., Ajubi, N. E., Nijweide, P. J. & Burger, E. H. (1995a) Pulsating fluid flow increases nitric oxide (NO) synthesis by osteocytes but not periosteal fibroblasts--correlation with prostaglandin upregulation. *Biochemical and Biophysical Research Communications,* **217,** 640-648.

Klein-Nulend, J., van der, P. A., Semeins, C. M., Ajubi, N. E., Frangos, J. A., Nijweide, P. J. & Burger, E. H. (1995b) Sensitivity of osteocytes to biomechanical stress in vitro. *FASEB Journal,* **9,** 441-445.

Knippenberg, M., Helder, M. N., Blieck-Hogervorst, J. M., Wuisman, P. I. & Klein-Nulend, J. (2007) Prostaglandins Differentially Affect Osteogenic

Differentiation of Human Adipose Tissue-Derived Mesenchymal Stem Cells. *Tissue Engineering*, **13,** 2495-2503.

Krishnan, V. & Davidovitch, Z. (2006) Cellular, molecular, and tissue-level reactions to orthodontic force. *American Journal of Orthodontics and Dentofacial Orthopedics,* **129,** 469.e1-32.

Kyung, H. M., Park, H. S., Bae, S. M., Sung, J. H. & Kim, I. B. (2003) Development of orthodontic micro-implants for intraoral anchorage. *Journal of Clinical Orthodontics,* **37,** 321-328.

Liou, E. J., Pai, B. C. & Lin, J. C. (2004) Do miniscrews remain stationary under orthodontic forces? *American Journal of Orthodontics and Dentofacial Orthopedics,* **126,** 42-47.

Matsuura, M., Herr, Y., Han, K. Y., Lin, W. L., Genco, R. J. & Cho, M. I. (1995) Immunohistochemical expression of extracellular matrix components of normal and healing periodontal tissues in the beagle dog. *Journal of Periodontology,* **66,** 579-593.

Mehlbauer, M. J., Greenwell, H., Nouneh, I., Drisko, C., Wittwer, J. W., Yancey, J. & Rebitski, G. (2000) Improved closure rate of Class III furcations using a layered GTR technique. *The International Journal of Periodontics & Restorative Dentistry*, **20,** 285-295.

Meinel, L., Karageorgiou, V., Fajardo, R., Snyder, B., Shinde-Patil, V., Zichner, L., Kaplan, D., Langer, R. & Vunjak-Novakovic, G. (2004) Bone tissue engineering using human mesenchymal stem cells: effects of scaffold material and medium flow. *Annals of Biomedical Engineering,* **32,** 112-122.

Melsen, B. (2001) Tissue reaction to orthodontic tooth movement--a new paradigm. *European Journal of Orthodontics,* **23,** 671-681.

Melsen, B. & Agerbaek, N. (1991) [Can attachment gain be achieved by means of orthodontic measures?]. *Praktische Kieferorthopädie,* **5,** 11-16.

Melsen, B., Agerbaek, N., Eriksen, J. & Terp, S. (1988) New attachment through periodontal treatment and orthodontic intrusion. *American Journal of Orthodontics and Dentofacial Orthopedics*, **94,** 104-116.

Melsen, B., Agerbaek, N. & Markenstam, G. (1989) Intrusion of incisors in adult patients with marginal bone loss. *American Journal of Orthodontics and Dentofacial Orthopedics,* **96,** 232-241.

Miyawaki, S., Koyama, I., Inoue, M., Mishima, K., Sugahara, T. & Takano-Yamamoto, T. (2003) Factors associated with the stability of titanium screws placed in the posterior region for orthodontic anchorage. *American Journal of Orthodontics and Dentofacial Orthopedics,* **124,** 373-378.

Murakami, T., Yokota, S. & Takahama, Y. (1989) Periodontal changes after experimentally induced intrusion of the upper incisors in Macaca fuscata monkeys. *American Journal of Orthodontics and Dentofacial Orthopedics,* **95,** 115-126.

Nemcovsky, C. E., Beny, L., Shanberger, S., Feldman-Herman, S. & Vardimon, A. (2004) Bone apposition in surgical bony defects following orthodontic movement: a comparative histomorphometric study between root and periodontal ligament-damaged and periodontally intact rat molars. *Journal of Periodontology,* **75,** 1013-1019.

Nemcovsky, C. E., Zubery, Y., Artzi, Z. & Lieberman, M. A. (1996) Orthodontic tooth movement following guided tissue regeneration: report of three cases. *The International Journal of Adult Orthodontics and Orthognathic Surgery,* **11,** 347-355.

Ng, J., Major, P. W. & Flores-Mir, C. (2006) True molar intrusion attained during orthodontic treatment: a systematic review. *American Journal of Orthodontics and Dentofacial Orthopedics,* **130,** 709-714.

Ng, J., Major, P. W., Heo, G. & Flores-Mir, C. (2005) True incisor intrusion attained during orthodontic treatment: a systematic review and meta-analysis. *American Journal of Orthodontics and Dentofacial Orthopedics,* **128,** 212-219.

Noxon, S. J., King, G. J., Gu, G. & Huang, G. (2001) Osteoclast clearance from periodontal tissues during orthodontic tooth movement. *American Journal of Orthodontics and Dentofacial Orthopedics,* **120,** 466-476.

Nyman, S., Lindhe, J., Karring, T. & Rylander, H. (1982) New attachment following surgical treatment of human periodontal disease. *Journal of Clinical Periodontology,* **9,** 290-296.

Ohmae, M., Saito, S., Morohashi, T., Seki, K., Qu, H., Kanomi, R., Yamasaki, K. I., Okano, T., Yamada, S. & Shibasaki, Y. (2001) A clinical and histological evaluation of titanium mini-implants as anchors for orthodontic intrusion in the beagle dog. *American Journal of Orthodontics and Dentofacial Orthopedics,* **119,** 489-497.

Palioto, D. B., Joly, J. C., de Lima, A. F., Mota, L. F. & Caffesse, R. (2003) Clinical and radiographic treatment evaluation of class III furcation defects using GTR with and without inorganic bone matrix. *Journal of Clinical Periodontology,* **30,** 1-8.

Park, J. B., Matsuura, M., Han, K. Y., Norderyd, O., Lin, W. L., Genco, R. J. & Cho, M. I. (1995) Periodontal regeneration in class III furcation defects of beagle dogs using guided tissue regenerative therapy with platelet-derived growth factor. *Journal of Periodontology,* **66,** 462-477.

Parker, R. J. & Harris, E. F. (1998) Directions of orthodontic tooth movements associated with external apical root resorption of the maxillary central incisor. *American Journal of Orthodontics and Dentofacial Orthopedics,* **114,** 677-683.

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S. & Marshak, D. R. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science,* **284,** 143-147.

Polson, A., Caton, J., Polson, A. P., Nyman, S., Novak, J. & Reed, B. (1984) Periodontal response after tooth movement into intrabony defects. *Journal of Periodontology,* **55,** 197-202.

Pontoriero, R., Lindhe, J., Nyman, S., Karring, T., Rosenberg, E. & Sanavi, F. (1989) Guided tissue regeneration in the treatment of furcation defects in mandibular molars. A clinical study of degree III involvements. *Journal of Clinical Periodontology,* **16,** 170-174.

Re, S., Cardaropoli, D., Abundo, R. & Corrente, G. (2004) Reduction of gingival recession following orthodontic intrusion in periodontally compromised patients. *Orthodontics & Craniofacial Research,* **7,** 35-39.

Re, S., Corrente, G., Abundo, R. & Cardaropoli, D. (2000) Orthodontic treatment in periodontally compromised patients: 12-year report. *The International Journal of Periodontics & Restorative Dentistry,* **20,** 31-39.

Re, S., Corrente, G., Abundo, R. & Cardaropoli, D. (2002) Orthodontic movement into bone defects augmented with bovine bone mineral and fibrin sealer: a reentry case report. *The International Journal of Periodontics & Restorative Dentistry*, **22,** 138-145.

Reynolds, M. A., Aichelmann-Reidy, M. E., Branch-Mays, G. L. & Gunsolley, J. C. (2003) The efficacy of bone replacement grafts in the treatment of periodontal osseous defects. A systematic review. *Annals of Periodontolology,* **8,** 227-265.

Roberts, W. E. & Chase, D. C. (1981) Kinetics of cell proliferation and migration associated with orthodontically-induced osteogenesis. *Journal of Dental Research,* **60,** 174-181.

Roberts, W. E. & Jee, W. S. (1974) Cell kinetics of orthodontically-stimulated and non-stimulated periodontal ligament in the rat. *Archives of Oral Biology,* **19,** 17-21.

Roriz, V. M., Souza, S. L., Taba, M., Jr., Palioto, D. B. & Grisi, M. F. (2006) Treatment of Class III furcation defects with expanded polytetrafluoroethylene membrane associated or not with anorganic bone matrix/synthetic cell-binding peptide: a histologic and histomorphometric study in dogs. *Journal of Periodontology,***77,** 490-497.

Rossa, C. Jr., Marcantonio, E. Jr, Cirelli, J. A., Marcantonio, R. A. C., Spolidorio, L. C. & Fogo, J. C. (2000) Regeneration of Class III furcation defects with basic fibroblast growth factor (b-FGF) associated with GTR. A descriptive and histometric study in dogs. *Journal of Periodontology,* **71,** 775-784.

Rupprecht, R. D., Horning, G. M. & Towle, H. J. III (2001) A clinical evaluation of hydroxyapatite cement in the treatment of Class III furcation defects. *Journal of Periodontology,* **72,** 1443-1450.

Salina, S., Maiorana, C., Iezzi, G., Colombo, A., Fontana, F. & Piattelli, A. (2006) Histological evaluation, in rabbit tibiae, of osseointegration of mini-implants in sites prepared with Er:YAG laser versus sites prepared with traditional burs. *Journal of Long- Term Effects of Medical Implants,* **16,** 145-156.

Simion, M. & Fontana, F. (2004) Autogenous and xenogeneic bone grafts for the bone regeneration. A literature review. *Minerva Stomatologica,* **53,** 191-206.

Southard, T. E., Buckley, M. J., Spivey, J. D., Krizan, K. E. & Casko, J. S. (1995) Intrusion anchorage potential of teeth versus rigid endosseous implants: a clinical and radiographic evaluation. *American Journal of Orthodontics and Dentofacial Orthopedics,* **107,** 115-120.

Stelzel, M. J. & Flores-de-Jacoby, L. (1998) Guided tissue regeneration in a combined periodontal and orthodontic treatment: a case report. *The International Journal of Periodontics & Restorative Dentistry*, **18,** 189-195.

Thomas, G. P. & el Haj, A. J. (1996) Bone marrow stromal cells are load responsive in vitro. *Calcified Tissue International,* **58,** 101-108.

Vardimon, A. D., Nemcovsky, C. E. & Dre, E. (2001) Orthodontic tooth movement enhances bone healing of surgical bony defects in rats. *Journal of Periodontology,* **72,** 858-864.

Wikesjo, U. M., Selvig, K. A., Zimmerman, G. & Nilveus, R. (1991) Periodontal repair in dogs: healing in experimentally created chronic periodontal defects. *Journal of Periodontology,* **62,** 258-263.

Yao, C. C., Lee, J. J., Chen, H. Y., Chang, Z. C., Chang, H. F. & Chen, Y. J. (2005) Maxillary molar intrusion with fixed appliances and mini-implant anchorage studied in three dimensions. *Angle Orthodontist,* **75,** 754-760.

Yao, C. C., Wu, C. B., Wu, H. Y., Kok, S. H., Chang, H. F. & Chen, Y. J. (2004) Intrusion of the overerupted upper left first and second molars by mini-

implants with partial-fixed orthodontic appliances: a case report. *Angle Orthodontist,* **74,** 550-557.

Table 1: Horizontal furcation classification in lower third and fourth pre-molars in dogs, according to groups and time-points.

Groups	Cl III - R	Cl III - F	Cl I/II - F	0 - F
OFD	7	4	0	3
OFD+I	7	0*	4	3
GTR/BA	7	3	2	2
GTR/BA+I	7	0*	6	1

Data are expressed as the number of teeth with the presence of class I/II, III or absence (0) of furcation lesion at regeneration (R) and end of contention (F) time-point. * Significant effect of intrusion in the reduction of class III furcation lesions, between R and F time-points, $p < 0.05$ (n=7).

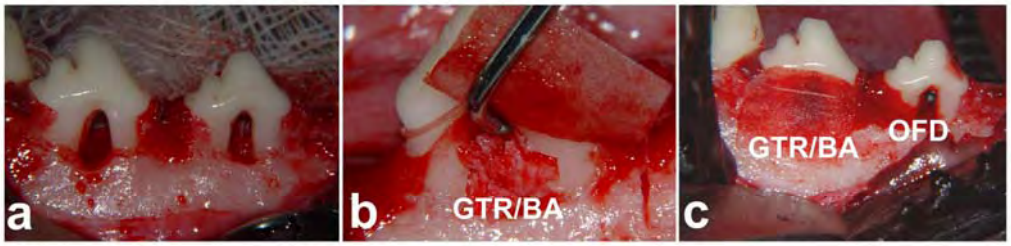

Fig. 1 Periodontal treatment of the class III furcation lesions. **(a)** Surgical access of Class III furcation lesions after cronification time-point. **(b)** Treatment with guided tissue regeneration using absorbable membrane and bone autograft (GTR/BA group). **(c)** Adjacent pre-molars from GTR/BA and OFD groups.

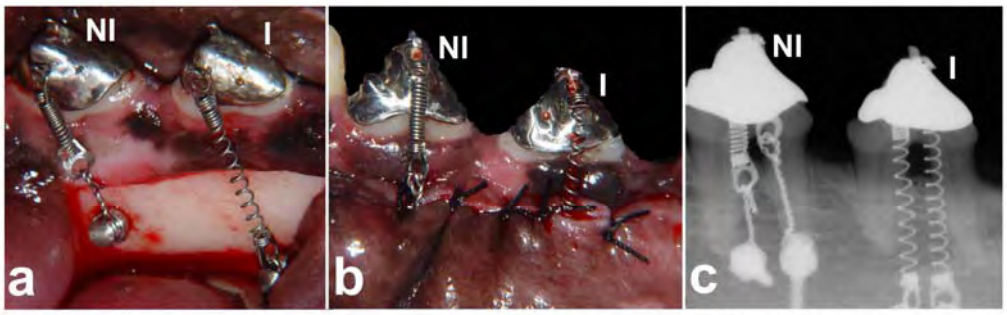

Fig. 2 Orthodontic intrusion after periodontal treatment. **(a)** Mini-implants placed in the apical cortical bone of the mandible connected to metallic crowns by nickel-titanium springs. These were activated on teeth of the intrusion groups (I) and passive in non intrusion groups (NI). **(b)** Suture after mini-implants installation. **(c)** Radiographic exam was taken immediately after mini-implants installation.

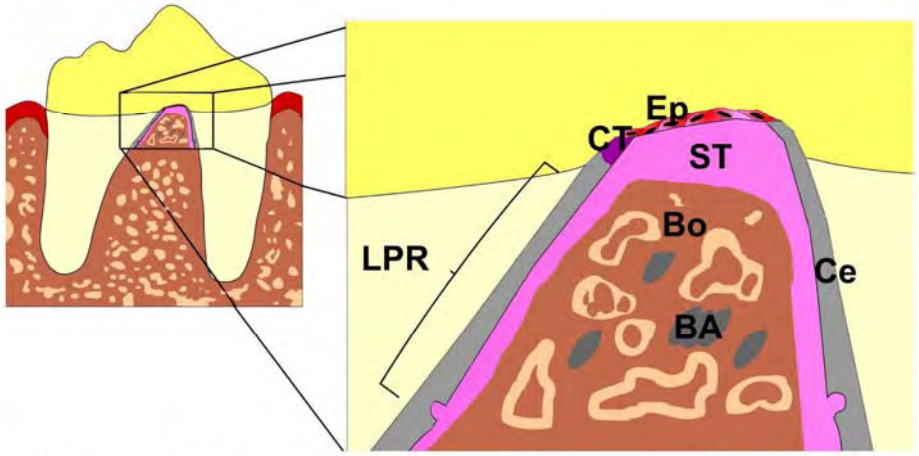

Fig. 3 Schematic representation of linear and area variables evaluated in the histometric analysis of the furcation defects. Ce - cementum formation; CT - connective tissue; LPR - linear periodontal regeneration; Ep - epithelium; Bo - bone tissue area; ST - soft tissue area; BA - bone autograft area.

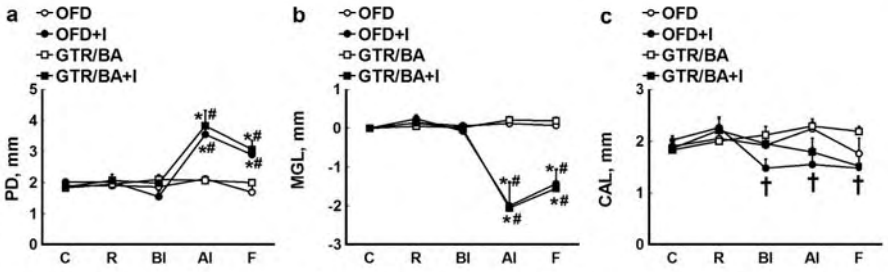

Fig. 4 Clinical measurement for each group, at five time-points. **(a)** Probing depth (PD), **(b)** Marginal gingival level (MGL) and **(c)** Clinical attachment level (CAL). Values are expressed as mean percentage ± SEM (n=7). * $p<0.01$ - significantly different from C, R and BI time-points (intra - treatment comparison); # $p<0.01$ - significantly different from OFD or GTR/BA groups in the same time-point (inter - treatment comparison); † $p<0.05$ - significantly different from C and R time-points (intra - treatment comparison).

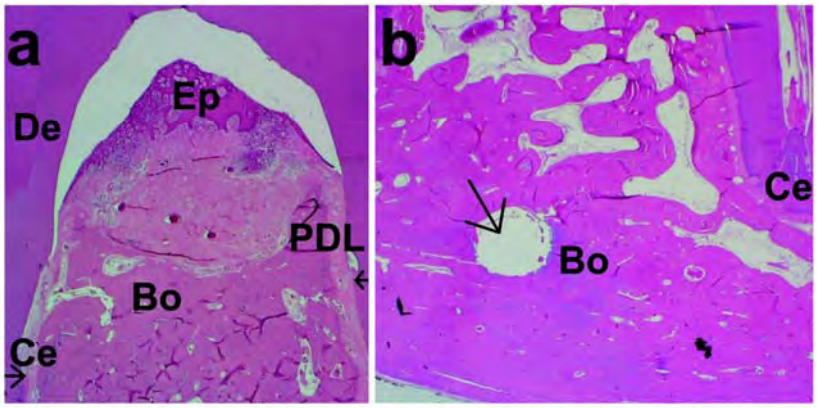

Fig. 5 Panoramic view of the furcation region and mini-implant site in the OFD group. **(a)** Healing of the furcation area with epithelial migration (Ep), new cementum (Ce), periodontal ligament (PDL), and alveolar bone formation (Bo). Arrows indicate the notches. Dentin (De). **(b)** Arrow indicates mini-implant site evolved by bone (Bo). Haematoxylin and eosin, original magnification x 20.

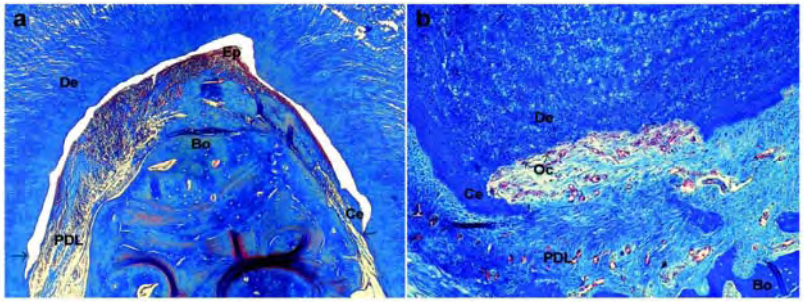

Fig. 6 Panoramic view of the furcation region and apical root resorption in the OFD+I group. **(a)** Healing of the furcation area with epithelial migration (Ep), new cementum (Ce), periodontal ligament (PDL) and alveolar bone formation (Bo). Dentin (De). Arrows indicate the notches. Masson trichrome, original magnification x 20. **(b)** Active apical root resorption. Arrows indicate osteoclasts (Oc). Masson trichrome, original magnification x 100.

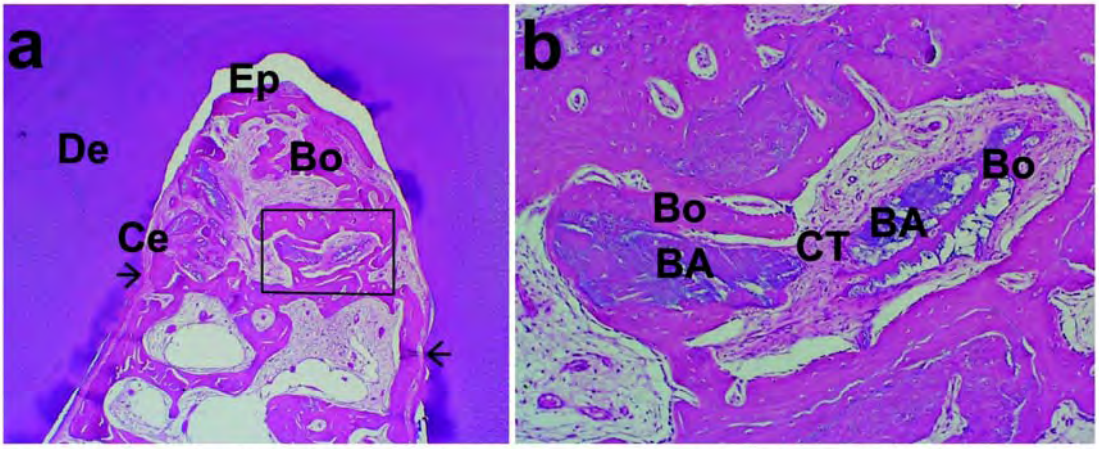

Fig. 7 Panoramic view of the furcation area and bone autograft in the GTR/BA group. **(a)** Healing of the furcation area with epithelial migration (Ep), new cementum (Ce), periodontal ligament (PDL) and alveolar bone formation (Bo). Dentin (De). Arrows indicate the notches and rectangle indicates figure 7b. Haematoxylin and eosin, original magnification x 20. **(b)** Bone autograft (BA) integrated to newly formed bone (Bo) and evolved by connective tissue (CT). BA is being substituted by newly formed bone (Bo). Haematoxylin and eosin, original magnification x 100.

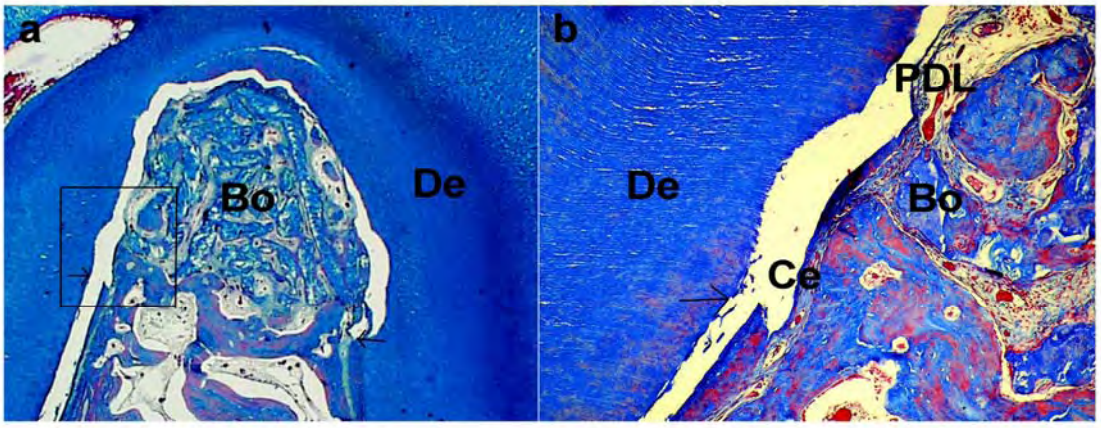

Fig. 8 Panoramic view of the furcation region in the GTR/BA+I group. **(a)** Healing of the furcation area with new cementum, periodontal ligament and alveolar bone formation (Bo). Dentin (De). Arrows indicate the notches and square indicates figure 8b. Masson trichrome, original magnification x 20. **(b)** Detail of the square in the figure 8a with new cementum formation (Ce), periodontal ligament (PDL) and alveolar bone (Bo) above the notch. Dentin (De). Masson trichrome, original magnification x 100.

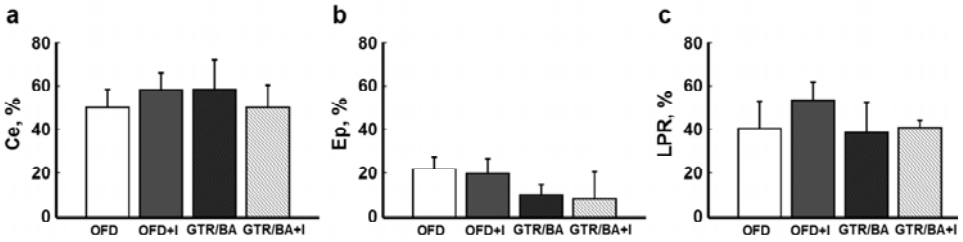

Fig. 9 Histometric analysis of root linear measurements in the furcation region between the notches. **(a)** Ce - cementum formation; **(b)** Ep - epithelial migration and **(c)** LPR - linear periodontal regeneration. There were not significant differences inter groups. Values are expressed as mean percentage ± SEM (n=7). OFD, open flap debridement; OFD+I, open flap debridement plus intrusion; GTR/BA, guided tissue regeneration and bone autograft; GTR/BA+I, guided tissue regeneration and bone autograft plus intrusion.

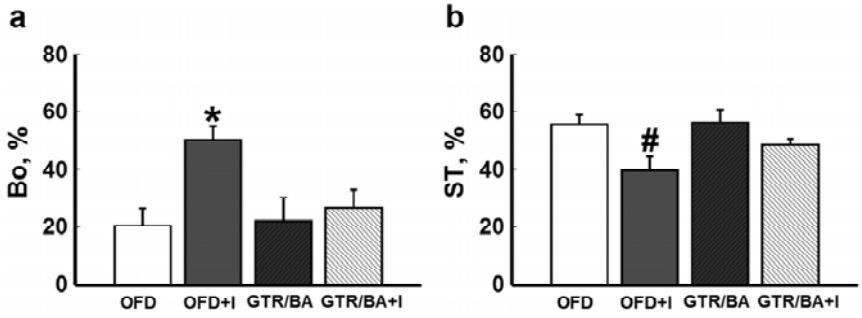

Fig. 10 Histometric analysis of area measurements in the furcation region between the notches. **(a,b)** OFD group presented smaller area of soft tissue and bigger area of bone tissue ($p < 0.05$). Values are expressed as mean percentage ± SEM (n=7). OFD, open flap debridement; OFD+I, open flap debridement plus intrusion; GTR/BA, guided tissue regeneration and bone autograft; GTR/BA+I, guided tissue regeneration and bone autograft plus intrusion; Bo = bone tissue area; ST = soft tissue area. * $p<0.05$ - significant difference from OFD and GTR/BA; # $p<0.05$ - significant difference from OFD.

4 CAPÍTULO 2

Este capítulo é constituído por uma introdução específica ao assunto "Eventos envolvidos na resposta celular à carga mecânica." e pelo seguinte artigo:

da Silva VC, Bakker AD, Blaauboer ME, Bacabac RG, Semeins CM, Marcantonio RAC, Cirelli JA, Klein-Nulend J. TNFα and IL-1β inhibit fluid shear stress-induced nitric oxide production by osteocytes and osteoblasts. Submetido para publicação no periódico Journal of Bone and Mineral Research.

No Anexo 3 está incluída a descrição detalhada da seção Material e Métodos.

Eventos envolvidos na resposta celular à carga mecânica

Durante o movimento dentário, as células e a matriz extracelular do ligamento periodontal e do osso alveolar respondem concomitantemente resultando em remodelação tecidual[78]. Durante as fases iniciais do movimento dentário, há um fluxo contínuo de fluidos no ligamento periodontal e tecido ósseo levando à distorção de células e matriz[78]. Em conseqüência, citocinas, fatores de crescimento e outras substâncias são produzidas e liberadas para iniciar e manter a remodelação óssea resultando no movimento dentário[78].

A formação óssea é resultado de eventos que envolvem diferenciação de precursores de osteoblastos das células mesenquimais, maturação de osteoblastos e formação de matriz, seguida pela sua mineralização[61]. Após a deposição de matriz óssea, osteoblastos gradualmente se achatam para tornarem-se células de revestimento ósseo enquanto outros ficam embebidos na matriz neoformada[61]. Osteoblastos envolvidos por matriz calcificada permanecendo em lacunas ósseas são conhecidos como osteócitos. Cerca de 95% do esqueleto adulto é composto por osteócitos e células de revestimento ósseo[106]. Osteócitos mantém contato entre si e com as células da superfície óssea via junções "gap" localizadas nos longos processos celulares dentro de canalículos na matriz óssea que permitem a passagem e trocas de íons e pequenas moléculas entre células comunicantes[41,128,129,148]. Recentes observações em microscopia eletrônica revelam uma matriz fibrosa não calcificada ao redor dos osteócitos preenchida por líquido intersticial[75,148,156]. Embora já estabelecido que o fluido intersticial atua no metabolismo (troca de nutrientes e liberação de subprodutos) e adaptação óssea, sua ultra-estrutura e constituição não são totalmente conhecidas.

Células ósseas reconhecem a aplicação da carga mecânica gerando uma remodelação óssea coordenada. Estudos recentes mostram que a mecanosensibilidade óssea é tarefa primária dos osteócitos[19]. Enquanto células musculares podem ser estiradas mais que 50% devido a função fisiológica, células ósseas não se deformam mais que 0.2 a 0.4% do tamanho original[20,121,122]. Contudo, para obter resposta de células ósseas in vitro são necessárias

deformações celulares na ordem de 1-3% o que não ocorre in vivo[93,94]. Portanto, diferentemente do tecido muscular, deformações nas células ósseas não são as responsáveis pela mecanoativação celular. O que ocorre é que a carga mecânica resulta em movimento do fluido na rede lacuno-canalicular do tecido ósseo[79,109], gerando um atrito na superfície celular que ativa mecanicamente os osteócitos [10,29,30,149]. Estudos in vivo observaram que moléculas injetadas intravascularmente estavam presentes no sistema lacuno-canalicular ósseo após a aplicação da carga mecânica devido ao movimento do fluido intersticial na matriz óssea mineralizada[75,76]. Vários estudos em animais e usando cultura de células suportam esse conceito observando maior formação óssea e/ou produção de substâncias sinalizadoras de formação óssea após a aplicação de carga mecânica e movimento do fluido intersticial[2,72,73,104,110,137,138,151]. Juntos eles sugerem que os osteócitos e a porosidade lacuno-canalicular são o sítio da mecanotransdução no tecido ósseo. Mecanotrandução é o processo pelo qual a energia mecânica gerada pelo movimento do fluido intersticial é convertida em sinais químicos e/ou elétricos pelas células ósseas[19]. Esse processo tem sido estudado por meio da técnica de fluxo de fluido pulsátil (PFF), que simula o movimento do fluido intersticial sobre as células ósseas[19,73].

As pesquisas usando PFF foram inicialmente aplicadas para estudo de células endoteliais dos vasos sangüíneos[47]. Células endoteliais são reconhecidas como células mecanosensoriais e respondem a variações de fluxo sangüíneo com a liberação de óxido nítrico e prostaglandinas para manter o tônus muscular constante e controlar a pressão arterial[59]. O movimento do fluido intersticial na rede lacuno-canalicular ativa os osteócitos e também assegura o transporte de nutrientes, moléculas sinalizadoras e produtos do metabolismo. Assim, a técnica do PFF passou a ser utilizada para análise da resposta de células ósseas à carga mecânica[72,73].

Os osteócitos são as células mecanosensíveis do tecido ósseo capazes de responder ao estresse mecânico por meio da liberação prolongada de moléculas sinalizadoras quando comparados a osteoblastos e fibroblastos periosteais embora estas células também respondam a carga mecânica[72,73,149]. Assim, os osteócitos

sinalizam para o recrutamento de osteoclastos e osteoblastos controlando a remodelação óssea.

Recentes estudos propõem um papel para o óxido nítrico (NO) como uma molécula sinalizadora de mecanotransdução durante o estágio inicial do movimento ortodôntico[3,33,57,130,154]. NO é produzido pela enzima óxido nítrico sintetase (NOS) a partir de oxigênio molecular e nitrogênio-guanidino terminal do aminoácido L-arginina[144]. Ele reage rapidamente com oxigênio formando metabólitos como nitrito (NO_2) e a produção de óxido nítrico pode ser acessada indiretamente medindo o acúmulo desses metabólitos usando reação de Griess[144]. O NO formado em presença de carga mecânica é produzido pela enzima NOS constitutiva endotelial (ecNOS)[144]. Esta é uma enzima unida à membrana plasmática que originalmente foi detectada apenas no endotélio, mas posteriormente foi também encontrada em osteócitos e osteoblastos[60,71,157] além de nervos e fibroblastos do ligamento periodontal[78]. ecNOS é constitutivamente expressada em baixos níveis nos tecidos de origem e sua atividade é principalmente regulada por mudanças na concentração de cálcio intracelular[144]. Essa enzima produz apenas NO relacionado ao atrito gerado pelo movimento do fluido, como ocorre nas células endoteliais em função do fluxo sangüíneo[21,140].

Durante as primeiras 6 horas após o movimento dental com aplicação de forças leves e contínuas, a atividade da enzima NOS aumenta nos tecidos periodontais[154] pois células ósseas e fibroblastos respondem à carga mecânica com a produção de NO[72,73]. Estudos em animais têm mostrado que NO é essencial à indução de formação óssea[139]. NO é capaz de inibir apoptose de osteócitos e osteoblastos[38,99,108,120] e atividade osteoclástica[85] favorecendo a formação óssea. Além disso, NO é um forte inibidor de reabsorção óssea que atua suprimindo a expressão de receptor ativador do fator nuclear-κB ligante (RANK-L) e aumentando a expressão de osteoprotegerina (OPG) nas células ósseas, reduzindo assim a formação e atividade de osteoclastos[44]. Assim, NO atua na remodelação óssea modulando atividade de osteoblasto e osteoclasto.

Durante o movimento ortodôntico, a carga mecânica aplicada nos tecidos periodontais gera uma compressão no ligamento periodontal no lado de pressão e

estiramento de fibras periodontais no lado de tensão[92]. No lado de tensão, o estiramento das fibras periodontais gera uma ligeira curvatura da parede alveolar que assume uma forma côncava[43,78,89]. Isso causa uma pequena deformação/pressão no tecido ósseo e movimento do fluido intersticial na rede lacuno-canalicular levando a produção de moléculas sinalizadoras principalmente pelos osteócitos, mas também por osteoblastos, fibroblastos e células de revestimento ósseo[137]. Essas moléculas, principalmente NO, causam inibição de reabsorção óssea estimulando a formação óssea[46,139].

No lado de pressão do ligamento periodontal, ocorre um afrouxamento das fibras periodontais e o tecido ósseo assume uma superfície convexa[43,78]. Desse modo, existe a hipótese que o movimento do fluido intersticial da rede canalicular dos osteócitos seria reduzido[19,89]. A baixa produção de NO leva a apoptose de osteócitos, que atrai osteoclastos e, conseqüentemente, aumenta a reabsorção óssea[19].

Além disso, nos três primeiros dias após a ativação da carga ortodôntica, ocorre vasodilação e migração de leucócitos que produzem citocinas pró-inflamatórias como TNFα e IL-1ß[13,34,56,84,141]. Estas interagem com as células locais estimulando a expressão de genes associados à reabsorção óssea[34,49]. TNFα e IL-1ß são produzidos por inúmeras células incluindo macrófagos, linfócitos T, fibroblastos, células endoteliais, osteoblastos, osteócitos, osteoclastos, cementoblastos e cementoclastos[5,16,125]. TNFα e IL-1ß estão presentes em maior quantidade no lado de pressão do ligamento periodontal quando comparado ao lado de tensão[13,49,56,84,141]. No entanto, a seqüência de eventos que ocorre após a aplicação de força ortodôntica envolve o movimento de fluidos do lado de pressão do LP para o lado de tensão[78,84] e com isso citocinas também estão presentes no lado de tensão do LP[13,84]. IL-1β afeta o metabolismo ósseo estimulando a reabsorção óssea[53,58] e inibindo a formação óssea[132]. TNFα e IL-1ß aumentam o número de células pré-osteoclásticas[62] e agem sinergicamente estimulando a reabsorção óssea[12,22,39,77,80]. Porém, ainda não está elucidado se durante a aplicação da carga mecânica, a presença de TNFα e/ou IL-1ß afeta a

mecanotransdução, que é um importante processo no controle da remodelação óssea.

TNFα AND IL-1β INHIBIT FLUID SHEAR STRESS-INDUCED NITRIC OXIDE PRODUCTION BY OSTEOCYTES AND OSTEOBLASTS

Vanessa C. da Silva[1,2], Astrid D. Bakker[1], Marjolein E. Blaauboer[1], Rommel G. Bacabac[1], Cornelis M. Semeins[1], Rosemary A.C. Marcantonio[2], Joni A. Cirelli[2], Jenneke Klein-Nulend[1]

[1] Department of Oral Cell Biology, ACTA-Universiteit van Amsterdam and Vrije Universiteit, Research Institute MOVE, Amsterdam, The Netherlands
[2] UNESP - São Paulo State University, Department of Periodontology, School of Dentistry at Araraquara – SP, Brazil

Funding: This work was supported by a grant of the Foundation for the Coordination of Higher Education and Graduate Training - Brazil (MEC/CAPES BEX0481/06-8), to V.C. da Silva, by a grant of the Research Institute MOVE of the Vrije Universiteit Amsterdam to A.D. Bakker, and by a grant of the Space Research Organization of The Netherlands to R.G. Bacabac (SRON grant MG-055) who also received financial assistance from the Netherlands Organization For International Cooperation In Higher Education (Physics Development Project PHL-146).

Running title: TNFα and IL-1β inhibit the mechanoresponse of bone cells

Author's email addresses: silvavan@uol.com.br; a.bakker@vumc.nl; me.blaauboer@vumc.nl; bacabac@amolf.nl; c.semeins@vumc.nl; adriana@foar.unesp.br; cirelli@foar.unesp.br; j.kleinnulend@vumc.nl

Corresponding author: Jenneke Klein-Nulend, PhD, Dept Oral Cell Biology, ACTA-Vrije Universiteit, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands.
tel: +31 20 4448660, fax: +31 20 4448683, email: j.kleinnulend@vumc.nl

Number of words/characters of text in abstract and manuscript: 6195/34365.
Number of figures: 0 colour, 4 black and white figures, 2 tables.
Conflict of interest: All authors have no conflict of interest.

MICROABSTRACT

We show that TNFα and IL-1ß inhibit the upregulation of NO production after mechanical stimulation, an essential chemical signal in bone remodeling. This inhibition was associated with a prevention of fluid shear stress-induced $[Ca^{2+}]_i$ upregulation. These data support the concept that cytokines affect the balance between bone resorption and formation *in vivo* by changing the mechanoresponsiveness of bone cells.

ABSTRACT

Introduction: Osteocytes translate loading-induced canalicular fluid flow into signals, such as nitric oxide (NO), that alter osteoclast and osteoblast recruitment and activity. In the presence of elevated cytokine levels the balance between bone resorption and formation is often disturbed. Hence we investigated whether TNFα and/or IL-1β affect the NO response of bone cells to mechanical loading.

Methods: MLO-Y4 osteocytes and MC3T3-E1 osteoblasts were incubated with TNFα (0.5-30 ng/ml), IL-1β (0.1-10 ng/ml) or calcium inhibitors TMB-8, $GdCl_3$, or EGTA for 30 min, followed by 30 min of pulsating fluid flow (PFF; 0.6±0.3 Pa, 5 Hz) with or without cytokines or calcium inhibitors. Apoptosis was quantified using the Caspase-Glo 3/7 assay. NO production was measured using Griess reagent. $[Ca^{2+}]_i$ was monitored using Fluo-4 AM.

Results: Neither TNFα nor IL-1ß affected apoptosis in MLO-Y4 and MC3T3-E1 cells. PFF stimulated NO production within 5 min (MLO-Y4, 4.4-fold; MC3T3-E1, 2.3-fold), up to 30 min. Within seconds PFF also stimulated $[Ca]_i^{2+}$ (MLO-Y4, 4.7-fold; MC3T3-E1, 5.8-fold). Both TNFα and IL-1ß decreased PFF-induced NO production at all time points, with maximal inhibition by 30 ng/ml TNFα at 30 min (MLO-Y4, 50%; MC3T3-E1, 69%), and by 1 ng/ml IL-1ß at 15 min (MLO-Y4, 56%; MC3T3-E1, 70%). Both TNFα and IL-1β abolished the PFF-induced rise in $[Ca^{2+}]_i$ in both cell types. In MLO-Y4 cells, PFF-induced NO production at 5 min was inhibited by TMB-8 (57%), $GdCl_3$ (70%), and EGTA (67%). In MC3T3-E1 cells, this effect was inhibited by TMB-8 (63%) and $GdCl_3$ (42%), but not EGTA.

Conclusions: TNFα and IL-1β inhibit fluid shear stress-induced NO production by MLO-Y4 and MC3T3-E1 cells, likely via prevention of the rapid rise in $[Ca^{2+}]_i$ associated with the onset of PFF. This suggests that the presence of TNFα and IL-1β affect the balance between bone resorption and formation *in vivo* by changing the mechanoresponsiveness of bone cells.

Key Words: mechanical loading; bone cells; cytokines; nitric oxide; calcium.

INTRODUCTION

Bone mass and structure are strongly affected by mechanical loading. Increased mechanical loading results in a gain of bone mass and mineral density *in vivo*[1-3], while unloading of bones is known to reduce bone formation, mineral content, and bone matrix protein production[4-6]. Using a computer model it has been demonstrated that the emergence and maintenance of trabecular architecture as an optimal mechanical structure is governed by the response of mechanosensitive bone cells to external loads[7]. Osteocytes are currently considered as the mechanosensory cells of bone, driving the process of bone mechanical adaptation[8,9].

Osteocytes are the most prevalent bone cells, and are uniquely positioned within the bone matrix, their cell bodies being located in lacunae, and their long interconnected cell processes being positioned in the canaliculi[10]. It is currently believed that loading on bone generates a flow of interstitial fluid through the lacuno-canalicular network[9]. This flow is sensed by the osteocytes which respond among others with a rapid increase in intracellular calcium concentration[11]. This response is followed by the release of signalling factors such as nitric oxide (NO) and prostaglandin E_2 (PGE_2), which may alter osteoblast and osteoclast activity[9,12-14]. Thus osteocytes orchestrate the balance between local bone resorption and bone formation, thereby driving the mechanical adaptation of bone under physiological conditions.

NO is essential for load-induced bone formation *in vivo*[15,16]. Several studies have shown that NO production is rapidly increased in response to mechanical stress in bone cells, including isolated osteocytes[8,17-20]. The mechanism of action of NO in bone mechanotransduction is not fully elucidated, but NO production has been used as a marker for studying the mechanosensitivity of osteocytes[8]. NO is produced when L-arginine is converted to L-citrulline in the presence of NO synthase (NOS) enzyme[21], molecular oxygen, NADPH, and other cofactors[22]. In bone, the constitutive endothelial NOS (eNOS) is known to produce NO as a result of fluid shear stress[19]. The activity of eNOS depends on substrate availability and binding to calmodulin, which is calcium dependent[23].

During inflammatory diseases like periodontitis and arthritis, as well as during orthodontic tooth movement, the balance between bone formation and resorption is often disturbed, resulting in a localized bone loss[24-26]. It is likely that cytokines such as tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β), which are readily present during inflammation[27,28] as well as during orthodontic tooth movement[29-33], are involved in this process. TNFα and IL-1β are known to affect bone metabolism, by direct stimulation of bone resorption[34-38] and inhibition of bone formation[39]. TNFα and IL-1ß increase the number of pre-osteoclastic cells[40], and act synergistically to stimulate bone resorption[34,35,41,42]. Cytokines stimulate PGE_2 production by osteoblasts, which activate osteoclast activity[43]. However, it is still unknown whether TNFα and/or IL-1β can serve as negative regulators of bone metabolism by affecting the response of bone cells to mechanical loading. The aim of this study was therefore to assess whether TNFα or IL-1β affect the bone cell response to fluid shear stress, using NO production as parameter for the bone cell response. Representative mechanosensitive cells of the osteogenic lineage were used, i.e. MLO-Y4 osteocyte-like cells and MC3T3-E1 osteoblast-like cells. We hypothesized that cytokines inhibit the fluid shear stress-induced production of NO, possibly via inhibition of $[Ca^{2+}]_I$ transients.

MATERIALS AND METHODS

Bone cell culture

MC3T3-E1 osteoblasts[44] were cultured up to near-confluency in 75-cm^2 flasks (Nunc, Roskilde, Denmark), using α-Modified Eagle's Medium (α-MEM; Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; Gibco), ascorbate (50 μg/ml; Merck, Darmstadt, Germany), l-glutamine (300 μg/ml; Merck), penicillin (300 μg/ml; Sigma-Aldrich, St. Louis, MO, USA), streptomycin (250 μg/ml; Sigma), fungizone (1.25 μg/ml; Gibco), at 37ºC with 5% CO_2 in air. Cells between passages 10-20 were harvested using 0.25% trypsin (Difco Laboratories, Detroit, MI, USA) and 0.1% EDTA (Sigma) in PBS and seeded at 3 x 10^5 cells/cm^2 on polylysine-coated (50 μg/ml; poly-L-lysine hydrobromide; Sigma) glass slides (25 cm^2) in α-MEM with 10% FBS and supplements. They were incubated overnight at 37ºC with 5% CO_2 in air to promote cell attachment prior to fluid shear stress experimental treatment as described below.

MLO-Y4 osteocytes (kindly provided by Dr Bonewald, San Antonio, TX, USA)[45] were also cultured up to near-confluency in 75-cm^2 culture flasks using α-MEM supplemented with 5% FBS, 5% calf serum (CS; Gibco), gentamicin (50 μg/ml), fungizone (1.25 μg/ml), penicillin (60 μg/ml), and streptomycin (50 μg/ml), at 37ºC and 5% CO_2 in air. Cells between passages 30-35 were harvested and seeded at 3 x 10^5 cells/cm^2 on polylysine-coated glass slides in α-MEM with 5% FBS and 5% calf serum and supplements as described for MC3T3-E1 cells.

Pulsatile fluid flow (PFF)

Pulsating fluid shear stress, at 5 Hz pulse frequency, was generated by pumping 13 ml of culture medium in a pulsatile manner through a parallel-plate flow chamber containing the bone cells, as described previously[8]. Mean fluid shear stress was 0.6 Pa with pulse amplitude of 0.3 Pa, and the estimated peak stress rate was 8.4 Pa/sec as described earlier[17,46,47]. Control cultures were kept under static conditions in a petridish, under similar conditions as the experimental cultures, i.e. at 37ºC with 5% CO_2 in air. TNFα (Sigma) at 0.5, 10, or 30 ng/ml, IL-1ß (Sigma)

at 0.1, 1, or 10 ng/ml, or calcium inhibitors (described below) were added 30 min before the start of the PFF or static treatment, and were also present during the entire treatment period. After 5, 15 and 30 min of treatment with or without PFF, medium was collected and assayed for NO concentrations.

Calcium inhibitors

To verify whether NO production is dependent of calcium, calcium inhibitors were used under static and PFF conditions in MLO-Y4 and MC3T3-E1 cells. To inhibit intracellular calcium mobilization from the endoplasmic reticulum 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate·HCl (TMB-8, Biomol Research Laboratories Inc, Plymouth Meeting, PA, USA) was used at a concentration of 20 μM[48]. Gadolinium chloride ($GdCl_3$, Sigma) was used at 10 μM to inhibit mechanosensitive calcium channels, and ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA, Sigma) as an extracellular calcium scavenger at a concentration of 2 mM[48].

Nitric oxide

The conditioned medium was assayed for NO, which was measured as nitrite (NO_2^-) accumulation in the conditioned medium, using Griess reagent (1% sulphanilamide, 0.1% naphthylethelene-diamine-dihydrochloride and 2.5 M H_3PO_4). The absorbance was measured at 540 nm. NO concentrations were determined using a standard curve derived from known concentrations of $NaNO_2$ in non-conditioned culture medium. Data from separate experiments were collected and normalized with respect to static controls, and expressed as treatment-over-control (PFF/Stat) ratios, or as absolute values where indicated.

Caspase-3/7 activity and DNA content

After 30 min of static and PFF conditions with or without TNFα or IL-1ß, cell-lysis-based reagent of the Caspase-Glo 3/7 Assay (Promega, Madison, WI, USA) was added to the cells for the assessment of caspase-3/7 activity with a luminometer (Berthold Technologies, Bad Wildbad, Germany), according to the

manufacturer's instructions. DNA in the cell lysate was determined by a CyQUANT Cell Proliferation Assay (Molecular Probes Inc., Eugene, OR, USA). Apoptosis was expressed in relative light units per ng DNA.

Intracellular calcium levels

To determine whether PFF in the presence or absence of TNFα or IL-1β cause alterations in intracellular calcium levels, MLO-Y4 and MC3T3-E1 cells were analyzed using confocal microscopy (Leica TCS-SP2, Mannheim, Germany). MC3T3-E1 cells between passages 10-20 and MLO-Y4 cells between passages 30-35 were seeded at 1 x 10^4 cells/cm^2 on polylysine-coated (50 µg/ml) glass slides (5 cm^2) in α-MEM with antibiotics and 10% FBS (MC3T3-E1 cells) or 5% FBS and 5% calf serum (MLO-Y4 cells). Cells were incubated overnight at 37ºC with 5% CO_2 in air to promote cell attachment prior to fluid shear stress experimental treatment as described below.

One hour before static and PFF treatment, cells were incubated with 10 µM Fluo-4 acetoxymethyl (AM) (Invitrogen, Eugene, OR, USA) in α-MEM at 37^{o}C for 30 min. Cells were gently washed with sterile Dulbecco's phosphate-buffered saline (Gibco). To study the interaction of mechanical loading and cytokines, TNFα (10 ng/ml) or IL-1ß (1 ng/ml) were added to α-MEM without supplements 30 min before static and PFF treatment. TNFα (10 ng/ml) or IL-1ß (1 ng/ml) was also present during PFF treatment as described below.

Pulsating fluid shear stress, at 5 Hz pulse frequency, was generated by pumping 13 ml of CO_2-independent medium without L-glutamine (Gibco) in a pulsatile manner through a small parallel-plate flow chamber (20 x 18 x 0.30 mm) containing the bone cells. The apparatus was placed directly onto the confocal microscope (Leica TCS-SP2) with the flowing culture medium. The glass slide, containing the monolayer of cells, served as the bottom of the flow chamber. Mean fluid shear stress was 0.6 Pa with pulse amplitude of 0.3 Pa, and the estimated peak stress rate 8.4 Pa/sec as described earlier[8,17,46]. PFF was monitored throughout the experiment using a small animal blood flow sensor (T206, Transonic Systems, Ithaca, NY, USA).

Intracellular calcium was monitored as fluorescence intensity in single Fluo4-AM (Invitrogen) loaded cells, using a 63 x 1.2 water correction objective. The excitation wavelength used was 520 nm, and emission spectra peaked at 488 nm.

Fluorescence images were recorded from cell cultures at each glass slide at one frame each 15s during 7 min. The fluorescence intensity was quantified using Leica Confocal Software (Leica Microsystems, Wetzlar, Germany). The background fluorescence intensity was subtracted from the fluorescence intensity in each bone cell. Data were obtained from 4 independent experiments. Per experiment 15-30 cells were analysed per treatment group.

Statistics

Wilcoxon and Friedman tests were used to compare groups in relation to NO production and caspase-3/7 activity. Differences were considered significant at a p value < 0.05.

RESULTS

Treatment of the cells with PFF in the presence or absence of TNFα, IL-1ß or calcium inhibitors did neither affect the total amount of DNA in MLO-Y4 cell cultures nor in MC3T3-E1 cell cultures (Table 1). PFF did not result in visible changes in cell shape or alignment of MLO-Y4 cells or MC3T3-E1 cells in the direction of the flow (data not shown).

Treatment with TNFα or IL-1ß, either in the presence or absence of PFF, did not cause apoptosis in MLO-Y4 or MC3T3-E1 cells (Table 2).

Effect of TNFα or IL-1ß on PFF-upregulated NO production

To determine the effect of cytokines on PFF-induced NO production in MLO-Y4 and MC3T3-E1 cells, TNFα (0.5, 10 and 30 ng/ml) or IL-1ß (0.1, 1 and 10 ng/ml) was added 30 minutes before, and during 30 min of treatment with or without PFF. Neither TNFα nor IL-1ß affected NO production under static culture conditions in MLO-Y4 cells (Fig. 1A, 2A) or MC3T3-E1 cells (Fig 1C, 2C). PFF rapidly (within 5 min) increased NO production (MLO-Y4, 3.8 to 4.9-fold; MC3T3-E1, 2.3-fold) compared to static cultures, and this stimulatory effect continued up to 30 min (Fig. 1B, D and 2B, D).

TNFα at 0.5-30 ng/ml prevented the upregulation of NO production by PFF in MLO-Y4 cells (Fig. 1B) and in MC3T3-E1 cells (Fig. 1D). TNFα at 30 ng/ml maximally inhibited the effect of PFF on NO production in MLO-Y4 osteocytes (without TNFα: PFF-treated-over-static control ratio (PFF/Stat), 6.3; with TNFα: PFF/Stat, 2.5, at 30 min, Fig. 1B). In MC3T3-E1 cells, 30 ng/ml TNFα caused maximal inhibition of PFF-upregulated NO production (w/o TNFα: PFF/Stat, 7.5; with TNFα, 2.2, at 30 min, Fig. 1D).

IL-1ß prevented the PFF-upregulated NO production in MLO-Y4 cells at 0.1-10 ng/ml (Fig. 2B), and in MC3T3-E1 cells at 1-10 ng/ml, but not at 0.1 ng/ml (Fig. 2D). IL-1ß at 1 ng/ml maximally inhibited the effect of PFF on NO production in MLO-Y4 osteocytes (without IL-1ß: PFF/Stat, 8.6; with IL-1ß: PFF/Stat, 2.5, at 15 min, Fig. 2B). In MC3T3-E1 cells, 1 ng/ml IL-1ß appeared to

maximally inhibit PFF-upregulated NO production (w/o IL-1ß: PFF/Stat, 5.5; with IL-1ß, 1.0, at 15 min, Fig. 2D).

Effect of calcium inhibitors on PFF-upregulated NO production

Treatment of MLO-Y4 cells with TMB-8, $GdCl_3$, or EGTA significantly inhibited the PFF-upregulated NO production after 5 min (Fig. 3A). Maximal inhibition of the effect of PFF on NO production was observed in the presence of $GdCl_3$ (70% decrease) or EGTA (67% decrease) ($p < 0.05$), while TMB-8 resulted in a 57% decrease ($p < 0.05$). No significant effect of calcium inhibitors on PFF-upregulated NO production was observed in MC3T3-E1 cells, although TMB-8 and $GdCl_3$, but not EGTA showed a trend towards inhibition of the PFF-upregulated NO production after 5 min (TMB-8, 63% decrease; GdCl3, 42% decrease; Fig. 3B).

Effect of TNFα or IL-1ß on PFF-stimulated $[Ca^{2+}]_i$

PFF immediately (within seconds) increased intracellular calcium levels in MLO-Y4 cells (range 125-367% increase) and in MC3T3-E1 cells (range 293-480% increase) (Fig. 4A, B). During 5 minutes of PFF, the calcium levels did not return to basal levels, but remained elevated up to 1 minute after termination of PFF (Fig. 4A, B). In the presence of TNFα at 10 ng/ml, PFF caused a sharp decline in baseline $[Ca^{2+}]_i$ in MLO-Y4 cells (87-95% decrease) and in MC3T3-E1 cells (98-100% decrease) (Fig. 4C, D). $[Ca^{2+}]_i$ returned to baseline values within approximately 30 sec after onset of PFF in MLO-Y4 cells (Fig. 4C), and within 60 sec in MC3T3-E1 cells (Fig. 4D). In the presence of IL-1ß at 1 ng/ml, also caused a sharp decline in baseline $[Ca^{2+}]_i$ in MLO-Y4 cells (47-51% decrease) and in MC3T3-E1 cells (84-94% decrease) (Fig. 4E, F). $[Ca^{2+}]_i$ returned to baseline values within seconds after cessation of PFF in MLO-Y4 cells (Fig. 4E), while in MC3T3-E1 cells the return to baseline values occurred within approximately 15 sec after the onset of PFF (4F).

DISCUSSION

In the present study we investigated whether TNFα and/or IL-1ß affect the NO response of bone cells to mechanical loading. The data shows that both TNFα and IL-1ß inhibit the upregulation of NO production after mechanical stimulation. This inhibition was associated with a prevention of fluid shear stress-induced $[Ca^{2+}]_I$ upregulation.

Localized bone loss resulting from a disturbed balance between bone resorption and formation often occurs during inflammatory diseases such as periodontitis and arthritis, as well as during orthodontic tooth movement[24-26]. Proinflammatory cytokines such as TNFα and IL-1ß play a role in this bone loss, and are produced during inflammation[27,28] as well as during orthodontic tooth movement[29-33]. TNFα and IL-1ß directly stimulate bone resorption[34-38] and inhibit bone formation[39]. However, it is still unknown whether TNFα and/or IL-1ß can serve as negative regulators of bone metabolism by affecting the response of bone cells to mechanical loading. The aim of this study was therefore to assess whether TNFα and IL-1ß affect the bone cell response to fluid shear stress, using NO production as a parameter for the bone cell response, since NO is an essential chemical signal in bone remodelling. MLO-Y4 osteocyte-like cells and MC3T3-E1 osteoblast-like cells were used as representative mechanosensitive cells of the osteogenic lineage. It is known that osteocytes respond stronger to fluid shear stress than less differentiated cells of the osteogenic lineage, such as osteoblasts and periosteal fibroblasts, but osteoblasts also release signalling factors in response to fluid flow[49,50]. We have tested TNFα and IL-1ß at concentrations as observed *in vivo* during orthodontic tooth movement[32] for their effect on the mechanoresponse of bone cells.

Both TNFα and IL-1ß promote apoptosis of bone cells[51,52]. Tan et al.[53] demonstrated that 16 h of treatment with TNFα induced apoptosis in osteocytes and osteoblasts, while Tsuboi et al.[52] observed an effect of TNFα and IL-1 ß on bone cell apoptosis after 48 h of incubation. In the present study, cytokines did not affect apoptosis in MLO-Y4 and MC3T3-E1 cells, since the bone cells were only incubated with IL-1ß or TNFα for a limited time period of 60 minutes. Therefore it

is unlikely that the reduction in PFF-upregulated NO production by cytokine-treated bone cells was due to bone cell apoptosis.

We have quantified the response of osteocytes and osteoblasts to PFF in terms of NO production, since NO has been shown to be essential for the adaptive response of bone to mechanical loading[15,16]. NO is a very reactive, short-lived radical, which is formed during the conversion of L-arginine by the enzyme nitric oxide synthase (NOS)[54]. Of the different isoforms of NOS, endothelial constitutive NOS (eNOS) is the isoform likely responsible for the fluid flow-induced NO upregulation in osteocytes[19]. eNOS is constitutively expressed and its activity seems to be partially regulated by binding of both calmodulin and substrate, although eNOS requires binding of calcium/calmodulin to achieve maximum NO synthase activity and NO production[23]. Therefore the upregulation of NO production in response to PFF might be dependent on increased $[Ca^{2+}]_i$ resulting from PFF treatment. We observed that PFF rapidly increased both $[Ca^{2+}]_I$ and NO production in MLOY4 and MC3T3-E1 cells. In addition inhibition of $[Ca^{2+}]_i$ resulted in a decrease in PFF-induced NO production by both cell types, suggesting that PFF initiates Ca^{2+}-dependent NO production. Ca^{2+}-dependent NO production, resulting from mechanical loading, has also been observed in cardiomyocytes and arterial endothelial cells[55-57]. Fluid flow-induced NO production by osteoblasts could be inhibited by the intracellular Ca chelator Quin 2/AM[58]. In addition, Ca^{2+}-dependent NO production occurs in whole bones loaded, where inhibition of calcium channels by $GdCl_3$ resulted in an inhibition of the loading-induced NO production[59].

PFF induced a rapid (within seconds) increase in $[Ca^{2+}]_i$ in MLO-Y4 and MC3T3-E1 cells. During the 5 minutes of PFF treatment, calcium levels did not return to basal levels, but remained elevated up to at least 1 minute after termination of PFF. This Ca^{2+}-response to PFF was comparable in magnitude and duration between multiple MLO-Y4 and MC3T3-E1 cells, suggesting that this calcium dynamics is characteristic for the response of bone cells to PFF. The rise in $[Ca^{2+}]_i$ as observed in MC3T3-E1 cells that are subjected to oscillatory flow (2 Pa, 1Hz) or steady shear flow (2 Pa) seems to be more transient in nature[60,61], i.e.

$[Ca^{2+}]_I$ returns to baseline levels within approximately 1 minute after initiation of fluid flow. Interestingly, when gap junction transfer was blocked in MC3T3-E1 cells, a sustained high $[Ca^{2+}]_i$ was observed in response to a steady laminar fluid flow, resembling the response of MC3T3-E1 cells to PFF[61].

We found that both TNFα and IL-1ß decreased PFF-induced NO production at all time points, with maximal inhibition by TNFα at 30 ng/ml and by IL-1ß at 1-10 ng/ml. It has been shown that IL-1ß also reduces NO production by dynamically compressed chondrocytes[62], underscoring that IL-1ß affects loading-induced NO production by cells from the mesenchymal lineage. Considering that PFF initiates Ca^{2+}-dependent NO production, we inferred that cytokines such as TNFα and/or IL-1ß affect PFF-upregulated NO production because they affect fluxes in $[Ca^{2+}]_i$ in bone cells. Using fluorescence analysis we observed that TNFα as well as IL-1ß decrease the rise in $[Ca^{2+}]_i$ normally associated with the onset of PFF in MLO-Y4 and MC3T3-E1 cells. These results are in accordance with other studies showing that TNFα decreases $[Ca^{2+}]_i$ in adult cardiac myocytes[63] and in mechanically stimulated rat brain endothelial cells[64]. Thus, TNFα and IL-1ß might reduce PFF-stimulated NO production at least in part by decreasing $[Ca^{2+}]_i$.

In summary, the results of this study show that TNFα and IL-1ß decrease the mechanoresponsiveness of osteocytes and osteoblasts in terms of changes in intracellular calcium levels and NO production. This provides a new mechanism by in which cytokines might interfere in bone remodeling during inflammatory diseases or during orthodontic tooth movement.

ACKNOWLEDGMENTS

The work of V.C. da Silva was supported by a grant of the Foundation for the Coordination of Higher Education and Graduate Training - Brazil (MEC/CAPES BEX0481/06-8), A.D. Bakker was supported by a grant of the Research Institute MOVE of the Vrije Universiteit Amsterdam, and R.G. Bacabac was supported by a grant of the Space Research Organization of The Netherlands (SRON grant MG-055), and he also received financial assistance from the Netherlands Organization For International Cooperation In Higher Education (Physics Development Project PHL-146). The authors wish to thank JMA de Blieck for excellent technical assistance.

REFERENCES

1. Lanyon LE, Rubin CT 1984 Static vs dynamic loads as an influence on bone remodeling. J Biomech **17:**897-905.
2. Turner CH, Owan I, Takano Y 1995 Mechanotransduction in bone: role of strain rate. Am J Physiol **269:**E438-E442.
3. Forwood MR 1996 Inducible cyclo-oxygenase (COX-2) mediates the induction of bone formation by mechanical loading in vivo. J Bone Miner Res **11:**1688-1693.
4. Rubin CT, Lanyon LE 1984 Regulation of bone formation by applied dynamic loads. J Bone Joint Surg Am **66:**397-402.
5. Vico L, Alexandre C 1992 Microgravity and bone adaptation at the tissue level. J Bone Miner Res **7:**S445-S447.
6. Westerlind KC, Turner RT 1995 The skeletal effects of spaceflight in growing rats: tissue-specific alterations in mRNA levels for TGF-beta. J Bone Miner Res **10:**843-848.
7. Huiskes R, Ruimerman R, van Lenthe GH, Janssen JD 2000 Effects of mechanical forces on maintenance and adaptation of form in trabecular bone. Nature **405:**704-706.
8. Klein-Nulend J, Semeins CM, Ajubi NE, Nijweide PJ, Burger EH 1995 Pulsating fluid flow increases nitric oxide (NO) synthesis by osteocytes but not periosteal fibroblasts--correlation with prostaglandin upregulation. Biochem Biophys Res Commun **217:**640-648.
9. Burger EH, Klein-Nulend J 1999 Mechanotransduction in bone--role of the lacuno-canalicular network. FASEB J **13** Suppl**:**S101-S112.
10. Parfitt AM 1977 The cellular basis of bone turnover and bone loss: a rebuttal of the osteocytic resorption - bone flow theory. Clin Orthop Relat Res **127:**236-247.
11. Mikuni-Takagaki Y 1999 Mechanical responses and signal transduction pathways in stretched osteocytes. J Bone Mineral Metabol **17:**57-60.

12. Vezeridis PS, Semeins CM, Chen Q, Klein-Nulend J 2006 Osteocytes subjected to pulsating fluid flow regulate osteoblast proliferation and differentiation. Biochem Biophys Res Commun **29:**1082-1088.
13. Vatsa A, Mizuno D, Smit TH, Schmidt CF, MacKintosh FC, Klein-Nulend,J 2006 Bio imaging of intracellular NO production in single bone cells after mechanical stimulation. J Bone Miner Res **21:**1722-1728.
14. Tan SD, de Vries,TJ, Kuijpers-Jagtman AM, Semeins CM, Everts V, Klein-Nulend J 2007 Osteocytes subjected to fluid flow inhibit osteoclast formation and bone resorption. Bone **41:**745-751.
15. Chow JW, Fox SW, Lean JM, Chambers TJ 1998 Role of nitric oxide and prostaglandins in mechanically induced bone formation J Bone Miner Res **13:**1039-1044.
16. Turner CH, Takano Y, Owan I, Murrell GA 1996 Nitric oxide inhibitor L-NAME suppresses mechanically induced bone formation in rats. Am J Physiol **270:**E634-E639.
17. Bacabac RG, Smit TH, Mullender MG, Dijcks SJ, Van Loon JJ, Klein-Nulend J 2004 Nitric oxide production by bone cells is fluid shear stress rate dependent. Biochem Biophys Res Commun **315:**823-829.
18. Bacabac RG, Smit TH, Van Loon JJWA, Zandieh Doulabi B, Helder MN, Klein-Nulend J 2006 Bone cell responses to high-frequency vibration stress: does the nucleus oscillate within the cytoplasm? FASEB J **20**:858-864.
19. Klein-Nulend J, Helfrich MH, Sterck JG, MacPherson H, Joldersma M, Ralston SH 1998 Nitric oxide response to shear stress by human bone cell cultures is endothelial nitric oxide synthase dependent. Biochem Biophys Res Commun **250:**108-114.
20. Pitsillides AA, Rawlinson SC, Suswillo RF, Bourrin S, Zaman G, Lanyon LE 1995 Mechanical strain-induced NO production by bone cells: a possible role in adaptive bone (re)modeling? FASEB J **9:**1614-1622.
21. Zaman G, Pitsillides AA, Rawlinson SC, Suswillo RF, Mosley JR, Cheng MZ, Platts LA, Hukkanen M, Polak JM, Lanyon LE 1999 Mechanical

strain stimulates nitric oxide production by rapid activation of endothelial nitric oxide synthase in osteocytes. J Bone Miner Res **14:**1123-1131.

22. Marletta MA 1994 Nitric oxide synthase: aspects concerning structure and catalysis. Cell **78:**927-930.
23. Presta A, Liu J, Sessa WC, Stuehr DJ 1997 Substrate binding and calmodulin binding to endothelial nitric oxide synthase coregulate its enzymatic activity. Nitric Oxide **1:**74-87.
24. Verna C, Zaffe D, Siciliani G 1999 Histomorphometric study of bone reactions during orthodontic tooth movement in rats. Bone **24:**371-379.
25. Kinane DF, Lappin DF 2002 Immune processes in periodontal disease: a review. Ann Periodontol **7:**62-71.
26. Bettica P, Cline G, Hart DJ, Meyer J, Spector TD 2002 Evidence for increased bone resorption in patients with progressive knee osteoarthritis: longitudinal results from the Chingford study. Arthritis Rheum **46:**3178-3184.
27. Fernandes JC, Martel-Pelletier J, Pelletier JP 2002 The role of cytokines in osteoarthritis pathophysiology. Biorheology **39:**237-246.
28. Holmlund A, Hänstrom L, Lerner UH 2004 Bone resorbing activity and cytokine levels in gingival crevicular fluid before and after treatment of periodontal disease. J Clin Periodontol **31:**475-482.
29. Alhashimi N, Frithiof L, Brudvik P, Bakhiet M 2001 Orthodontic tooth movement and de novo synthesis of proinflammatory cytokines. Am J Orthod Dentofacial Orthop **119:**307-312.
30. Bletsa A, Berggreen E, Brudvik P 2006 Interleukin-1alpha and tumor necrosis factor-alpha expression during the early phases of orthodontic tooth movement in rats. Eur J Oral Sci **114:**423-429.
31. Grieve WG III, Johnson GK, Moore RN, Reinhardt RA, DuBois LM 1994 Prostaglandin E (PGE) and interleukin-1 beta (IL-1 beta) levels in gingival crevicular fluid during human orthodontic tooth movement. Am J Orthod Dentofacial Orthop **105:**369-374.

32. Lowney JJ, Norton LA, Shafer DM, Rossomando EF 1995 Orthodontic forces increase tumor necrosis factor alpha in the human gingival sulcus. Am J Orthod Dentofacial Orthop **108:**519-524.
33. Uematsu S, Mogi M, Deguchi T 1996 Interleukin (IL)-1 beta, IL-6, tumor necrosis factor-alpha, epidermal growth factor, and beta 2-microglobulin levels are elevated in gingival crevicular fluid during human orthodontic tooth movement. J Dent Res **75:**562-567.
34. Bertolini DR, Nedwin GE, Bringman TS, Smith DD, Mundy GR 1986 Stimulation of bone resorption and inhibition of bone formation in vitro by human tumour necrosis factors. Nature **319:**516-518.
35. Canalis E 1987 Effects of tumor necrosis factor on bone formation in vitro. Endocrinology **121:**1596-1604.
36. Li YP, Stashenko P 1992 Proinflammatory cytokines tumor necrosis factor-alpha and IL-6, but not IL-1, down-regulate the osteocalcin gene promoter. J Immunol **148:**788-794.
37. Gowen M, Wood DD, Ihrie EJ, McGuire MK, Russell RG 1983 An interleukin 1 like factor stimulates bone resorption in vitro. Nature **306:**378-380.
38. Heath JK, Saklatvala J, Meikle MC, Atkinson SJ, Reynolds JJ 1985 Pig interleukin 1 (catabolin) is a potent stimulator of bone resorption in vitro. Calcif Tissue Int **37:**95-97.
39. Stashenko P, Obernesser MS, Dewhirst FE 1989 Effect of immune cytokines on bone. Immunol Invest **18:**239-249.
40. Hofbauer LC, Lacey DL, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S 1999 Interleukin-1beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. Bone **25:**255-259.
41. Dinarello CA 1989 Interleukin-1 and its biologically related cytokines. Adv Immunol **44:**153-205.
42. Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Morinaga

T, Higashio K, Martin TJ, Suda T 2000 Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. J Exp Med **191:**275-286.

43. Akatsu T, Takahashi N, Udagawa N, Imamura K, Yamaguchi A, Sato K, Nagata N, Suda T 1991 Role of prostaglandins in interleukin-1-induced bone resorption in mice in vitro. J Bone Miner Res **6:**183-189.
44. Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S 1983 In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol **96:**191-198.
45. Kato Y, Windle JJ, Koop BA, Mundy GR, Bonewald LF 1997 Establishment of an osteocyte-like cell line, MLO-Y4. J Bone Miner Res **12:**2014-2023.
46. Bakker AD, Soejima K, Klein-Nulend J, Burger EH 2001 The production of nitric oxide and prostaglandin E(2) by primary bone cells is shear stress dependent. J Biomech **34:**671-677.
47. Bacabac RG, Smit TH, Cowin SC, Van Loon JJ, Nieuwstadt FT, Heethaar R, Klein-Nulend J 2005 Dynamic shear stress in parallel-plate flow chambers. J Biomech **38:**159-167.
48. Ajubi NE, Klein-Nulend J, Alblas MJ, Burger EH, Nijweide PJ 1999 Signal transduction pathways involved in fluid flow-induced PGE2 production by cultured osteocytes. Am J Physiol **276:**E171-E178.
49. Klein-Nulend J, van der Plas A, Semeins CM, Ajubi NE, Frangos JA, Nijweide PJ, Burger EH 1995 Sensitivity of osteocytes to biomechanical stress in vitro. FASEB J **9:**441-445.
50. Mullender MG, Dijcks SJ, Bacabac RG, Semeins CM, Van Loon JJ, Klein-Nulend J 2006 Release of nitric oxide, but not prostaglandin E_2, by bone cells depends on fluid flow frequency. J Orthop Res **24:**1170-1177.
51. Ahuja SS, Zhao S, Bellido T, Plotkin LI, Jimenez F, Bonewald LF 2003 CD40 ligand blocks apoptosis induced by tumor necrosis factor alpha, glucocorticoids, and etoposide in osteoblasts and the osteocyte-like cell line murine long bone osteocyte-Y4. Endocrinology **144:**1761-1769.

52. Tsuboi M, Kawakami A, Nakashima T, Matsuoka N, Urayama S, Kawabe Y, Fujiyama K, Kiriyama T, Aoyagi T, Maeda K, Eguchi K 1999 Tumor necrosis factor-alpha and interleukin-1beta increase the Fas-mediated apoptosis of human osteoblasts. J Lab Clin Med **134:**222-231.
53. Tan SD, Kuijpers-Jagtman AM, Semeins CM, Bronckers AL, Maltha JC, Von den Hoff JW, Everts V, Klein-Nulend J 2006 Fluid shear stress inhibits TNFalpha-induced osteocyte apoptosis. J Dent Res **85:**905-909.
54. Hibbs JB Jr, Taintor RR, Vavrin Z 1987 Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. Science **23:**473-476.
55. Fleming I and Busse R 2003 Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. Am J Physiol Regul Integr Comp Physiol **284:**R1-12.
56. Liao X, Liu JM, Du L, Tang A, Shang Y, Wang SQ, Chen LY, Chen Q 2006 Nitric oxide signaling in stretch-induced apoptosis of neonatal rat cardiomyocytes. FASEB J **20:**1883-1885.
57. Takeda H, Komori K, Nishikimi N, Nimura Y, Sokabe M, Naruse K 2006 Bi-phasic activation of eNOS in response to uni-axial cyclic stretch is mediated by differential mechanisms in BAECs. Life Sci **79:**233-239.
58. McAllister TN, Frangos JA 1999 Steady and transient fluid shear stress stimulate NO release in osteoblasts through distinct biochemical pathways. J Bone Miner Res **14:**930-936.
59. Rawlinson SC, Pitsillides AA, Lanyon LE 1996 Involvement of different ion channels in osteoblasts' and osteocytes' early responses to mechanical strain. Bone **19:**609-614.
60. Godin LM, Suzuki S, Jacobs CR, Donahue HJ, Donahue SW 2007 Mechanically induced intracellular calcium waves in osteoblasts demonstrate calcium fingerprints in bone cell mechanotransduction. Biomechan Model Mechanobiol **6:**391–398.
61. Huo BB, Lu XL, Guo XE 2007 Cellular network on bone cell response to fluid shear. Abstract of the 8th International Bone Fluid Flow Workshop

[serial online: page 35] Available at: http://www.bme.sunysb.edu/8ibff/program_fin3d_ym4_FINAL.pdf. Accessed November 15, 2007.

62. Chowdhury TT, Bader DL, Lee DA 2001 Dynamic compression inhibits the synthesis of nitric oxide and PGE_2 by IL-1beta-stimulated chondrocytes cultured in agarose constructs. Biochem Biophys Res Commun **285:**1168-1174.
63. Yokoyama T, Vaca L, Rossen RD, Durante W, Hazarika P, Mann DL 1993 Cellular basis for the negative inotropic effects of tumor necrosis factor-alpha in the adult mammalian heart. J Clin Invest **92:**2303-2312.
64. Vandamme W, Braet K, Cabooter L, Leybaert L 2004 Tumour necrosis factor alpha inhibits purinergic calcium signalling in blood-brain barrier endothelial cells. J Neurochem **88:**411-421.

Table 1: DNA content of MLO-Y4 osteocytes and MC3T3-E1 osteoblasts cultured in the presence or absence of TNFα or IL-1ß after 30 min with or without PFF treatment.

	DNA, µg				
		MLO-Y4 cells		MC3T3-E1 cells	
		Stat	PFF	Stat	PFF
TNFα	0 ng/ml	1.1 ± 0.2	1.2 ± 0.2	1.7 ± 0.1	1.6 ± 0.1
	0.5 ng/ml	1.7 ± 0.5	1.7 ± 0.6	1.6 ± 0.1	1.7 ± 0.1
	10 ng/ml	1.6 ± 0.4	1.8 ± 0.7	1.9 ± 0.2	1.5 ± 0.2
	30 ng/ml	2.0 ± 0.3	1.8 ± 0.2	1.9 ± 0.1	2.1 ± 0.4
IL-1ß	0 ng/ml	2.7 ± 0.8	2.0 ± 0.4	1.9 ± 0.3	1.9 ± 0.5
	0.1 ng/ml	3.4 ± 0.8	2.7 ± 0.2	1.8 ±0.1	2.1 ± 0.3
	1 ng/ml	3.2 ± 1.0	2.7 ± 0.2	1.6 ± 0.3	2.1 ± 0.3
	10 ng/ml	2.1 ± 0.3	2.4 ± 0.6	2.2 ± 0.3	2.1 ± 0.2

Data are expressed as the mean ± SEM. Stat: static; PFF: pulsating fluid flow. No significant differences were found.

Table 2: Apoptosis in MLO-Y4 osteocytes and MC3T3-E1 osteoblasts in the presence or absence of TNFα or IL-1ß after 30 min with or without PFF treatment.

		Caspase-3/7, au/ng DNA			
		MLO-Y4 cells		MC3T3-E1 cells	
		Stat	PFF	Stat	PFF
TNFα	0 ng/ml	8.9 ± 3.3	8.8 ± 2.5	5.4 ± 0.3	6.9 ± 0.8
	0.5 ng/ml	4.8 ± 0.7	4.6 ± 1.4	4.8 ± 0.9	5.7 ± 0.5
	10 ng/ml	3.4 ± 0.5	6.4 ± 0.4	7.1 ± 0.7	8.1 ± 1.3
	30 ng/ml	9.8 ± 4.6	12.3 ± 4.5	6.3 ± 0.8	6.5 ± 0.9
IL-1ß	0 ng/ml	8.6 ± 3.5	9.0 ± 2.9	8.66 ± 0.2	7.93 ± 1.0
	0.1 ng/ml	5.1 ± 2.1	5.0 ± 1.9	10.5 ± 2.3	8.9 ± 0.5
	1 ng/ml	3.7 ± 1.4	6.0 ± 2.3	10.3 ± 1.9	6.3 ± 0.5 [a]
	10 ng/ml	7.7 ± 3.2	10.3 ± 4.2	6.3 ± 0.7	7.2 ± 0.7

Apoptosis is expressed as caspase-3/7 activity. Data are expressed as the mean ± SEM. au: arbitrary units; Stat: static; PFF: pulsating fluid flow. a: Significant effect of PFF, $p < 0.05$.

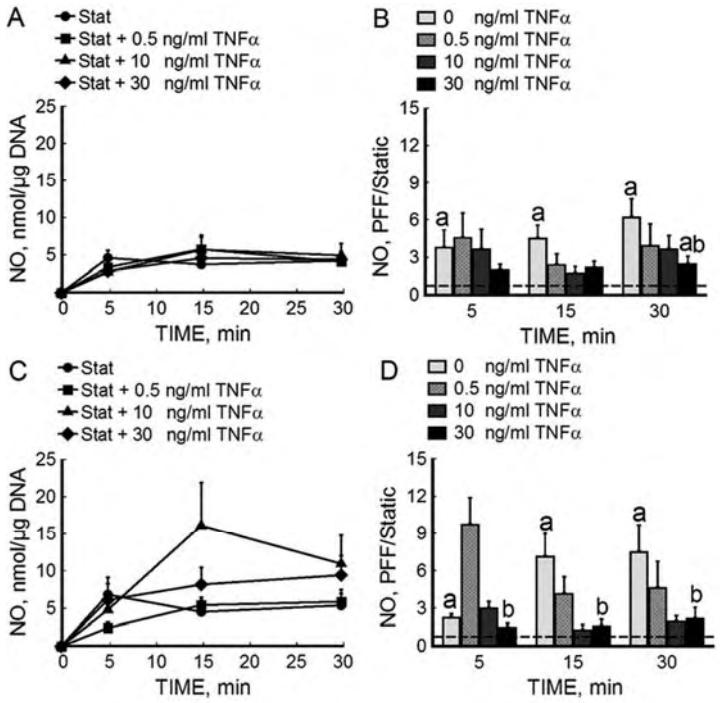

Fig. 1 Effect of TNFα on NO production by MLO-Y4 osteocytes and MC3T3-E1 osteoblasts. **(A)** Cumulative NO production during 30 min of static culture conditions in the absence or presence of TNFα at 0.5, 10 or 30 ng/ml in MLO-Y4 cells. TNFα did not change NO production under static culture conditions. **(B)** NO response by MLO-Y4 cells, expressed as PFF-treated-over-control values (PFF/Stat) in the absence or presence of TNFα at 0.5, 10 or 30 ng/ml. PFF stimulated NO production by MLO-Y4 cells ($p<0.05$). TNFα dose-dependently inhibited the NO response to PFF. **(C)** Cumulative NO production during 30 min of static culture conditions in the absence or presence of TNFα at 0.5, 10 or 30 ng/ml in MC3T3-E1 cells. TNFα did not change NO production in static culture conditions. **(D)** NO response by MC3T3-E1 cells, expressed as PFF-treated-over-control values (PFF/Stat) in the absence or presence of TNFα at 0.5, 10 or 30 ng/ml. PFF stimulated NO production by MC3T3-E1 cells ($p<0.05$). TNFα dose-dependently inhibited the NO response to PFF. Values are mean ± SEM of 7 independent experiments. Stat, stationary control culture; PFF, pulsating fluid flow; NO, nitric oxide. Dashed line, PFF treatment-over-control values = 1 (no effect). a: Significant effect of PFF, $p<0.05$. b: Significant effect of TNFα, $p<0.05$.

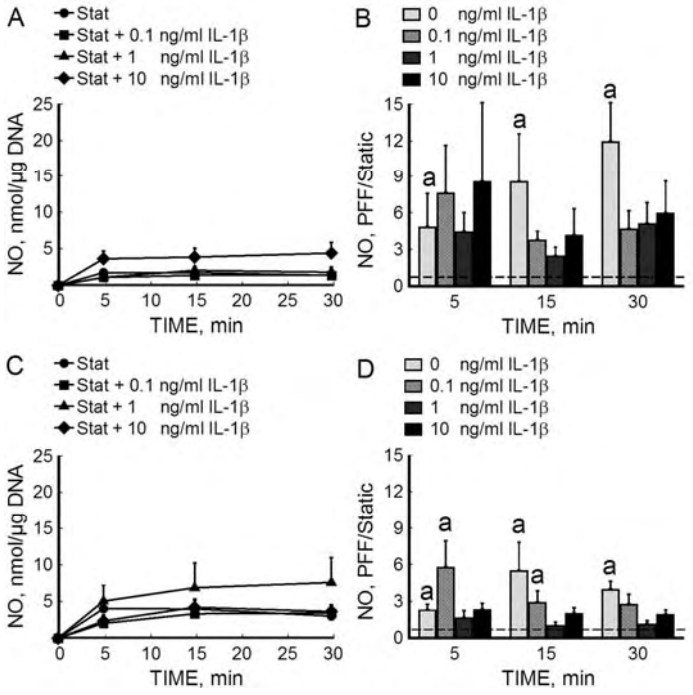

Fig. 2 Effect of IL-1ß on NO production by MLO-Y4 osteocytes and MC3T3-E1 osteoblasts. **(A)** Cumulative NO production during 30 min of static culture conditions in the absence or presence of IL-1ß at 0.1, 1 or 10 ng/ml in MLO-Y4 cells. Il-1ß did not change NO production under static conditions. **(B)** NO response by MLO-Y4 cells, expressed as PFF-treated-over-control values (PFF/Stat) in the absence or presence of IL-1β at 0.1, 1 or 10 ng/ml. PFF stimulated the NO response ($p<0.05$) by MLO-Y4 cells ($p<0.05$). IL-1β inhibited the NO response to PFF. **(C)** Cumulative NO production during 30 min of static culture conditions in the absence or presence of IL-1ß at 0.1, 1 or 10 ng/ml in MC3T3-E1 cells. Il-1ß did not change NO production under static conditions. **(D)** NO response by MC3T3-E1, expressed as PFF-treated-over-control values (PFF/Stat) in the absence or presence of IL-1β at 0.1, 1 or 10 ng/ml. PFF stimulated NO production by MC3T3-E1 cells ($p<0.05$). IL-1β at 1 and 10ng/ml inhibited the NO response to PFF. Values are mean ± SEM of 7 independent experiments. Stat, stationary control culture; PFF, pulsating fluid flow; NO, nitric oxide. Dashed line, PFF treatment-over-control values = 1 (no effect). a: Significant effect of PFF, $p<0.05$. b: Significant effect of IL-1β, $p<0.05$.

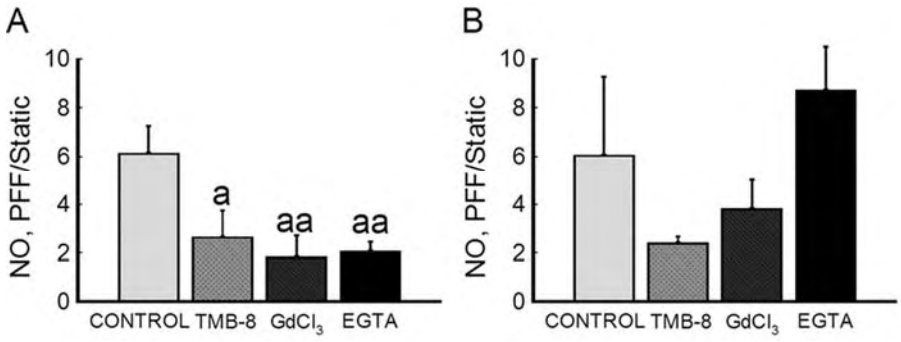

Fig. 3 Effect of inhibition of intracellular calcium signals on PFF-induced NO production in MLO-Y4 osteocytes and MC3T3-E1 osteoblasts. **(A)** TMB-8 decreased the PFF-induced NO production by MLO-Y4 cells at 5 minutes (n = 8, $p < 0.05$), as well as $GdCl_3$ and EGTA (n = 7, $p < 0.01$ and n = 7, $p < 0.01$ respectively). **(B)** None of the calcium inhibitors had a significant effect on PFF-induced NO production by MC3T3-E1 cells ($p > 0,05$; TMB-8 n=4, $GdCl_3$ n=3 and EGTA n=2). Values are mean ± SEM. Stat, stationary control culture; PFF, pulsating fluid flow. a: Significant effect of calcium inhibitor, $p<0.05$. aa: Significant effect of calcium inhibitor, $p<0.01$.

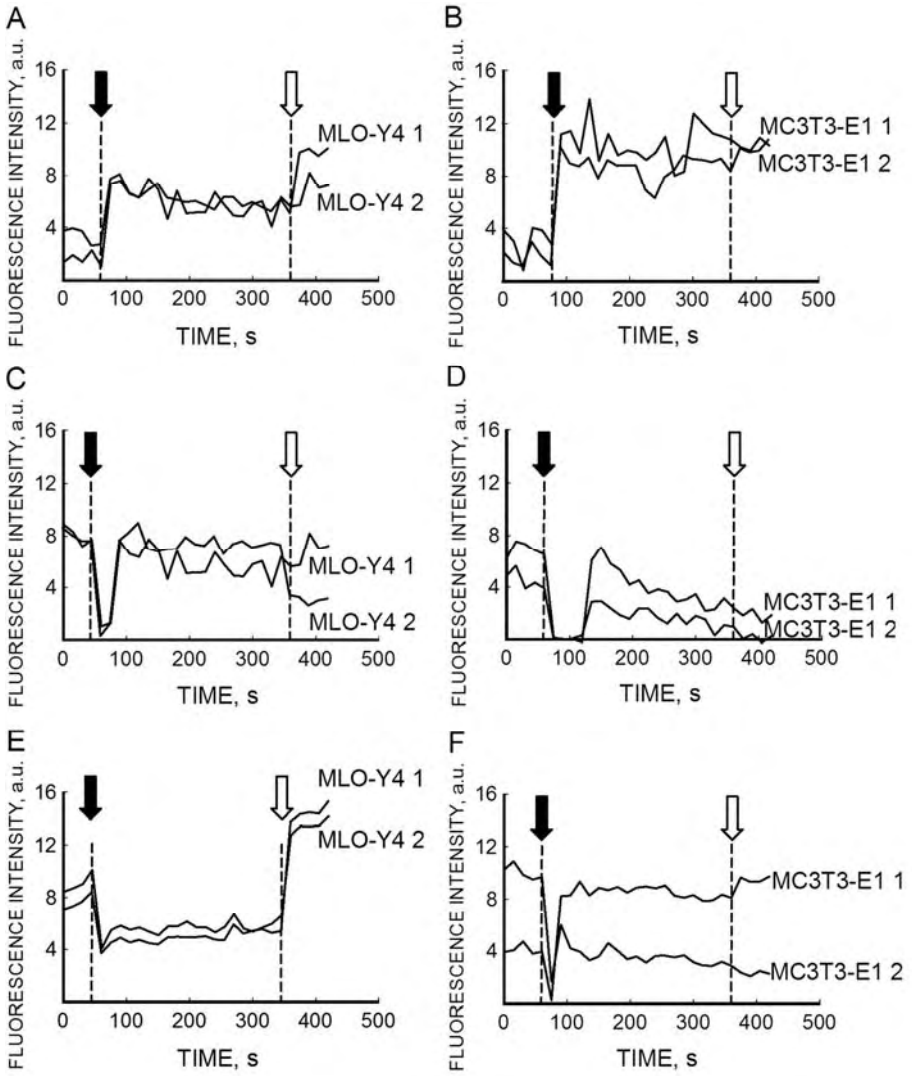

Fig. 4 Effect of 5 minutes of PFF on $[Ca^{2+}]_i$ in MLO-Y4 osteocytes and MC3T3-E1 osteoblasts loaded with Fluo4-AM chromophore. **(A)** Fluorescence intensity increased immediately after the start of PFF in MLO-Y4 cells, indicating a rise in $[Ca^{2+}]_i$. **(B)** PFF increased fluorescence intensity in MC3T3-E1 cells. **(C)** Fluorescence intensity in MLO-Y4 cells under Stat and PFF conditions in the presence of TNFα at 10 ng/ml showed a decrease of calcium levels during 30s of the PFF returning to baseline values under stat conditions in MLO-Y4 cells. **(D)** PFF caused an initial drop in fluorescence intensity in MC3T3-E1 cells in the presence of TNFα at 10 ng/ml. **(E)** PFF caused a decrease in fluorescence intensity

in MLO-Y4 cells in the presence of IL-1β at 1ng/ml. This indicates a decrease in $[Ca^{2+}]_i$ during PFF. After cessation of PFF, the calcium levels returned to baseline values. **(F)** PFF caused an initial drop in fluorescence intensity in MC3T3-E1 cells in the presence of IL-1β at 1 ng/ml returning to baseline values under stat conditions. Values represent the average fluorescence intensity in the cell body of 2 cells after correction for background fluorescence. Values shown are representative for 20-30 cells that were measured in 4 independent experiments. Black arrow: start of PFF treatment; White arrow: end of PFF treatment. Stat, stationary control culture; PFF, pulsating fluid flow; a.u., arbitrary units.

5 CONSIDERAÇÕES GERAIS

O primeiro estudo dessa tese teve como propósito avaliar em cães, a resposta tecidual após intrusão de lesão de furca grau III previamente tratada com retalho para raspagem (OFD) associado ou não a membrana (GTR) e enxerto ósseo autógeno (BA).

Regeneração periodontal ainda representa um desafio na Periodontia principalmente em lesões extensas, como os defeitos de furca grau III[83,113,114]. O objetivo do uso de técnicas regenerativas nesse estudo foi favorecer a regeneração periodontal; no entanto, não foram observadas diferenças significativas nas variáveis histométricas avaliadas entre os grupos OFD e GTR/BA sem movimento ortodôntico.

Com o objetivo de favorecer a regeneração periodontal, o movimento ortodôntico em áreas com defeitos periodontais tem sido largamente estudado enfocando a reação de biomateriais e a reparação de defeitos ósseos quando submetidos à carga mecânica[1,7,23,32,37,63,115,133]. Desta forma, movimento ortodôntico de intrusão com forças leves foi aplicado no presente estudo visando a redução da altura vertical do defeito e a ativação de células locais indiferenciadas, podendo conseqüentemente melhorar o prognóstico das lesões de furca grau III[95,96,145].

O uso de forças de baixa magnitude durante o movimento de intrusão diminue o risco de reabsorção radicular[90] e de danos pulpares uma vez que esses somente foram observados quando forças maiores que 60g foram usadas[134]. O deslocamento apical dental durante a intrusão com aplicação de forças leves e adequado controle de placa não promove a migração apical do epitélio, pois quando a força é direcionada próxima ao centro de resistência dental (para evitar movimento de inclinação) ocorre baixo estresse no ligamento periodontal cervical e estiramento das fibras colágenas que estimulam as células do ligamento periodontal próximo à crista óssea alveolar[88]. Assim, mesmo durante a intrusão, forças de tensão do LP estão sendo aplicadas, estimulando a regeneração periodontal. No presente estudo, miniimplantes foram utilizados como ancoragem

com o objetivo de favorecer a intrusão dentária pura[27,54,69,142] evitando o uso de dentes para ancoragem, os quais poderiam sofrer extrusão e/ou inclinação[4].

A análise dos efeitos do movimento intrusivo neste estudo ocorreu em duas situações periodontais: após o tratamento da doença periodontal no grupo OFD+I e após o tratamento da doença e do defeito periodontal no grupo GTR/BA+I. No grupo OFD+I, o tratamento periodontal cirúrgico prévio à ortodontia teve por objetivo apenas o controle da doença, uma vez que dentes com doença periodontal ativa apresentam, quando movimentados, um aumento na atividade osteoclástica que acelera a taxa de destruição periodontal[42,150]. Portanto, nesse grupo, o objetivo da intrusão foi promover uma redução da altura vertical do defeito de furca. Esse tratamento poderia favorecer o prognóstico de um tratamento periodontal regenerativo posterior se necessário, embora não estudado no presente trabalho.

No grupo GTR/BA, a intervenção periodontal visou o tratamento da doença e a correção do defeito ósseo resultante da mesma. Em virtude do baixo prognóstico ao tratamento regenerativo das lesões de furca grau III[83,113,114], a intrusão ortodôntica foi realizada após 30 dias do tratamento periodontal regenerativo com o objetivo de estimular as células locais indiferenciadas e favorecer a regeneração periodontal[36]. De acordo com Araujo et al.[6], após 30 dias da GTR em cães, 89% do defeito se encontra preenchido por tecido conjuntivo, 7% por tecido de granulação (células inflamatórias e vasos sangüíneos associados) e 4% por tecido ósseo imaturo na base da furca. Em um estudo piloto usando dois cães com um total de 8 pré-molares inferiores com lesão de furca grau III e intrusão após 30 dias da GTR com membrana absorvível, observou-se um ganho de inserção e regeneração periodontal em 4 dentes, sugerindo que a intrusão é um movimento promissor na resolução de defeitos críticos como as lesões de furca grau III, se um adequado controle de placa for realizado[37]. Em nosso estudo, uma significante redução clínica das lesões de furca grau III para grau II/I ou ausência da lesão foi observada para ambos os grupos, GTR/BA e OFD, quando a intrusão foi realizada.

Pela análise histométrica dos grupos com intrusão, observou-se que OFD+I apresentou maior área de preenchimento ósseo e menor área de tecido conjuntivo

comparado ao grupo GTR/BA+I. Esta observação nos levou a questionar a possibilidade de que o processo de degradação da membrana e/ou enxerto ósseo interagiria negativamente sobre o processo de reparo quando associado a forças ortodônticas. Durante a degradação da membrana e enxerto ósseo, uma reação inflamatória está presente[17], podendo ter intensificado a presença de citocinas pró-inflamatórias já liberadas em função do movimento ortodôntico no grupo GTR/BA+I[101,141]. Já foi evidenciado que células inflamatórias envolvidas no processo de degradação da membrana são capazes de expressar citocinas inflamatórias[17,55,68,147]. Assim, a remodelação óssea local poderia ter sido prejudicada o que nos leva a sugerir que a presença da membrana absorvível e/ou enxerto ósseo autógeno interferiram negativamente nos resultados obtidos após a intrusão.

O objetivo do estudo in vivo foi avaliar as respostas teciduais e a formação óssea após a contenção ortodôntica. Contudo, sabe-se que a alteração tecidual proveniente da movimentação ortodôntica é a resultante de eventos celulares desencadeados por mediadores químicos sobre as células dos tecidos periodontais. Em nível celular, quando uma força ortodôntica é aplicada, ocorre um aumento na liberação de NO que é responsável pela indução da neoformação óssea[72,123,135]. Similarmente, citocinas liberadas pelas células do tecido conjuntivo e ósseo participam do processo de remodelação óssea por meio da indução à reabsorção óssea[12,22,53,58,80] ou inibição de formação óssea[132]. Vários estudos clínicos têm evidenciado a presença de elevados níveis de citocinas (IL-1ß, TNFα e IL-6) no fluido gengival crevicular em pacientes durante a fase inicial do movimento ortodôntico mesmo quando toda inflamação gengival é eliminada antes e durante a ortodontia[56,84,141]. Também, TNFα e IL-1ß foram imunolocalizadas no ligamento periodontal e tecido ósseo após movimento ortodôntico em ratos e gatos[13,34]. Assim, nós hipotetizamos que a presença já elevada de TNFα e IL-1ß em função da carga ortodôntica pode ter sido amplificada no grupo GTR/BA+I devido ao processo de degradação da membrana e enxerto ósseo associado, influenciando negativamente a resposta tecidual in vivo. Desta forma, no estudo in vitro (capítulo 2) buscou-se averiguar os efeitos de TNFα e/ou IL-1ß na resposta de

células com característica de osteócitos e osteoblastos submetidas a carga mecânica com a pretensão de auxiliar no entendimento dos resultados clínicos obtidos pelo estudo in vivo.

O movimento ortodôntico resulta da adaptação do tecido ósseo submetido à carga mecânica. Durante esse processo ocorre o movimento do fluido intersticial na rede lacuno-canalicular do tecido ósseo que é sentido principalmente por osteócitos[18,19,135,136]. O PFF (pulsatile fluid flow) é um modelo largamente utilizado para simular in vitro o movimento do líquido intersticial na rede lacuno-canalicular do tecido ósseo[72,73]. O regime de PFF (pressão + freqüência) capaz de simular o fluxo de fluido intersticial derivado de uma carga mecânica foi determinado a partir de estudos preliminares in vivo onde magnitudes de pressão de até 3 Pa são capazes de causar deformações da matriz óssea da ordem de 0.1 a 0.3% (1000-3000με)[31,149]. Deformações da matriz óssea dessa ordem são suficientes para causar a modelação do osso lamelar in vivo e in vitro[48,65,67,72,73]. Desta forma, o regime de 0.6 ± 0.3 Pa e freqüência de 5Hz utilizado no presente estudo é suficiente para estimular as células ósseas e iniciar a remodelação do tecido ósseo in vivo em situações como andar, correr, pular, mastigar e pode também ser aplicado na simulação de forças ortodônticas[9,10,135,136].

Os resultados do estudo in vitro demonstram que, em presença de TNFα e IL-1ß, a produção de NO foi significantemente reduzida por células com características de osteócitos e osteoblastos. Desta forma, considerando-se que a resposta celular à carga mecânica na ausência de citocinas se caracteriza pelo aumento na produção de NO[10,72,73], é possível concluir que a mecanoresposta das células ósseas foi afetada em presença de TNFα e IL-1ß.

Outra questão levantada no estudo in vitro foi o entendimento do mecanismo pelo qual TNFα e IL-1ß agem diminuindo a produção de NO em presença de carga mecânica. Na ausência de TNFα e IL-1ß, células ósseas submetidas à carga mecânica promovem um aumento imediato dos níveis de cálcio intracelular[91]. O cálcio intracelular possui um importante papel na produção de NO induzido pela carga mecânica, pois a enzima óxido nítrico sintetase é cálcio dependente[126]. Assim, nós hipotetizamos que TNFα e/ou IL-1ß poderiam diminuir

a produção de NO por interferir com os níveis de cálcio intracelular nas células ósseas submetidas ao PFF. Já foi demonstrado que TNFα diminui os níveis de cálcio intracelular em miócitos cardíacos em adultos[153] e em células endoteliais cerebrais de rato que foram mecano-estimuladas[143]. No presente estudo, os níveis de cálcio foram avaliados usando microscopia de fluorescência em células com característica de osteoblastos e osteócitos em presença de TNFα, IL-1ß e carga mecânica. Observamos que os níveis de cálcio aumentaram em presença de carga mecânica, mas diminuíram quando TNFα e IL-1ß estavam presentes. Portanto, de acordo com nossos resultados, TNFα e IL-1ß interferem na regulação local do cálcio citoplasmático alterando a mecanotransdução nas células ósseas.

O uso da ortodontia como coadjuvante no tratamento de defeitos periodontais poderia beneficiar os pacientes previamente afetados por doença periodontal. De acordo com nossos resultados, o movimento de intrusão promove a redução e/ou fechamento das lesões de furca grau III em cães. A presença da membrana e enxerto ósseo autógeno ainda em fase de degradação pode ter potencializado a presença de substâncias inflamatórias liberadas durante a reação ortodôntica e com isso reduzido a formação óssea nesse grupo. In vitro, citocinas como TNFα e IL-1ß inibem o efeito da carga mecânica em estimular a formação de substâncias indutoras de formação óssea como o cálcio e NO. Contudo vários fatores devem ser melhores estudados como o intervalo ideal para aplicação da força ortodôntica após o tratamento periodontal, interações com os diversos mediadores biológicos presentes durante o movimento ortodôntico, além do uso de cargas dinâmicas representada pela amplitude e freqüência da carga mecânica nos estudos in vitro que favorecem a resposta celular quando comparadas com cargas constantes[9,10]. Estudos adicionais são necessários para melhor elucidar essas questões.

6 CONCLUSÕES

De acordo com o que foi proposto e dentro dos limites desse estudo, podemos concluir que:

1- clinicamente a intrusão ortodôntica com mini-implantes promoveu uma redução ou eliminação de lesões de furca grau III em pré-molares de cães quando aplicada em fase recente de cicatrização após tratamento periodontal.

2- a intrusão ortodôntica resultou em significante preenchimento ósseo e redução dos tecidos moles na área do defeito de furca grau III quando associada ao grupo raspagem com retalho (OFD); porém, a presença de membrana absorvível e enxerto ósseo autógeno (GTR/BA) interferiram negativamente na regeneração periodontal.

3- in vitro, TNFα e IL-1ß diminuíram a resposta de osteócitos e osteoblastos durante a aplicação de carga mecânica por reduzir o nível de cálcio intracelular e a produção de NO, sugerindo um novo mecanismo para explicar como as citocinas interferem na remodelação óssea em presença de carga mecânica.

7 REFERÊNCIAS[1]

1. Aguirre-Zorzano LA, Bayona JM, Remolina A, Castanos J, Diez R, Estefania, E. Postorthodontic stability of the new attachment achieved by guided tissue regeneration following orthodontic movement: report of 2 cases. Quintessence Int. 1999; 30: 769-74.
2. Ajubi NE, Klein-Nulend J, Nijweide PJ, Vrijheid-Lammers T, Alblas MJ, Burger EH. Pulsating fluid flow increases prostaglandin production by cultured chicken osteocytes--a cytoskeleton-dependent process. Biochem Biophys Res Commun. 1996; 225:62-8.
3. Akin E, Gurton AU, Olmez H. Effects of nitric oxide in orthodontic tooth movement in rats. Am J Orthod Dentofacial Orthop. 2004; 126: 608-14.
4. Alessandri BG, Giunta D. Molar intrusion with a removable appliance. J Clin Orthod. 1996; 30: 434-7.
5. Alhashimi N, Frithiof L, Brudvik P, Bakhiet M. Orthodontic tooth movement and de novo synthesis of proinflammatory cytokines. Am J Orthod Dentofacial Orthop. 2001; 119: 307-12.
6. Araújo MG, Berglundh T, Lindhe J. On the dynamics of periodontal tissue formation in degree III furcation defects. An experimental study in dogs. J Clin Periodontol. 1997; 24: 738-46.
7. Araujo MG, Carmagnola D, Berglundh T, Thilander B, Lindhe J. Orthodontic movement in bone defects augmented with Bio-Oss. An experimental study in dogs. J Clin Periodontol. 2001; 28: 73-80.
8. Araujo MG, Lindhe J. GTR treatment of degree III furcation defects following application of enamel matrix proteins. An experimental study in dogs. J Clin Periodontol. 1998; 25: 524-30.

[1] De acordo com estilo Vancouver. Disponível em: http://www.nlm.nih.gov/bsd/uniform_requirements.html

9. Bacabac RG, Smit TH, Mullender MG, Dijcks SJ, Van Loon JJ, Klein-Nulend J. Nitric oxide production by bone cells is fluid shear stress rate dependent. Biochem Biophys Res Commun. 2004; 315: 823-9.
10. Bakker AD, Soejima K, Klein-Nulend J, Burger EH. The production of nitric oxide and prostaglandin E(2) by primary bone cells is shear stress dependent. J Biomech. 2001; 34: 671-7.
11. Becker W, Becker BE, Berg L, Prichard J, Caffesse R, Rosenberg E. New attachment after treatment with root isolation procedures: report for treated Class III and Class II furcations and vertical osseous defects. Int J Periodontics Restorative Dent. 1988; 8:8-23.
12. Bertolini DR, Nedwin GE, Bringman TS, Smith DD, Mundy GR. Stimulation of bone resorption and inhibition of bone formation in vitro by human tumour necrosis factors. Nature. 1986; 319: 516-8.
13. Bletsa A, Berggreen E, Brudvik P. Interleukin-1alpha and tumor necrosis factor-alpha expression during the early phases of orthodontic tooth movement in rats. Eur J Oral Sci. 2006; 114: 423-9.
14. Bondevik O. Tissue changes in the rat molar periodontium following application of intrusive forces. Eur J Orthod. 1980; 2: 41-9.
15. Brunsvold MA, Mellonig JT. Bone grafts and periodontal regeneration. Periodontol 2000. 1993; 1: 80-91.
16. Bu R, Borysenko CW, Li Y, Cao L, Sabokbar A, Blair HC. Expression and function of TNF-family proteins and receptors in human osteoblasts. Bone. 2003; 33:760-70.
17. Buchmann R, Hasilik A, Heinecke A, Lange DE. PMN responses following use of 2 biodegradable GTR membranes. J Clin Periodontol. 2001; 28: 1050-7.
18. Burger EH, Klein-Nulend J, Smit TH. Strain-derived canalicular fluid flow regulates osteoclast activity in a remodelling osteon--a proposal. J Biomech. 2003; 36: 1453-9.
19. Burger EH, Klein-Nulend J. Mechanotransduction in bone--role of the lacuno-canalicular network. FASEB J. 1999; 13 Suppl: S101-S112.

20. Burr DB, Milgrom C, Fyhrie D, Forwood M, Nyska M, Finestone A, Hoshaw S, Saiag E, Simkin A. In vivo measurement of human tibial strains during vigorous activity. Bone. 1996; 18: 405-10.
21. Busse R, Fleming I. Pulsatile stretch and shear stress: physical stimuli determining the production of endothelium-derived relaxing factors. J Vasc Res. 1998; 35: 73-84.
22. Canalis E. Effects of tumor necrosis factor on bone formation in vitro. Endocrinology. 1987; 121: 1596-1604.
23. Cirelli CC, Cirelli JA, Rosa Martins JC, Comelli Lia RC, Rossa C Jr, Marcantonio E Jr. Orthodontic movement of teeth with intraosseous defects: Histologic and histometric study in dogs. Am J Orthod Dentofacial Orthop. 2003: 123; 666-73.
24. Cirelli JA, Cirelli CC, Holzhausen M, Martins LP, Brandao CH. Combined periodontal, orthodontic, and restorative treatment of pathologic migration of anterior teeth: a case report. Int J Periodontics Restorative Dent. 2006; 26: 501-6.
25. Conti P, Kempuraj D, Kandere K, Di Gioacchino M, Barbacane RC, Castellani ML, Felaco M, Boucher W, Letourneau R, Theoharides TC. IL-10, an inflammatory/inhibitory cytokine, but not always. Immunol Lett. 2003; 86: 123–9.
26. Cortellini P, Pini PG, Baldi C, Clauser C. Guided tissue regeneration with different materials. Int J Periodontics Restorative Dent. 1990; 10; 136-51.
27. Costa A, Raffainl M, Melsen B. Miniscrews as orthodontic anchorage: a preliminary report. Int J Adult Orthodon Orthognath Surg. 1998; 13: 201-9.
28. Costa MR, Silverio KG, Rossa CJ, Cirelli JA. Periodontal conditions of teeth presenting pathologic migration. Braz Oral Res. 2004; 18: 301-5.
29. Cowin SC, Moss-Salentijn L, Moss ML. Candidates for the mechanosensory system in bone. J Biomech Eng. 1991; 113: 191-7.

30. Cowin SC, Weinbaum S, Zeng Y. A case for bone canaliculi as the anatomical site of strain generated potentials. J Biomech. 1995; 28: 1281-97.
31. Cowin SC. Bone poroelasticity. J Biomech. 1999; 32: 217-38.
32. da Silva VC, Cirelli CC, Ribeiro FS, Costa MR, Lia RCC, Cirelli JA. Orthodontic movement after periodontal regeneration of class II furcation: a pilot study in dogs. J Clin Periodontol. 2006; 33: 440-8.
33. D'Attillio M, Di Maio F, D'Arcangela C, Filippi MR, Felaco M, Lohinai Z, Festa F, Perinetti G. Gingival endothelial and inducible nitric oxide synthase levels during orthodontic treatment: a cross-sectional study. Angle Orthod. 2004; 74: 851-8.
34. Davidovitch Z, Nicolay OF, Ngan PW, Shanfeld JL. Neurotransmitters, cytokines, and the control of alveolar bone remodeling in orthodontics. Dent Clin North Am. 1988; 32: 411-35.
35. Dellinger EL. A histologic and cephalometric investigation of premolar intrusion in the Macaca speciosa monkey. Am J Orthod. 1967; 53: 325-55.
36. Diedrich P, Fritz U, Kinzinger G, Angelakis J. Movement of periodontally affected teeth after guided tissue regeneration (GTR)--an experimental pilot study in animals. J Orofac Orthop. 2003; 64: 214-27.
37. Diedrich P, Wehrbein H, Schneider B. Zur problematik der orthodontischen intrusion parodontal erkrankter zähne. Parodontologie 1992; 2: 87-102.
38. Dimmeler S, Zeiher AM. Nitric oxide and apoptosis: another paradigm for the double-edged role of nitric oxide. Nitric Oxide. 1997; 1: 275-81.
39. Dinarello CA. Interleukin-1 and its biologically related cytokines. Adv Immunol. 1989: 44: 153-205.
40. Donos N, Sculean A, Glavind L, Reich E, Karring T. Wound healing of degree III furcation involvements following guided tissue regeneration and/or Emdogain. A histologic study. J Clin Periodontol. 2003; 30: 1061-8.

41. Doty SB. Morphological evidence of gap junctions between bone cells. Calcif Tissue Int. 1981; 33: 509-12.
42. Eliasson LA, Hugoson A, Kurol J, Siwe H. The effects of orthodontic treatment on periodontal tissues in patients with reduced periodontal support. Eur J Orthod. 1982; 4: 1-9.
43. Epker BN, Frost HM. Correlation of bone resorption and formation with the physical behavior of loaded bone. J Dent Res. 1965; 44: 33-41.
44. Fan X, Roy E, Zhu L, Murphy TC, Ackert-Bicknell C, Hart CM, Rosen C, Nanes MS, Rubin J. Nitric oxide regulates receptor activator of nuclear factor-kappaB ligand and osteoprotegerin expression in bone marrow stromal cells. Endocrinology. 2004; 145:751–9
45. Fernandes JM, Rego RO, Spolidorio LC, Marcantonio RA, Marcantonio JE, Cirelli JA. Enamel matrix proteins associated with GTR and bioactive glass in the treatment of class III furcation in dogs. Braz Oral Res. 2005; 19: 169-75.
46. Forwood MR. Inducible cyclo-oxygenase (COX-2) mediates the induction of bone formation by mechanical loading in vivo. J Bone Miner Res. 1996; 11: 1688-93
47. Frangos JA, Eskin SG, McIntire LV, Ives CL. Flow effects on prostacyclin production by cultured human endothelial cells. Science. 1985; 227: 1477-79.
48. Frost HM. The role of changes in mechanical usage set points in the pathogenesis of osteoporosis. J Bone Miner Res. 1992; 7: 253-61.
49. Garlet TP, Coelho U, Silva JS, Garlet GP. Cytokine expression pattern in compression and tension sides of the periodontal ligament during orthodontic tooth movement in humans. Eur J Oral Sci. 2007; 115: 355-62.
50. Garrett S, Gantes B, Zimmerman G, Egelberg J. Treatment of mandibular class III periodontal furcation defects. Coronally positioned flaps with and without expanded polytetrafluoroethylene membranes. J Periodontol. 1994; 65: 592-7.

51. Geraci TF, Nevins M, Crossetti HW, Drizen K, Ruben MP. Reattachment of the periodontium after tooth movement into an osseous defect in a monkey. Int J Periodontics Restorative Dent. 1990; 10: 184-97.
52. Gottlow J, Nyman S, Karring T, Lindhe J. New attachment formation as the result of controlled tissue regeneration. J Clin Periodontol. 1984; 11: 494-503.
53. Gowen M, Wood DD, Ihrie EJ, McGuire MK, Russell RG. An interleukin 1 like factor stimulates bone resorption in vitro. Nature. 1983; 306: 378-80.
54. Gray JB, Smith R. Transitional implants for orthodontic anchorage. J Clin Orthod. 2000; 34: 659-66.
55. Grayson AC, Voskerician G, Lynn A, Anderson JM, Cima MJ, Langer R. Differential degradation rates in vivo and in vitro of biocompatible poly(lactic acid) and poly(glycolic acid) homo- and co-polymers for a polymeric drug-delivery microchip. J Biomater Sci Polym Ed. 2004; 15: 1281-1304.
56. Grieve WG III, Johnson GK, Moore RN, Reinhardt RA, DuBois LM. Prostaglandin E (PGE) and interleukin-1 beta (IL-1 beta) levels in gingival crevicular fluid during human orthodontic tooth movement. Am J Orthod Dentofacial Orthop. 1994; 105: 369-74.
57. Hayashi K, Igarashi K, Miyoshi K, Shinoda H, Mitani H. Involvement of nitric oxide in orthodontic tooth movement in rats. Am J Orthod Dentofacial Orthop. 2002; 122: 306-9.
58. Heath JK, Saklatvala J, Meikle MC, Atkinson SJ, Reynolds JJ. Pig interleukin 1 (catabolin) is a potent stimulator of bone resorption in vitro. Calcif Tissue Int. 1985; 37: 95-7.
59. Hecker M, Mulsch A, Bassenge E, Busse R. Vasoconstriction and increased flow: two principal mechanisms of shear stress-dependent endothelial autacoid release. Am J Physiol. 1993; 265: H828-H833.
60. Helfrich MH, Evans DE, Grabowski PS, Pollock JS, Ohshima H, Ralston SH. Expression of nitric oxide synthase isoforms in bone and bone cell cultures. J Bone Miner Res. 1997; 12: 1108-15.

61. Hill PA. Bone remodelling. Br J Orthod. 1998; 25: 101-7.
62. Hofbauer LC, Lacey DL, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S. Interleukin-1beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. Bone. 1999; 25: 255-9.
63. Hossain MZ, Kyomen S, Tanne K. Biologic responses of autogenous bone and beta-tricalcium phosphate ceramics transplanted into bone defects to orthodontic forces. Cleft Palate Craniofac J. 1996; 33: 277-83.
64. Ingman T, Apajalahti S, Mantyla P, Savolainen P, Sorsa T. Matrix metalloproteinase-1 and -8 in gingival crevicular fluid during orthodontic tooth movement: a pilot study during 1 month of follow-up after fixed appliance activation. Eur J Orthod. 2005; 27: 202–7.
65. Jacobs CR, Yellowley CE, Davis BR, Zhou Z, Cimbala JM, Donahue HJ. Differential effect of steady versus oscillating flow on bone cells. J Biomech. 1998; 31: 969-76.
66. Jager A, Zhang D, Kawarizadeh A, Tolba R, Braumann B, Lossdorfer S, Gotz W. Soluble cytokine receptor treatment in experimental orthodontic tooth movement in the rat. Eur J Orthod. 2005; 27: 1–11.
67. Johnson DL, McAllister TN, Frangos JA. Fluid flow stimulates rapid and continuous release of nitric oxide in osteoblasts. Am J Physiol. 1996; 271: E205-E208.
68. Jones JA, Chang DT, Meyerson H, Colton E, Kwon IK, Matsuda T, Anderson JM. Proteomic analysis and quantification of cytokines and chemokines from biomaterial surface-adherent macrophages and foreign body giant cells. J Biomed Mater Res A. 2007; 83: 585-96.
69. Kanomi R. Mini-implant for orthodontic anchorage. J Clin Orthod. 1997; 31: 763-7.
70. Kazandjian G, Scopp IW, Stahl S. Combined orthodontic-periodontal treatment of an infrabony defect. A case report. J Periodontol. 1979; 50: 479-82.

71. Klein-Nulend J, Helfrich MH, Sterck JG, MacPherson H, Joldersma M, Ralston SH, Semeins CM, Burger EH. Nitric oxide response to shear stress by human bone cell cultures is endothelial nitric oxide synthase dependent. Biochem Biophys Res Commun. 1998; 250: 108-14.
72. Klein-Nulend J, Semeins CM, Ajubi NE, Nijweide PJ, Burger EH. Pulsating fluid flow increases nitric oxide (NO) synthesis by osteocytes but not periosteal fibroblasts--correlation with prostaglandin upregulation. Biochem Biophys Res Commun. 1995; 217: 640-8.

73. Klein-Nulend J, van der Plas A, Semeins CM, Ajubi NE, Frangos JA, Nijweide PJ, Burger EH. Sensitivity of osteocytes to biomechanical stress in vitro. FASEB J. 1995; 9: 441-5.

74. Knippenberg M, Helder MN, Blieck-Hogervorst JM, Wuisman PI, Klein-Nulend J. Prostaglandins Differentially Affect Osteogenic Differentiation of Human Adipose Tissue-Derived Mesenchymal Stem Cells. Tissue Engineering. 2007; 13: 2495-3.

75. Knothe Tate ML, Knothe U. An ex vivo model to study transport processes and fluid flow in loaded bone. J Biomech. 2000; 33: 247-54.
76. Knothe Tate ML, Niederer P, Knothe U. In vivo tracer transport through the lacunocanalicular system of rat bone in an environment devoid of mechanical loading. Bone. 1998; 22: 107-17.
77. Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Morinaga T, Higashio K, Martin TJ, Suda T. Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL–RANK interaction. J Exp Med. 2000; 191: 275–86.
78. Krishnan V, Davidovitch Z. Cellular, molecular, and tissue-level reactions to orthodontic force. Am J Orthod Dentofacial Orthop. 2006; 129: 469-32.

79. Kufahl RH, Saha S. A theoretical model for stress-generated fluid flow in the canaliculi-lacunae network in bone tissue. J Biomech. 1990; 23: 171-80.
80. Li YP, Stashenko P. Proinflammatory cytokines tumor necrosis factor-alpha and IL-6, but not IL-1, down-regulate the osteocalcin gene promoter. J Immunol. 1992; 148: 788-94.
81. Lian JB, Stein GS. The developmental stages of osteoblast growth and differentiation exhibit selective responses of genes to growth factors (TGF beta 1) and hormones (vitamin D and glucocorticoids). J Oral Implantol. 1993; 19: 95–105.
82. Lin WL, McCulloch CA, Cho MI. Differentiation of periodontal ligament fibroblasts into osteoblasts during socket healing after tooth extraction in the rat. Anat Rec. 1994; 240: 492-506.
83. Lindhe J, Pontoriero R, Berglundh T, Araujo M. The effect of flap management and bioresorbable occlusive devices in GTR treatment of degree III furcation defects. An experimental study in dogs. J Clin Periodontol. 1995; 22: 276-83.
84. Lowney JJ, Norton LA, Shafer DM, Rossomando EF. Orthodontic forces increase tumor necrosis factor alpha in the human gingival sulcus. Am J Orthod Dentofacial Orthop. 1995; 108: 519-24.
85. MacIntyre I, Zaidi M, Alam AS, Datta HK, Moonga BS, Lidbury PS, Hecker M, Vane JR. Osteoclastic inhibition: an action of nitric oxide not mediated by cyclic GMP. Proc Natl Acad Sci USA. 1991; 88: 2936-40.
86. Mayer T, Basdra EK. A combined surgical and orthodontic treatment of Class III furcations. Report of a case. J Clin Periodontol. 1997; 24: 233-6.
87. Mehlbauer MJ, Greenwell H, Nouneh I, Drisko C, Wittwer JW, Yancey J, Rebitski G. Improved closure rate of Class III furcations using a layered GTR technique. Int J Periodontics Restorative Dent. 2000; 20: 285-95.

88. Melsen B, Agerbaek N, Eriksen J, Terp S. New attachment through periodontal treatment and orthodontic intrusion. Am J Orthod Dentofacial Orthop. 1988; 94: 104-16.
89. Melsen B. Biological reaction of alveolar bone to orthodontic tooth movement. Angle Orthod. 1999; 69: 151-8.
90. Melsen B. Tissue reaction following application of extrusive and intrusive forces to teeth in adult monkeys. Am J Orthod. 1986; 89: 469-75.
91. Mikuni-Takagaki Y, Naruse K, Azuma Y, Miyauchi A. The role of calcium channels in osteocyte function. J Musculoskelet Neuronal Interact. 2002; 2: 252-5.
92. Mostafa YA, Weaks-Dybvig M, Osdoby P. Orchestration of tooth movement. Am J Orthod. 1983; 83: 245-50.
93. Murray DW, Rushton N. The effect of strain on bone cell prostaglandin E2 release: a new experimental method. Calcif Tissue Int. 1990; 47: 35-9.
94. Neidlinger-Wilke C, Stalla I, Claes L, Brand R, Hoellen I, Rubenacker S, Arand M, Kinzl L. Human osteoblasts from younger normal and osteoporotic donors show differences in proliferation and TGF beta-release in response to cyclic strain. J Biomech. 1995; 28: 1411-8.
95. Nemcovsky CE, Beny L, Shanberger S, Feldman-Herman S, Vardimon A. Bone apposition in surgical bony defects following orthodontic movement: a comparative histomorphometric study between root- and periodontal ligament-damaged and periodontally intact rat molars. J Periodontol. 2004; 75: 1013-9.
96. Nemcovsky CE, Sasson M, Beny L, Weinreb M, Vardimon AD. Periodontal healing following orthodontic movement of rat molars with intact versus damaged periodontia towards a bony defect. Eur J Orthod. 2007; 29: 338-44.
97. Nemcovsky CE, Zubery Y, Artzi Z, Lieberman MA. Orthodontic tooth movement following guided tissue regeneration: report of three cases. Int J Adult Orthodon Orthognath Surg. 1996; 11: 347-55.

98. Nevins M, Wise RJ. Use of orthodontic therapy to alter infrabony pockets. Int J Periodontics Restorative Dent. 1990; 10: 198-207.

99. Noble BS, Reeve J. Osteocyte function, osteocyte death and bone fracture resistance. Mol Cell Endocrinol. 2000; 159: 7-13.

100. Nohutcu RM, McCauley LK, Koh AJ, Somerman MJ. Expression of extracellular matrix proteins in human periodontal ligament cells during mineralization in vitro. J Periodontol. 1997; 68: 320-7.

101. Noxon SJ, King GJ, Gu G, Huang G. Osteoclast clearance from periodontal tissues during orthodontic tooth movement Am J Orthod Dentofacial Orthop. 2001; 120: 466-76.

102. Nyman S, Lindhe J, Karring T, Rylander H. New attachment following surgical treatment of human periodontal disease. J Clin Periodontol. 1982; 9: 290-6.

103. Ohmae M, Saito S, Morohashi T, Seki K, Qu H, Kanomi R, Yamasaki KI, Okano T, Yamada S, Shibasaki Y. A clinical and histological evaluation of titanium mini-implants as anchors for orthodontic intrusion in the beagle dog. Am J Orthod Dentofacial Orthop. 2001; 119: 489-97.

104. Owan I, Burr DB, Turner CH, Qiu J, Tu Y, Onyia JE, Duncan RL. Mechanotransduction in bone: osteoblasts are more responsive to fluid forces than mechanical strain. Am J Physiol. 1997; 273: C810-15.

105. Palioto DB, Joly JC, de Lima AF, Mota LF, Caffesse R. Clinical and radiographic treatment evaluation of class III furcation defects using GTR with and without inorganic bone matrix. J Clin Periodontol. 2003; 30: 1-8.

106. Parfitt AM. The cellular basis of bone turnover and bone loss: a rebuttal of the osteocytic resorption--bone flow theory. Clin Orthop Relat Res. 1977; 236-47.

107. Park, H. S., Bae, S. M., Kyung, H. M. & Sung, J. H. Micro-implant anchorage for treatment of skeletal Class I bialveolar protrusion. J Clin Orthod. 2001; 35: 417-22.

108. Pavalko FM, Gerard RL, Ponik SM, Gallagher PJ, Jin Y, Norvell SM. Fluid shear stress inhibits TNF-alpha-induced apoptosis in osteoblasts: a role for fluid shear stress-induced activation of PI3-kinase and inhibition of caspase-3. J Cell Physiol. 2003; 194: 194-205.

109. Piekarski K, Munro M. Transport mechanism operating between blood supply and osteocytes in long bones. Nature. 1977; 269: 80-2.

110. Pitsillides AA, Rawlinson SC, Suswillo RF, Bourrin S, Zaman G, Lanyon LE. Mechanical strain-induced NO production by bone cells: a possible role in adaptive bone (re)modeling? FASEB J. 1995; 9: 1614-22.

111. Polson A, Caton J, Polson AP, Nyman S, Novak J, Reed B. Periodontal response after tooth movement into intrabony defects. J Periodontol. 1984; 55: 197-202.

112. Polson AM, Reed BE. Long-term effect of orthodontic treatment on crestal alveolar bone levels. J Periodontol. 1984; 55: 28-4.

113. Pontoriero R, Lindhe J, Nyman S, Karring T, Rosenberg E, Sanavi F. Guided tissue regeneration in the treatment of furcation defects in mandibular molars. A clinical study of degree III involvements. J Clin Periodontol. 1989; 16: 170-4.

114. Pontoriero R, Lindhe J. Guided tissue regeneration in the treatment of degree III furcation defects in maxillary molars. J Clin Periodontol. 1995; 22: 810-2.

115. Re S, Corrente G, Abundo R, Cardaropoli D. Orthodontic movement into bone defects augmented with bovine bone mineral and fibrin sealer: a reentry case report. Int J Periodontics Restorative Dent. 2002; 22: 138-45.

116. Reynolds MA, Aichelmann-Reidy ME., Branch-Mays GL, Gunsolley JC. The efficacy of bone replacement grafts in the treatment of periodontal osseous defects. A systematic review. Ann Periodontol. 2003; 8: 227-65.

117. Robling AG, Castillo AB, Turner CH. Biomechanical and molecular regulation of bone remodeling. Annu Rev Biomed Eng. 2006; 8: 455-98.

118. Roriz VM, Souza SL, Taba M Jr, Palioto DB, Grisi MF. Treatment of Class III furcation defects with expanded polytetrafluoroethylene

membrane associated or not with anorganic bone matrix/synthetic cell-binding peptide: a histologic and histomorphometric study in dogs. J Periodontol. 2006; 77: 490-7.

119. Rossa C Jr, Marcantonio E Jr, Cirelli JA, Marcantonio RA, Spolidorio LC, Fogo JC. Regeneration of Class III furcation defects with basic fibroblast growth factor (b-FGF) associated with GTR. A descriptive and histometric study in dogs. J Periodontol. 2000; 71: 775-84.
120. Rossig L, Haendeler J, Hermann C, Malchow P, Urbich C, Zeiher AM, Dimmeler S. Nitric oxide down-regulates MKP-3 mRNA levels: involvement in endothelial cell protection from apoptosis. J Biol Chem. 2000; 275: 25502-7.
121. Rubin CT, Lanyon LE. Limb mechanics as a function of speed and gait: a study of functional strains in the radius and tibia of horse and dog. J Exp Biol. 1982; 101: 187-211.
122. Rubin CT, Lanyon LE. Regulation of bone formation by applied dynamic loads. J Bone Joint Surg Am. 1984; 66: 397-402.
123. Rubin J, Fan X, Biskobing DM, Taylor WR, Rubin CT. Osteoclastogenesis is repressed by mechanical strain in an in vitro model. J Orthop Res. 1999; 17: 639-45.
124. Samorodnitzky-Naveh GR, Geiger SB, Levin L. Patients' satisfaction with dental esthetics. J Am Dent Assoc. 2007; 138: 805-8.
125. Schling P, Rudolph C, Heimerl S, Fruth S, Schmitz G. Expression of tumor necrosis factor alpha and its receptors during cellular differentiation. Cytokine. 2006; 33: 239-45.
126. Schmidt HH, Pollock JS, Nakane M, Forstermann U, Murad F. Ca2+/calmodulin-regulated nitric oxide synthases. Cell Calcium, 1992; 13: 427-34.
127. Scott P, Fleming P, DiBiase A. An update in adult orthodontics. Dent Update. 2007; 34: 427-34, 436.

128. Shapiro F. Cortical bone repair. The relationship of the lacunar-canalicular system and intercellular gap junctions to the repair process. J Bone Joint Surg Am. 1988; 70: 1067-81.

129. Shapiro F. Variable conformation of GAP junctions linking bone cells: a transmission electron microscopic study of linear, stacked linear, curvilinear, oval, and annular junctions. Calcif Tissue Int. 1997; 61: 285-93.

130. Shirazi M, Nilforoushan D, Alghasi H, Dehpour AR. The role of nitric oxide in orthodontic tooth movement in rats. Angle Orthod. 2002; 72: 211-5.

131. Smit TH, Burger EH, Huyghe JM. A case for strain-induced fluid flow as a regulator of BMU-coupling and osteonal alignment. J Bone Miner Res. 2002; 17: 2021-9.

132. Stashenko P, Obernesser MS, Dewhirst FE. Effect of immune cytokines on bone. Immunol Invest. 1989; 18: 239-49.

133. Stelzel MJ, Flores-de-Jacoby L. Guided tissue regeneration in a combined periodontal and orthodontic treatment: a case report. Int J Periodontics Restorative Dent. 1998; 18: 189-95.

134. Stenvik A, Mjor IA. Pulp and dentine reactions to experimental tooth intrusion. A histologic study of the initial changes. Am J Orthod. 1970; 57: 370-85.

135. Tan SD, de Vries TJ, Kuijpers-Jagtman AM, Semeins CM, Everts V, Klein-Nulend J. Osteocytes subjected to fluid flow inhibit osteoclast formation and bone resorption. Bone. 2007; 41: 745-51.

136. Tan SD, Kuijpers-Jagtman AM, Semeins CM, Bronckers AL, Maltha JC, Von den Hoff JW, Everts V, Klein-Nulend J. Fluid shear stress inhibits TNFalpha-induced osteocyte apoptosis. J Dent Res. 2006; 85: 905-9.

137. Turner CH, Forwood MR, Otter MW. Mechanotransduction in bone: do bone cells act as sensors of fluid flow? FASEB J. 1994; 8: 875-8.

138. Turner CH, Owan I, Takano Y. Mechanotransduction in bone: role of strain rate. Am J Physiol. 1995; 269: E438-42.

139. Turner CH, Takano Y, Owan I, Murrell GA. Nitric oxide inhibitor L-NAME suppresses mechanically induced bone formation in rats. Am J Physiol. 1996; 270: E634-9.

140. Uematsu M, Ohara Y, Navas JP, Nishida K, Murphy TJ, Alexander RW, Nerem RM, Harrison DG. Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress. Am J Physiol. 1995; 269: C1371-8.

141. Uematsu S, Mogi M, Deguchi T. Interleukin (IL)-1 beta, IL-6, tumor necrosis factor-alpha, epidermal growth factor, and beta 2-microglobulin levels are elevated in gingival crevicular fluid during human orthodontic tooth movement. J Dent Res. 1996; 75: 562-7.

142. Umemori M, Sugawara J, Mitani H, Nagasaka H, Kawamura H. Skeletal anchorage system for open-bite correction. Am J Orthod Dentofacial Orthop. 1999; 115: 166-74.

143. Vandamme W, Braet K, Cabooter L, Leybaert L. Tumour necrosis factor alpha inhibits purinergic calcium signalling in blood-brain barrier endothelial cells. J Neurochem. 2004; 88: 411-21.

144. van't Hof RJ, Ralston SH. Nitric oxide and bone. Immunology. 2001; 103: 255-61.

145. Vardimon AD, Nemcovsky CE, Dre E. Orthodontic tooth movement enhances bone healing of surgical bony defects in rats. J Periodontol. 2001; 72: 858-64.

146. Waerhaug J. The furcation problem. Etiology, pathogenesis, diagnosis, therapy and prognosis. J Clin Periodontol. 1980; 7: 73-95.

147. Wakabayashi RC, Iha DK, Niu JJ, Johnson PW. Cytokine production by cells adherent to regenerative membranes. J Periodontal Res. 1997; 32: 215-24.

148. Wang L, Ciani C, Doty SB, Fritton SP. Delineating bone's interstitial fluid pathway in vivo. Bone. 2004; 34: 499-09.

149. Weinbaum S, Cowin SC, Zeng Y. A model for the excitation of osteocytes by mechanical loading-induced bone fluid shear stresses. J Biomech. 1994; 27: 339-60.

150. Wennstrom JL, Stokland BL, Nyman S, Thilander B. Periodontal tissue response to orthodontic movement of teeth with infrabony pockets. Am J Orthod Dentofacial Orthop. 1993; 103: 313-9.

151. Westbroek I, Ajubi NE, Alblas MJ, Semeins CM, Klein-Nulend J, Burger EH, Nijweide PJ. Differential stimulation of prostaglandin G/H synthase-2 in osteocytes and other osteogenic cells by pulsating fluid flow. Biochem Biophys Res Commun. 2000; 268: 414-9.

152. Yao CC, Wu CB, Wu HY, Kok SH, Chang HF, Chen YJ. Intrusion of the overerupted upper left first and second molars by mini-implants with partial-fixed orthodontic appliances: a case report. Angle Orthod. 2004; 74: 550-7.

153. Yokoyama T, Vaca L, Rossen RD, Durante W, Hazarika P, Mann DL. Cellular basis for the negative inotropic effects of tumor necrosis factor-alpha in the adult mammalian heart J Clin Invest. 1993; 92: 2303-12.

154. Yoo SK, Warita H, Soma K. Duration of orthodontic force affecting initial response of nitric oxide synthase in rat periodontal ligaments. J Med Dent Sci. 2004; 51: 83-8.

155. Yoshimatsu M, Shibata Y, Kitaura H, Chang X, Moriishi T, Hashimoto F, Yoshida N, Yamaguchi A. Experimental model of tooth movement by orthodontic force in mice and its application to tumor necrosis factor receptor-deficient mice. J Bone Miner Metab. 2006; 24: 20–7.

156. You LD, Weinbaum S, Cowin SC, Schaffler MB. Ultrastructure of the osteocyte process and its pericellular matrix. Anat Rec A Discov Mol Cell Evol Biol. 2004; 278: 505-13.

157. Zaman G, Pitsillides AA, Rawlinson SC, Suswillo RF, Mosley JR, Cheng MZ, Platts LA, Hukkanen M, Polak JM, Lanyon LE. Mechanical strain stimulates nitric oxide production by rapid activation of endothelial nitric oxide synthase in osteocytes. J Bone Miner Res. 1999; 14: 1123-31.

158. Zhao M, Xiao G, Berry JE, Franceschi RT, Reddi A, Somerman MJ. Bone morphogenetic protein 2 induces dental follicle cells to differentiate toward a cementoblast/osteoblast phenotype. J Bone Miner Res. 2002; 17: 1441-51.

8 Anexos

Anexo 1

Tabela A1 – Estatísticas descritivas: médias e desvio-padrão dos dados clínicos de profundidade de sondagem, em mm nos diferentes grupos avaliados. Médias e desvio-padrão seguidos por letras minúsculas iguais na linha ou maiúsculas iguais na coluna não são significativamente diferentes pelo teste de Tukey, ao nível de 5%

	C		**R**		**BI**		**AI**		**F**		**ANOVA (p)**
OFD	1.90±0.1	$^{A}_{a}$	1.90±0.4	$^{A}_{a}$	1.86±0.5	$^{AB}_{a}$	2.12±0.2	$^{A}_{a}$	1.69±0.7	$^{A}_{a}$	0.57 – ns
OFD+I	2.02±0.2	$^{A}_{a}$	2.02±0.2	$^{A}_{a}$	1.55±0.4	$^{A}_{a}$	3.55±0.9	$^{B}_{b}$	2.90±0.5	$^{BC}_{b}$	*0.0002 - s*
GTR/BA	1.83±0.3	$^{A}_{a}$	1.95±0.2	$^{A}_{a}$	2.10±0.4	$^{B}_{a}$	2.07±0.3	$^{A}_{a}$	2.00±0.2	$^{AB}_{a}$	0.50- ns
GTR/BA+I	1.86±0.2	$^{A}_{a}$	2.07±0.5	$^{A}_{a}$	1.98±0.3	$^{AB}_{a}$	3.83±1.3	$^{B}_{b}$	3.07±0.8	$^{C}_{b}$	*0.0008 - s*
ANOVA (p)	0.22	ns	0.66	ns	*0.02*	*s*	*0.0007*	*s*	*0.001*	*s*	

OFD = raspagem com acesso via retalho;

OFD+I = raspagem com acesso via retalho e intrusão;

GTR/BA = regeneração tecidual guiada e enxerto ósseo autógeno;

GTR/BA+I = regeneração tecidual guiada e enxerto ósseo autógeno e intrusão;

C = cronificação;

R = imediatamente antes do tratamento periodontal;

BI = antes da intrusão;

AI = após a intrusão;

F = final da contenção;

s, ns = significante e não significante.

Tabela A2 – Estatísticas descritivas: médias e desvio-padrão do nível gengival, em mm nos diferentes grupos avaliados. Médias e desvio-padrão seguidos por letras minúsculas iguais na linha ou maiúsculas iguais na coluna não são significativamente diferentes pelo teste de Tukey, ao nível de 5%

	R		**BI**		**AI**		**F**		**ANOVA (p)**
OFD	0.14±0.2	$^{A}_{a}$	0.06±0.2	$^{A}_{a}$	0.12±0.3	$^{A}_{a}$	0.07±0.4	$^{A}_{a}$	0.94 – ns
OFD+I	0.24±0.3	$^{A}_{a}$	-0.07±0.2	$^{A}_{a}$	-2.00±1.6	$^{B}_{b}$	-1.43±0.9	$^{B}_{b}$	*0.00003 - s*
GTR/BA	0.05±0.3	$^{A}_{a}$	0.02±0.2	$^{A}_{a}$	0.21±0.2	$^{A}_{a}$	0.19±0.2	$^{A}_{a}$	0.15 - ns
GTR/BA+I	0.14±0.2	$^{A}_{a}$	-0.02±0.2	$^{A}_{a}$	-2.05±1.78	$^{B}_{b}$	-1.55±1.3	$^{B}_{b}$	*0.000001 - s*
ANOVA (p)	0.08	ns	0.28	ns	*0.0004*	*s*	*0.001*	*s*	

OFD = raspagem com acesso via retalho;

OFD+I = raspagem com acesso via retalho e intrusão;

GTR/BA = regeneração tecidual guiada e enxerto ósseo autógeno;

GTR/BA+I = regeneração tecidual guiada e enxerto ósseo autógeno e intrusão;

R = imediatamente antes do tratamento periodontal;

BI = antes da intrusão;

AI = após a intrusão;

F = final da contenção;

s, ns = significante e não significante.

Tabela A3 – Estatísticas descritivas: médias e desvio-padrão do nível de inserção, em mm nos diferentes grupos avaliados. Médias e desvio-padrão seguidos por letras minúsculas iguais na linha ou maiúsculas iguais na coluna não são significativamente diferentes pelo teste de Tukey, ao nível de 5%

	C		**R**		**BI**		**AI**		**F**		**ANOVA (p)**
OFD	1.90±0.1	$^{A}_{a}$	2.05±0.5	$^{A}_{a}$	1.92±0.6	$^{AB}_{a}$	2.24±0.3	$^{A}_{a}$	1.76±0.8	$^{A}_{a}$	0.57 – ns
OFD+I	2.02±0.2	$^{A}_{a}$	2.26±0.5	$^{A}_{a}$	1.48±0.5	$^{A}_{b}$	1.55±0.9	$^{A}_{b}$	1.48±0.6	$^{A}_{b}$	*0.02 - s*
GTR/BA	1.83±0.3	$^{A}_{a}$	2.0±0.4	$^{A}_{a}$	2.12±0.4	$^{B}_{a}$	2.29±0.4	$^{A}_{a}$	2.19±0.2	$^{A}_{a}$	0.18 - ns
GTR/BA+I	1.86±0.2	$^{A}_{a}$	2.21±0.7	$^{A}_{a}$	1.95±0.3	$^{AB}_{a}$	1.79±0.7	$^{A}_{a}$	1.52±0.7	$^{A}_{a}$	0.34 - ns
ANOVA (p)	0.22	ns	0.31	ns	*0.02*	*s*	0.14	ns	0.11	ns	

OFD = raspagem com acesso via retalho;

OFD+I = raspagem com acesso via retalho e intrusão;

GTR/BA = regeneração tecidual guiada e enxerto ósseo autógeno;

GTR/BA+I = regeneração tecidual guiada e enxerto ósseo autógeno e intrusão;

C = cronificação;

R = imediatamente antes do tratamento periodontal;

BI = antes da intrusão;

AI = após a intrusão;

F = final da contenção;

s, ns = significante e não significante.

Tabela A4 – Estatísticas descritivas: médias, desvio-padrão (DP) e mediana (valor mínimo - Mín e máximo - Máx) do número de faces por grupo com placa visível nos diferentes grupos avaliados. Médias e desvio-padrão seguidos por letras minúsculas iguais na linha ou maiúsculas iguais na coluna não são significativamente diferentes (ns) pelo teste de comparações múltiplas, ao nível de 5%

	C		**R**		**BI**		**AI**		**F**		**Friedman (p)**
OFD											
Média±DP	4.14±0.9	$^{A}_{a}$	1.86±1.8	$^{A}_{a}$	2.29±2.6	$^{A}_{a}$	3.29±1.6	$^{A}_{a}$	3.57±2.5	$^{A}_{a}$	0.40 - ns
Mediana (Mín e Máx)	4 (3-6)		2 (0-4)		1 (0-6)		4 (0-5)		4 (1-6)		
OFD+I											
Média±DP	4.43±0.5	$^{A}_{a}$	1.71±1.7	$^{A}_{a}$	3.00±2.0	$^{A}_{a}$	3.71±0.7	$^{A}_{a}$	3.86±2.7	$^{A}_{a}$	0.16 - ns
Mediana (Mín e Máx)	4 (4-5)		1 (0-4)		3 (0-6)		4 (3-5)		6 (0-6)		
GTR/BA											
Média±DP	4.43±0.5	$^{A}_{a}$	1.71±1.8	$^{A}_{a}$	3.00±1.8	$^{A}_{a}$	3.14±0.1	$^{A}_{a}$	3.86±2.7	$^{A}_{a}$	0.15 - ns
Mediana (Mín e Máx)	4 (4-5)		2 (0-4)		3 (1-6)		3 (3-4)		6 (0-6)		
GTR/BA+I											
Média±DP	4.43±1.1	$^{A}_{a}$	2.43±1.9	$^{A}_{a}$	3.14±2.7	$^{A}_{a}$	3.00±1.4	$^{A}_{a}$	4.00±2.5	$^{A}_{a}$	0.32 - ns
Mediana (Mín e Máx)	4 (3-6)		4 (0-4)		2 (0-6)		3 (0-4)		5 (0-6)		
Friedman (p)	0.94	ns	0.70	ns	0.81	ns	0.50	ns	0.99	ns	

OFD = raspagem com acesso via retalho;

OFD+I = raspagem com acesso via retalho e intrusão;

GTR/BA = regeneração tecidual guiada e enxerto ósseo autógeno;

GTR/BA+I = regeneração tecidual guiada e enxerto ósseo autógeno e intrusão;

C = cronificação;

R = imediatamente antes do tratamento periodontal;

BI = antes da intrusão;

AI = após a intrusão;

F = final da contenção.

Tabela A5 – Estatísticas descritivas: médias, desvio-padrão (DP) e mediana (valor mínimo e máximo) do número de faces por grupo com sangramento gengival marginal nos diferentes grupos avaliados. Médias e DP seguidos por letras minúsculas iguais na linha ou maiúsculas iguais na coluna não são significativamente diferentes (ns) pelo teste de comparações múltiplas, ao nível de 5%

	C		**R**		**BI**		**AI**		**F**		**Friedman (p)**
OFD											
Média±DP	1.71±1.9	$^{A}_{a}$	1.57±1.3	$^{A}_{a}$	2.29±1.6	$^{A}_{a}$	1.29±1.4	$^{A}_{a}$	1.00±1.0	$^{A}_{a}$	0.90 - ns
Mediana (Mín e Máx)	1 (0-4)		1 (0-4)		3 (0-4)		1 (0-3)		1 (0-3)		
OFD+I											
Média±DP	0.86±1.5	$^{A}_{a}$	1.43±1.4	$^{A}_{a}$	1.14±1.5	$^{A}_{a}$	2.57±2.1	$^{A}_{a}$	1.29±1.1	$^{A}_{a}$	0.36 - ns
Mediana (Mín e Máx)	0 (0-4)		1 (0-4)		1 (0-4)		3 (0-6)		1 (0-3)		
GTR/BA											
Média±DP	1.14±1.5	$^{A}_{a}$	1.29±1.4	$^{A}_{a}$	2.00±1.3	$^{A}_{a}$	0.29±0.5	$^{A}_{a}$	0.57±0.8	$^{A}_{a}$	0.18 - ns
Mediana (Mín e Máx)	1 (0-4)		1 (0-4)		2 (0-4)		0 (0-1)		0 (0-2)		
GTR/BA+I											
Média±DP	1.00±1.5	$^{A}_{a}$	1.57±1.3	$^{A}_{a}$	2.7±1.6	$^{A}_{a}$	2.43±2.3	$^{A}_{a}$	1.71±2.1	$^{A}_{a}$	0.52 - ns
Mediana (Mín e Máx)	0 (0-4)		1 (0-4)		3 (0-4)		2 (0-6)		1 (0-6)		
Friedman (p)	0.98	ns	0.81	ns	0.13	ns	0.07	ns	0.61	ns	

OFD = raspagem com acesso via retalho;

OFD+I = raspagem com acesso via retalho e intrusão;

GTR/BA = regeneração tecidual guiada e enxerto ósseo autógeno;

GTR/BA+I = regeneração tecidual guiada e enxerto ósseo autógeno e intrusão;

C = cronificação;

R = imediatamente antes do tratamento periodontal;

BI = antes da intrusão;

AI = após a intrusão;

F = final da contenção.

Mín, Máx: valor mínimo e máximo

Tabela A6 – Estatísticas descritivas: médias, desvio-padrão (DP) e mediana (valor mínimo e máximo) do número de sítios por grupo com sangramento a sondagem nos diferentes grupos avaliados. Médias e DP seguidas por letras minúsculas iguais na linha ou maiúsculas iguais na coluna não são significativamente diferentes (ns) pelo teste de comparações múltiplas, ao nível de 5%

	C		R		BI		AI		F		Friedman (p)
OFD											
Média±DP	0.86±1.2	$^{A}_{a}$	2.00±2.3	$^{A}_{a}$	1.29±1.2	$^{A}_{a}$	1.14±1.3	$^{A}_{a}$	1.00±1.1	$^{A}_{a}$	0.78 - ns
Mediana (Mín e Máx)	0 (0-3)		2 (0-6)		1 (0-3)		1 (0-4)		1 (0-3)		
OFD+I											
Média±DP	0.43±1.1	$^{A}_{a}$	2.14±2.1	$^{A}_{a}$	2.29±1.9	$^{A}_{a}$	2.28±0.9	$^{A}_{a}$	1.71±1.5	$^{A}_{a}$	0.07 - ns
Mediana (Mín e Máx)	0 (0-3)		1 (0-6)		3 (0-5)		2 (1-4)		2 (0-4)		
GTR/BA											
Média±DP	0.14±0.4	$^{A}_{a}$	1.71±1.8	$^{A}_{a}$	1.71±0.7	$^{A}_{a}$	1.57±1.5	$^{A}_{a}$	0.43±0.8	$^{A}_{a}$	0.07 - ns
Mediana (Mín e Máx)	0 (0-1)		1 (0-5)		2 (1-3)		1 (0-4)		0 (0-2)		
GTR/BA+I											
Média±DP	1.00±1.7	$^{A}_{a}$	1.86±2.1	$^{A}_{a}$	2.14±1.3	$^{A}_{a}$	2.85±1.9	$^{A}_{a}$	1.42±0.8	$^{A}_{a}$	0.06 - ns
Mediana (Mín e Máx)	0 (0-4)		1 (0-6)		2 (1-4)		4 (0-5)		1 (1-3)		
Friedman (p)	0.70	ns	0.76	ns	0.605	ns	0.19	ns	0.09	ns	

OFD = raspagem com acesso via retalho;

OFD+I = raspagem com acesso via retalho e intrusão;

GTR/BA = regeneração tecidual guiada e enxerto ósseo autógeno;

GTR/BA+I = regeneração tecidual guiada e enxerto ósseo autógeno e intrusão;

C = cronificação;

R = imediatamente antes do tratamento periodontal;

BI = antes da intrusão;

AI = após a intrusão;

F = final da contenção.

Mín, Máx: valor mínimo e máximo

Tabela A7 – Estatísticas descritivas das medidas histométricas de osso e tecidos moles, em porcentagens, obtidas na área da lesão nos diferentes grupos avaliados: média, desvio padrão (DP), mediana (valor mínimo e máximo) e o valor de probabilidade p correspondente ao teste de Friedman

	OFD	**OFD+I**	**GTR/BA**	**GTR/BA+I**	**Friedman (p)**
Osso (%)					
Média±DP	20.45±16.2	50.26± 12.3 *	22.04±21.5	26.75±16.2	*0.01 - s*
Mediana (Mín e Máx)	20.62 (0.97-37.82)	50.32 (30.32-72.21)	14.11 (0.73-49.01)	34.74 (1.86-41.23)	
Tecidos Moles (%)					
Média±DP	55.70± 8.7	39.64±12.6 *	56.32±11.9	48.60±5.9	*0.01 - s*
Mediana (Mín e Máx)	55.38 (43.16-72.23)	35.05 (20.13-57.66)	53.75 (42.28-80.79)	51.21 (42.19-55.61)	

OFD = raspagem com acesso via retalho;

OFD+I = raspagem com acesso via retalho e intrusão;

GTR/BA = regeneração tecidual guiada e enxerto ósseo autógeno;

GTR/BA+I = regeneração tecidual guiada e enxerto ósseo autógeno e intrusão;

Mín: valor mínimo;

Máx: valor máximo;

s = significante.

Tabela A8 – Estatísticas descritivas das medidas histométricas de cemento, epitélio e regeneração periodontal linear (RPL), em porcentagens, obtidas na extensão linear da lesão nos diferentes grupos avaliados: média, desvio padrão (DP), mediana (valor mínimo e máximo) e o valor de probabilidade p correspondente ao teste de Friedman

	OFD	**OFD+I**	**GTR/BA**	**GTR/BA+I**	**Friedman (p)**
Cemento (%)					
Média±DP	50.08±22.4	58.25±20.9	58.53±36.1	50.14±27.3	
Mediana (Mín e Máx)	45.36 (22.56-85.66)	56.41 (37.15-100)	70.03 (12.22-100)	41.05 (24.21-100)	0.960 - ns
Epitélio (%)					
Média±DP	21.96±13.4	19.64±17.7	10.17±11.1	7.81±8.5	
Mediana (Mín e Máx)	20.81 (4.6-44.49)	15.14 (0-50.35)	6.88 (0-16.93)	3.97 (0-21.21)	0.147 - ns
RPL (%)					
Média±DP	40.51±33.0	53.45±21.7	38.49±37.6	40.84±33.4	
Mediana (Mín e Máx)	37.02 (5.62-100)	49.65 (35.64-100)	27.72 (2.55-94.66)	40.29 (1.68-100)	0.170 - ns

OFD = raspagem com acesso via retalho;

OFD+I = raspagem com acesso via retalho e intrusão;

GTR/BA = regeneração tecidual guiada e enxerto ósseo autógeno;

GTR/BA+I = regeneração tecidual guiada e enxerto ósseo autógeno e intrusão;

Mín: valor mínimo;

Máx: valor máximo;

ns = não significante.

UNIVERSIDADE ESTADUAL PAULISTA
Faculdade de Odontologia
Campus de Araraquara

Anexo 2

Comitê de Ética em Experimentação Animal-CEEA

CERTIFICADO

Certicamos que o protocolo nr. *14/2005* referente à pesquisa *"Intrusão ortodôntica de pré-molares com lesão de furca Grau III. Estudo histológico e histométrico em cães"* sob a responsabilidade de *Vanessa Camila da Silva* e *Rosemary Adriana Chiérici Marcantonio* está de acordo com os Princípios Éticos em Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pelo Comitê de Ética em Experimentação Animal (CEEA) da Faculdade de Odontologia de Araraquara-UNESP em reunião de *16/abril/2007.*

CERTIFICATE

We certify that the protocol *14/2005* referring to the research *"Orthodontic intrusion of premolars with class III furcation lesions. Histologic and histometric study in dogs "* under responsibility of *Vanessa Camila da Silva e Rosemary Adriana Chiérici Marcantonio* is in agreement with the Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation (COBEA) and was approved by the Araraquara Dental School-UNESP Ethical Committee for Animal Research (CEEA) in *April 16, 2007.*

Araraquara, 16 de abril de 2007

Profa. Dra. Maria Rita Brancini de Oliveira
Coordenadora do CEEA/FOAr/UNESP

Anexo 3

Descrição detalhada da metodologia do artigo: "TNFα and IL-1β inhibit fluid shear stress-induced nitric oxide production by osteocytes and osteoblasts".

1. Metodologia do fluxo de fluido pulsátil (PFF)

MC3T3-E1 osteoblastos[5] foram cultivados até 80% de confluência em frascos de 75-cm^2 (Nunc, Roskilde, Dinamarca), usando meio de Eagle modificado (α-MEM; Gibco, Paisley, Inglaterra) suplementado com 10% de soro fetal bovino (FBS; Gibco), ácido ascórbico (50 μg/ml; Merck, Darmstadt, Alemanha), l-glutamina (300 μg/ml; Merck), penicilina (300 μg/ml; Sigma-Aldrich, St. Louis, MO, EUA), estreptomicina (250 μg/ml; Sigma), fungizona (1.25 μg/ml; Gibco), a 37ºC com atmosfera de 5% de CO_2.

MC3T3-E1 osteoblastos entre as passagens 10-20 foram coletados usando trypsina a 0.25% (Difco Laboratories, Detroit, MI, EUA) e EDTA a 0.1% (Sigma). Células foram semeadas a 3 x 10^5 células/cm^2 sobre lâminas de vidro (7.5 x 3.5 cm) previamente preparadas com polilisina (50 μg/ml; poly-L-lysine hydrobromide; Sigma) usando α-MEM com 10% FBS e suplementos descritos acima (Fig. A1a). As lâminas de vidro foram preparadas com polilisina conforme a seqüência: 30 min em 5 ml HCl + 495 ml álcool (96%), 1 min em 500 ml de água estéril, 15 min em 25 mg polilisina (Sigma) + 500 ml de água esterilizada e finalmente por 4 seg em 500 ml de álcool 70% e colocadas para secar em estufa a 37^0C por 12h.

A lâmina de vidro com as células foi acondicionada em placa de Petri plástica esterilizada (Greiner Bio-One, Frickenhausen, Alemanha) em estufa a 37ºC com 5% de CO_2. Após 45 min, as células estavam inicialmente aderidas na lâmina de vidro e foram adicionados 13 ml de meio de cultura (Fig. A1b, c). As células foram incubadas durante 24h a 37ºC com atmosfera de 5% de CO_2 para promover inserção celular antes da aplicação do fluxo de fluido pulsátil como descrito a seguir (Fig. A1d).

MLO-Y4 osteócitos (gentilmente fornecida por Dr. Bonewald, San Antonio, TX, EUA)[4] foram também cultivadas até 80% confluência em frascos de cultura de 75-cm^2 usando α-MEM suplementado com 5% FBS, 5% soro de bezerro (CS; Gibco), gentamicina (50 μg/ml), fungizone (1.25 μg/ml), penicilina (60 μg/ml), e estreptomicina (50 μg/ml), a 37ºC e atmosfera de 5% de CO_2. Células entre as passagens 30-35 foram coletadas e semeadas a 3 x 10^5 células/cm^2 sobre lâminas de vidro preparadas com polilisina usando α-MEM com 5% FBS e 5% CS e suplementos.

TNFα (0.5, 10 e 30 ng/ml), IL-1ß (0.1, 1 e 10 ng/ml) ou inibidores de cálcio como 8-(NN-dietilamino) octil-3,4,5-trimetoxibenzoato HCl (TMB-8, 20 μM, Biomol Research Laboratories Inc, Plymouth Meeting, PA, USA), cloreto de gadolínio ($GdCl_3$, 10 μM, Sigma) ou etilenoglicol-bis (2-aminoetiléter)-N,N,N',N'-ácido tetraacético (EGTA, 2 mM, Sigma) foram adicionados 30 min antes dos tratamentos estático e de fluxo de fluido pulsátil. As substâncias acima estavam também presentes durante o experimento descrito a seguir.

A lâmina de vidro com as células foi posicionada na câmara de fluxo (Fig. A2-4). O movimento pulsátil do fluido é gerado bombeando o meio de cultura através da câmara de fluxo contendo a monocamada celular (Fig. A5a). Todos os componentes foram colocados em uma estufa a 37^oC e um reservatório com água destilada foi conectado a um sistema gasoso que manteve a atmosfera úmida com 5% de CO_2 no ar (Fig. A5b). A freqüência (5 Hz) e a amplitude (0.3 Pa) do fluxo do fluido pulsátil foram geradas com uma bomba circular giratória de 24V (Masterflex, Cole-Parmer Instrument Co., Vernon Hills, Illinois, USA) (Fig. A5a). A velocidade do fluxo de 20 ml/min foi monitorada com um sensor (T206, Transonic Systems, Ithaca, NY, EUA) (Fig. A5a, b). A pressão média sobre a célula gerada pelo atrito produzido pelo movimento do líquido foi de 0.6 ± 0.3 Pa^2. O cálculo dessa pressão foi obtido com a fórmula: $\tau=6\mu Q/(bh^2)$ onde μ é a viscosidade do líquido (0.0069 dina/cm^2*seg), Q é a velocidade do fluxo (20ml/min=0.333333cm^3/seg), b é a largura da câmara onde o líquido se movimenta sobre as células (2.4 cm) e h é a altura da câmara onde o líquido se

movimenta sobre as células (0.03cm). O valor da pressão em dina/cm^2 foi convertido para Pascal.

2. Análise de óxido nítrico

O meio de cultura coletado durante o experimento foi usado para quantificação do óxido nítrico (NO) nos grupos com fluxo de fluido pulsátil e estático (Fig. A5c). NO foi medido como nitrito acumulado (NO_2^-) usando o reagente de Griess (1% sulfanilamida, 0.1% naftiletileno-diamina-dihidrocloridro e 2.5 M H_3PO_4). A absorbância foi medida a 540 nm com software Microplate Manager® versão 5.2 (Bio-Rad Laboratories, Hercules, CA, EUA). Concentrações de NO foram determinadas com uma curva padrão derivada de concentrações conhecidas de $NaNO_2$ (nitrito de sódio) em um meio de cultura não condicionado.

3. Avaliação de apoptose celular

A morte celular programada ou apoptose é um processo fisiológico pelos quais as células que perderam suas funções ou apresentam defeitos são eliminadas do organismo. Quando a apoptose é desencadeada, o resultado imediato é a ativação de enzimas proteases da família caspase. As caspases 3 e 7 são executoras e responsáveis pela clivagem de dezenas de proteínas vitais ao metabolismo e à estrutura da célula[3]. TNFα e IL-1ß podem provocar apoptose de células ósseas[1,6]. Portanto, o teste de apoptose foi realizado com o ensaio luminescente Caspase-Glo 3/7 (Promega, Madison, WI, EUA) para avaliar se as citocinas utilizadas estariam causando apoptose celular.

No final do experimento (após 30 min) nos grupos estático e de fluxo de fluido pulsátil com ou sem TNFα ou IL-1ß, foi posicionado um retângulo de borracha de 3 mm de espessura com abertura central de 1.5 x 5.5 cm preso com grampos ao redor da lâmina de vidro (Fig. A6). Partes iguais de meio de cultura e reagente Caspase-Glo 3/7 foi adicionado às células para avaliar a atividade das enzimas caspase 3 e 7 (Fig. 6). O ensaio foi realizado de acordo com as instruções do fabricante. Este ensaio contém o substrato pró-luminescente DEVD (seqüência de peptídeos) unido a aminoluciferina. A lise celular ocorre após a adição do

reagente Caspase-Glo 3/7. Células em apoptose liberam caspase 3 e/ou 7 que separam o substrato DEVD da aminoluciferina. A aminoluciferina livre é consumida pela enzima luciferase do kit gerando um sinal luminoso que é proporcional a atividade da caspase-3/7. Um período de 90 min foi esperado para a realização da leitura para que a máxima atividade da caspase fosse medida. Um grupo controle negativo (blank) usando meio de cultura e o reagente Caspase-Glo 3/7 (sem células) foi realizado e o valor obtido foi subtraído do valor de cada experimento para calibração. A leitora Wallac Victor 2 1420 Multilabel Counter (Berthold Technologies, Bad Wildbad, Alemanha) e o software WorkOut version 1.5 (DAZDAQ Ltda, Turku, Finlândia) foram usados para a medida da luminescência representando a atividade da caspase 3 e 7. Apoptose foi expressa em unidade arbitrária de luz por ng de DNA.

4. Quantificação do DNA total

O DNA celular foi determinado com o ensaio de proliferação celular CyQUANT® de acordo com instruções do fabricante (Molecular Probes Inc., Eugene, OR, EUA). A fluorescência emitida é proporcional ao valor total de DNA. Foi utilizada a leitora Wallac Victor 2 1420 Multilabel Counter contendo o filtro fluoresceína. Os valores obtidos foram usados para expressar o valor de NO e apoptose por ng de DNA.

5. Determinação de cálcio intracelular

1 x 10^4 células/cm^2 foram semeadas em lâminas de vidro (2 x 2.4 cm) da mesma forma como descrito inicialmente na metodologia do fluxo pulsátil. Uma hora antes do experimento, células foram incubadas com 10 μM Fluo-4 acetoximetil (AM) (Invitrogen, Eugene, OR, USA) em α-MEM a 37^{o}C, 5% de CO_2 por 30 min. Células foram lavadas delicadamente com DPBS esterilizada (salina fosfato tamponada de Dulbecco, Gibco). Para avaliar a interação da carga mecânica e citocinas, TNFα (10 ng/ml) ou IL-1ß (1 ng/ml) foram adicionados a α-MEM sem suplementos 30 min antes dos tratamentos estático e de fluxo de fluido pulsátil.

TNFα (10 ng/ml) ou IL-1ß (1 ng/ml) estavam também presentes durante o experimento descrito a seguir.

A câmara com as células usada para avaliar os níveis de cálcio intracelular teve as seguintes dimensões: 20 x 18 x 0.30 mm (Fig. A7a). A câmara contendo a lâmina de vidro com as células (Fig. A7b, c) foi colocada sobre o microscópio confocal (Leica TCS-SP2, Mannheim, Alemanha) (Fig. A8). A freqüência do fluxo do fluido pulsátil (5 Hz) foi produzido bombeando de modo pulsátil 13 ml de meio de cultura CO_2-independente sem L-glutamina (Gibco). A velocidade do fluxo foi monitorada com um sensor (T206, Transonic Systems, Ithaca, NY, EUA) no valor de 2.8 ml/min. A pressão média sobre a célula gerada pelo atrito produzido pelo movimento do líquido foi de 0.6 ± 0.3 Pa^2.

Cálcio intracelular foi monitorado como intensidade de fluorescência em cada célula corada com Fluo4-AM (Invitrogen), usando uma objetiva de correção com água 63 x 1.2. O comprimento de onda de excitação usado foi 520 nm, e o espectro de emissão 488 nm.

Imagens fluorescentes foram registradas em cada lâmina de vidro a cada 15 s durante 7 min. No primeiro minuto não houve fluxo de fluido pulsátil. Em seguida e por 5 min o fluxo de fluido pulsátil foi acionado e no minuto final ele foi desligado. A intensidade do sinal fluorescente foi quantificada usando Leica Confocal Software (Leica Microsystems, Wetzlar, Alemanha). A intensidade de fluorescência extracelular foi quantificada e subtraída do valor obtido em cada célula óssea para evitar interferências. Os dados foram obtidos de 4 experimentos independentes por grupo (sem citocinas, com TNFα (10 ng/ml) e com IL-1ß (1 ng/ml). Em cada experimento 15-30 células foram analisadas.

Referências

1- Ahuja SS, Zhao S, Bellido T, Plotkin LI, Jimenez F, Bonewald LF. CD40 ligand blocks apoptosis induced by tumor necrosis factor alpha, glucocorticoids, and etoposide in osteoblasts and the osteocyte-like cell line murine long bone osteocyte-Y4. Endocrinology. 2003; 144: 1761-9.

2- Bakker AD, Soejima K, Klein-Nulend J, Burger EH. The production of nitric oxide and prostaglandin E(2) by primary bone cells is shear stress dependent. J Biomech. 2001; 34: 671-7.

3- Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem. 1999; 68: 383-424.

4- Kato Y, Windle JJ, Koop BA, Mundy GR, Bonewald LF. Establishment of an osteocyte-like cell line, MLO-Y4. J Bone Miner Res. 1997; 12: 2014-23.

5- Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol. 1983; 96: 191-8.

6- Tsuboi M, Kawakami A, Nakashima T, Matsuoka N, Urayama S, Kawabe Y, et al. Tumor necrosis factor-alpha and interleukin-1beta increase the Fas-mediated apoptosis of human osteoblasts. J Lab Clin Med. 1999; 134: 222-31.

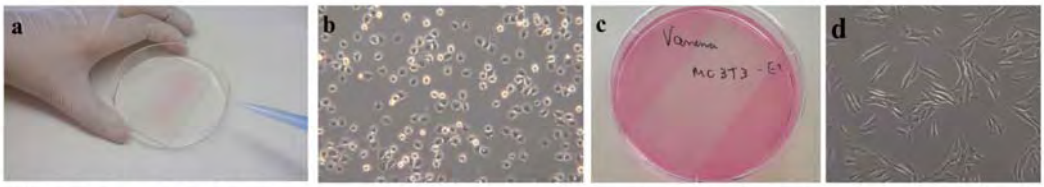

Figura A1: (a) Células em suspensão semeadas na lâmina de vidro. **(b)** MC3T3-E1 osteoblastos após 45 min em estufa a 37ºC com 5% de CO_2. **(c)** 13 ml de meio de cultura foram adicionados a placa de petri após 45 min de incubação. **(d)** MC3T3-E1 osteoblastos após 24 h semeados na lâmina de vidro em estufa a 37ºC com 5% de CO_2.

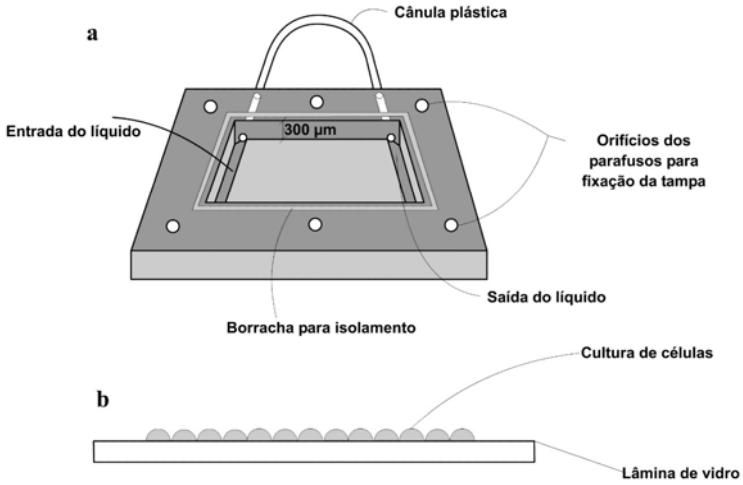

Figura A2: (a) Esquema da câmara de fluxo de fluido pulsátil. Vista frontal e superior. **(b)** Figura esquemática da lâmina de vidro com células na superfície.

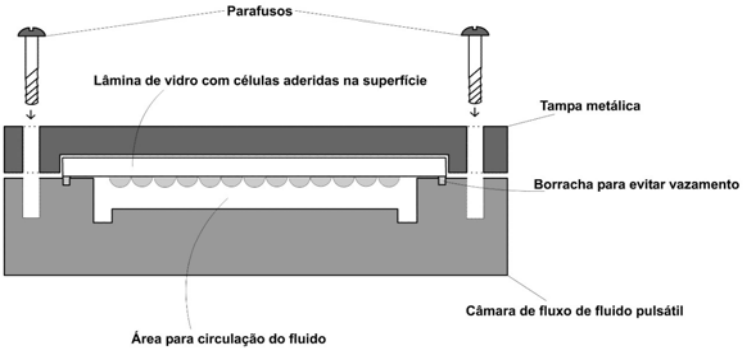

Figura A3: Esquema da câmara de fluxo de fluido pulsátil com lâmina de vidro, tampa e parafusos posicionados. Vista transversal.

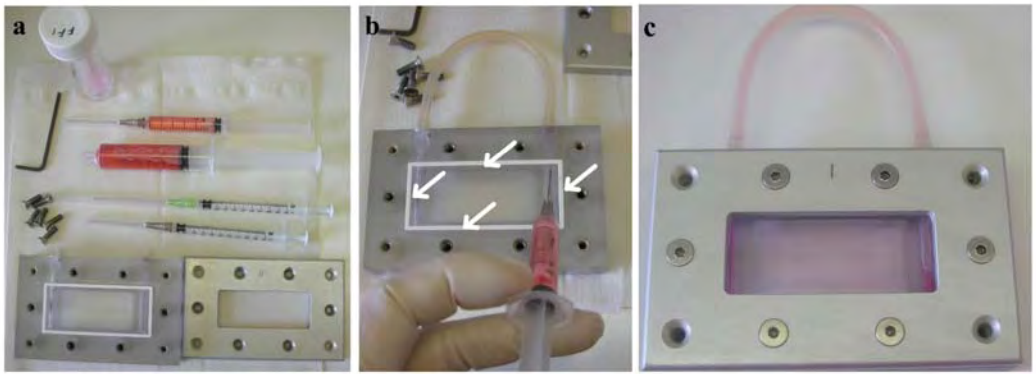

Figura A4: **(a)** Câmara para o teste de fluxo de fluido pulsátil e seringas com meio de cultura para preenchimento da câmara. Seringas de 1 ml para coleta de meio de cultura durante o experimento nos grupos teste e controle (sem fluxo de fluido pulsátil) para análise de óxido nítrico. **(b)** Preenchimento da câmara e cânula de borracha com 3 ml de meio de cultura. Setas indicam um degrau de 300 µm de altura entre a superfície superior e a base da câmara para que o meio de cultura circule sobre as células simulando o movimento do fluido intersticial. **(c)** Tampa em posição sobre a lâmina de vidro.

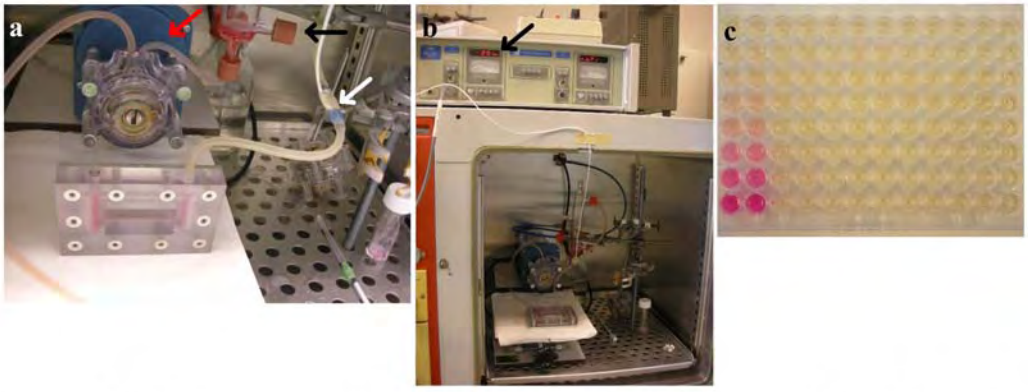

Figura A5: **(a)** Câmara com células sendo posicionada no sistema. Cânula de borracha na câmara foi solta em um dos lados da câmara e conectada a um sensor (seta branca) que irá medir a velocidade do fluxo. Bomba circular giratória (seta vermelha). Orifício para coleta de meio de cultura para análise de óxido nítrico (seta preta). **(b)** Equipamento do fluxo de fluido pulsátil com câmara posicionada em estufa a 37^0C e 5% de CO_2. Sensor conectado ao sistema de fluxo de fluido pulsátil para monitorar a velocidade do fluido (20 ml/min-seta preta). **(c)** Placa contendo amostras de meio de cultura dos experimentos com o reagente de Griess. As duas primeiras colunas correspondem a curva padrão de concentrações conhecidas de $NaNO_2$.

Figura A6: Lâmina de vidro com as células removida da câmara após o experimento com um retângulo de borracha colocado sobre a mesma e fixado com grampos. 250 µl de meio de cultura e 250 µl de Caspase-Glo 3/7 Assay foram dispensados sobre as células.

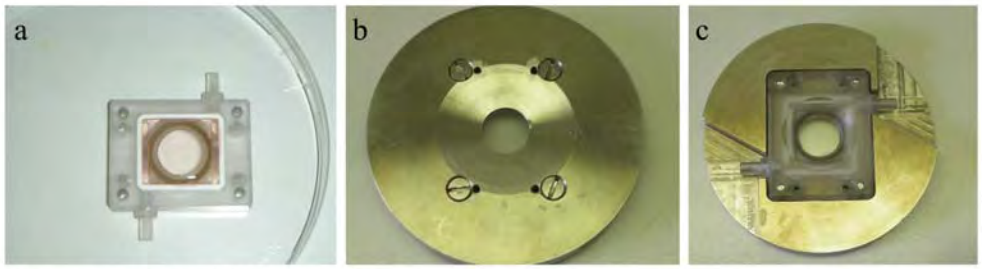

Figura A7: **(a)** Câmara de fluxo de fluido pulsátil usada para monitorar níveis de cálcio intracelular. Lâmina de vidro com células voltadas para baixo para permitir o contato com o meio de cultura na câmara. **(b)** Tampa metálica posicionada e parafusada sobre a câmara com as células. **(c)** Vista inferior da câmara posionada na tampa metálica. Células estão voltadas para cima.

Figura A8: **(a)** Base para encaixe da câmara. **(b)** Câmara posicionada no microscópio confocal Leica TCS-SP2 e acoplada a mangueiras de borracha para transporte do fluido. **(c)** Visão geral do sistema com bomba e mangueiras de borracha.

Araraquara, 27 de fevereiro de 2008.
VANESSA CAMILA DA SILVA